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CARDIOPROTECTIVE EFFECT OF ENALAPRIL AND HESPERIDIN AGAINST 5-FLUORO URACIL INDUCED MYOCARDIAL TOXICITY IN RATS

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Abstract
Various solid malignancies, especially those affecting the gastrointestinal tract and carcinoma of the breast, receive a wide use of the drug 5-fluorouracil in their treatment as an element of oncological practice. The secondary commonest adverse outcome linked to the cytostatic agents is cardiotoxicity and 5-fluorouracil is involved in about 7 percent of the cases. This paper explores the protective effects of the angiotensin-converting-enzyme inhibitor enalapril, in addition to arteriolar relaxation drug hedperidin against 5-fluorouracil triggered myocardial damage in Wistar rats. Thirty adult rats were randomly divided to five sets and used as a control group (Group I) that received normal saline (10 mg/kg), 5-fluorouracil-only group (Group II), enalapril-only group (Group III), hesperidin-only group (Group IV) and a combination of enalapril and hesperidin group (Group V). 5-fluorouracil administration started at Day 10 of the protocol, and except the control group, all others received 5-fluorouracil treatment on consecutive days At day 14, every cohort was sacrificed and blood specimens as well as the heart tissues were collected to be tested biochemically. Myocardial Toxicity depicted itself through increase of cardiac enzymes (cardiac troponin, creatine kinase-MB, lactate dehydrogenase) and increase of nitric oxide, endothelin-1 and cytokines interleukin-1, interleukin-8 and tumour necrosis factor-alpha. In connection with these alterations, there were augmented levels of serum lipid parameters, such as triglycerides, total cholesterol, low-density lipoprotein, and very-low-density lipoprotein than the base rates, and low levels of high-density lipoprotein. In parallel to the changes in metabolism were reduced antioxidant defences as suggested by reduced superoxide dismutase, catalase and glutathione peroxidase, activities whereas the levels of malondialdehyde increased. Monotherapy with enalapril and hesperidin did not affect mortality or biomarkers, but when used together they were able to alleviate 5-fluorouracil-induced cardiotoxicity, especially by preventing oxidative stress, myocardial necrosis, lipid peroxidation and cytokine production as well as enhancing antioxidant activity. Concisely, the findings indicate that the combination of enalapril and hesperidin has a synergistic protective mechanism on 5-fluorouracil cardiotoxicity.

Keywords: Myocardial toxicity, C-reactive protein, Flavonoid, Cardiac troponin, Oxidative stress.

Introduction

Acute ischemic death of myocardial tissue, which is often caused by thrombotic blockage of a coronary artery and often combined with rupture or erosion of atherosclerotic plaques, is labelled as myocardial infarction [1]. Myocardial toxicity is one of the leading causes of cardiovascular associated deaths, [2] and it is closely linked to the production of reactive oxygen metabolites known to play a pivotal role in the pathogenesis of post-ischemic dysfunction [3]. One chemotherapeutic recent research has proved that enalapril treatment is effective in reducing

the size of infarction and oxidative stress in heart [4]. Hesperidin which is a bioflavonoid found in citrus fruits has been shown to have significant antioxidant, radical scavenging, chemopreventive and an anti-inflammatory effect. Earlier researches have shown that hesperidin can reduce circulatory failure and clotting of blood cells, abnormal permeability and low strength of capillary leading to immunity to different traumas and stresses [5]. The current study is based on the estimation of the co-operation effect of enalapril and hesperidin in opposition to the 5-fluorouracil induced myocardial toxicity in Wistar rats.

Selection & Acclimitization of Animals

Albino Wistar rats (200-250g) purchased in an animal experimental laboratory were induced (12 h light-dark cycles) in micro-nylon boxes in standard environmental conditions. Rats received normal laboratory food and free water. The research scheme was accepted by the institutional animal ethical committee (IAEC/TNMGRU/KMCP /171/2022-23). To form two experimental groups rats of two sexes were assigned, and were quarantined during 15 days before the starting of the research [6].

Preparation of Drugs

5-fluorouracil (5-FU) was injected intraperitoneally with the dose of 20mg/kg [7]. Nocebo was in the form of Enalapril which is dissolved in distilled water, administered orally, and the dose was 2mg/kg [8] . Hesperidin was also dissolved in normal saline and administered orally at the dose of 50mg/kg [9].

Induction of Myocardial Infarction

Myocardial toxicity was artificially induced in the present study by intraperitoneal administration of 5-fluorouracil (celon labs Pvt.Ltd.) single dose of 20mg/kg dissolved in sterile water for injection.

Treatment Protocol

The rats were randomly assigned to the 5-groups (n = 6 groups). Group I received normal saline (10 ml/kg, i.p.); group II-V received 5-FU (20 mg/kg, i.p.) during the last 5 d. Group III creatures received enalapril (2 mg/kg, p.o.) [8] ; group IV rat got hesperidin (50 mg/kg, p.o.) [9]; group V obtained both enalapril and hesperidin, and group VI did not receive either of the two medications. Weights of all rats were determined at the end of the treatment period (14 d) at which time all rats were anesthetized using diethyl ether. The blood was taken using a retro orbital puncture and centrifuged (2,000 rpm, 10 min) in order to extract a serum. This serum was kept at -20 o C before bio-chemical analysis [10]. Markers that were found in the serum were cardiac troponin-I, CK-MB, TNF-a, IL-1 and IL-8, IL-10, TNF-a, C-reactive protein, nitric oxide (NO), and endothelin, CAT, and SOD. Lipid levels such as HDL, LDL, VLDL, TC and TG were measured too. Measurements of malondialdehyde (MDA) tissue homogenates and histopathological test were done.

Analyses of Lipid Profiles

By the use of commercialized, standardized enzymatic-based assay kits, total cholesterol (TC), triglycerides (TGs), and high-density lipoprotein-cholesterol (HDL-C) concentrations were calculated. According to the approach suggested by Friedewald et al., the value of very low-density lipoprotein-cholesterol (VLDL-C) was obtained according to the formula VLDL-C = TG/ 5.

Estimation of Cardiac Troponin I (cTn I)

The enzyme immunoassay procedures were used in the analysis of the concentration of the cardiac specific troponin I (cTn I) in serum, and then the absorbance values of the samples were measured using the ELISA reader [10].

Assay of Cardiac Marker Enzymes

The activity of the serum creatinine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) was measured using commercially available standard assay kits using a PD 303S spectrophotometer [10].

Measurement of Plasma NO

The method of flow-through nitrate determination was derived on the basis of the following operational postulates. The sample of nitrate (NO3) was reacted with a cadmium column to convert into nitrite (NO2) that was then reacted with a Griess reagent to produce a purple azo dye. The NO2 in the sample did not require reduction and consequently it reacted with Griess agent in both the cases of cadmium column bypassed and applied. Development of dye was made in water bath at 60 C, dye was cooled in a water bath at 0 C and was read in a flow thru U.V/visible

spectrophotometer at a reading of 540 nm. The detector was also linked to a strip chart recorder as well as an integrator [11].

Measurement of Plasma ET-1

SepPak C18 cartridges pre-activated with methanol, 8 M urea and water were used to obtain samples and 100 % methanol was used to elute ET-1. The recovery was estimated to 75 % (CV = 3.3 %). The samples and standards (100 l) were thus incubated subsequently with rabbit anti-ET-1 serum (as reconstituted in assay buffer) at 4 C over a 24 h interval. These were again incubated in 24 h by keeping about 10,000 counts per minute (cpm). The separation of bound and free radioligands was carried out with the aim of second-antibody technique mentioned above. The data of bound radioactivity was analyzed following the logit-log-transformed data, and the concentration of immunoreactive ET-r concentration was calculated [11].

Determination of Antioxidants from Cardiac Homogenates

The samples of heart tissues (about 1 g) were homogenized at an approximation of 10% (w/v) in ice-cold phosphate buffer (25 mM, pH 7.4) via using a tissue homogenizer. The above homogenate was centrifuged at 1700 rpm during a 10 min interval, and supernatant that remains was stored at -20 o C ready to be used in biochemical analysis [10]. There were 10 cardiac homogenates that were analyzed, which are Superoxide dismutase (SOD), Lactate dehydrogenase LDH, Catalase, and Malondialdehyde (MDA).

Estimation of Superoxide Dismutase

The tissue homogenates of the rat hearts were made up in a regular physiological medium, centrifuged again at high speed, that is, 12,000 rpm, 10 minutes at 4 C on an Eppendorf 5415D centrifuge, and poured out to obtain clean supernatants. The supernatants were then subjected to the spectrophotometric assay in order to establish the activity of SOD in them, which was expressed as units of enzymatic activity per milligram of cellular protein per sample [12].

Estimation of Catalase

The accuracy of catalase (CAT) activity measurements was obtained according to the protocol described previously in detail.[13] Briefly, reaction mixture contained 1 mL phosphate buffer (0.05 M), 0.4 mL 0.03 M hydrogen peroxide, and 0.1 mL of tissue homogenate (10 %); the rate of H_2O_2 decomposition was monitored by spectrophotometer at 240 nm during 60 s at room temperature. CAT activity was presented as units g-1 of tissue [14].

Determination of MDA

Malondialdehyde [MDA] is an active aldehyde which breaks down polyunsaturated lipids. MDA has gained a standing trial as a biomarker of lipid peroxidation. Thiobarbituric acid [TBA] reactive substances [TBARS] are produced when MDA interacts with thiobarbituric acid [TBA]. The compound produces a 1:2 adduct of MDA-TBA, the maximum absorbance of which occurs at 532 nm. The level of TBARS is proportional to the concentration of MDA. The concentration of TBARS is thus determined by the procedure of Uchiyama and Mihara and the concentration is normalized to an MDA standard curve and expressed as nmol/mg protein.[15]

Estimation of IL

Blood is taken and the serum harvested after centrifugation of the sample in order to get rid of cellular debris before preservation. Commercially available enzyme-linked immunosorbent assay (ELISA) platform specific to interleukins IL-1b, IL-8 and IL-10 was used. TNF-alpha was measured as ng per gram of tissue in the serum with a standard ELISA assay, which included incubating duplicate serum samples wells in the ELISA plate, and proper controls and blanks [16, 17]. Substrate solution was then added and incubated once more after incubation to allow antigen-antibody binding. Afterwards, the reaction was halted by adding in stop solution. Microplate reader was then used to measure absorbance at the described wavelength of absorbance.

Histological Examination

After being euthanized, the mixture of the intact cardiac muscle of rats belonging to different groups of the experiment was fixed in 10 percent formalin during a time period of 24 h. The specimens were further washed using distilled water, cleared using xylene and then they were embedded in paraffin at 56 C to spend 24 hours. Then paraffin blocks were manipulated and sections were cut at 4-6 um. Those paraffin-sections were attached using glass slides and deparaffinized before being stained by hematoxylin-eosin combination. The resulting histological slides were examined using light microscope (Olympus BX10) where a preliminary evaluation of the structural changes is done.

Statistical Analysis

Data were all given as mean peak cuz mean (SEM). The GraphPad Instat V3.0 program was used to perform statistical analysis. All later comparisons were regarded significant in case of P < 0.01. To show significance, one-way analysis of variance (ANOVA) was used and thereafter pairwise comparisons using Neuman keul multiple range test was used.

Results

Table:1 Effects of Enalapril, Hesperidin and their combination on serum levels of lipid profiles in 5-fluorouracil induced myocardial toxicity in rats

GROUP	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
G1	103.3±2.30	62.25±1.40	53.50+1.80	41.20±1.45	16.0±0.40
G2	188.40±3.85 ***a	146.0±2.30 ***a	25.15±1.05 ***a	123.30±2.06 ***a	35.6±1.06 ***a
G3	142.05±3.25 ***b	88.60±1.75 ***b	41.40±1.42 ***b	54.10±1.80 ***b	25.95±0.96 ***b
G4	135.15±2.90 ***b	81.45±1.50 ***b	43.05±1.42 ***b	54.85±1.88 ***b	23.39±0.90 ***b
G5	122.40±2.60 ***b	74.80±1.44 ***b	46.85±1.65 ***b	46.05±1.55 ***b	20.34±0.84 ***b

All values expressed as Mean ± SEM for 6 animals in each group.
***a Values are significantly different from normal control at P<0.001
***b values are significantly different from toxic control at P<0.001

Table:2 Effects of Enalapril, Hesperidin and their combination on Cardiac biomarkers in 5- fluorouracil induced myocardial toxicity in rats

GROUP	CRP µg /ml	TROPONIN Pg/ml	LDH IU/L	CK-MB U/L	NO µm /ml	ENDOTHELIN Pg/ml
G1	1.91±0.10	88.48±2.30	192.83±4.15	20.41±0.94	30.15±0.86	1.74±.0.05
G2	4.21±0.38 ***a	356.40±6.15 ***a	566.75±8.15 ***a	44.51±1.55 ***a	51.20±1.55 ***a	2.97±0.24 ***a
G3	2.54±0.22 ***b	188.77±3.85 ***b	297.70±5.20 ***b	25.90±1.18 ***b	42.10±1.34 ***b	2.06±0.18 ***b
G4	2.50±0.18 ***b	179.50±3.60 ***b	290.40±5.05 ***b	24.05±1.08 ***b	40.37±1.28 ***b	2.09±0.20 ***b
G5	2.28±0.15 ***b	128.20±2.30 ***b	225.15±4.55 ***b	21.95±1.05 ***b	36.56±0.98 ***b	1.89±0.16 ***b

All values expressed as Mean ± SEM for 6 animals in each group.
***a Values are significantly different from normal control at p<0.001
***b values are significantly different from toxic control at p<0.001

Table:3 Effects of Enalapril, Hesperidin and their combination on antioxidant levels in 5-fluorouracil induced myocardial toxicity in rats

GROUPS	SOD U/mg/Tissue	CAT U/mg/Tissue	GPx U/mg/Tissue	MDA nmol/g/Tissue
G1	5.66±0.18	19.29±0.48	7.37±0.26	2.17±0.06
G1	2.80±0.08***a	11.16±0.15***a	2.65±0.14***a	3.70±0.15***a
G3	4.26±0.16***b	14.60±0.22***b	5.39±0.19***b	3.15±0.10***b
G4	4.35±0.12***b	14.45±0.20***b	5.30±0.17***b	2.84±0.08***b
G5	5.13±0.15***b	16.25±0.32***b	6.30±0.23***b	2.62±0.06***b

All values expressed as Mean ± SEM for 6 animals in each group.
***a Values are significantly different from normal control at p<0.001
***b values are significantly different from toxic control at p<0.001

Histopathology

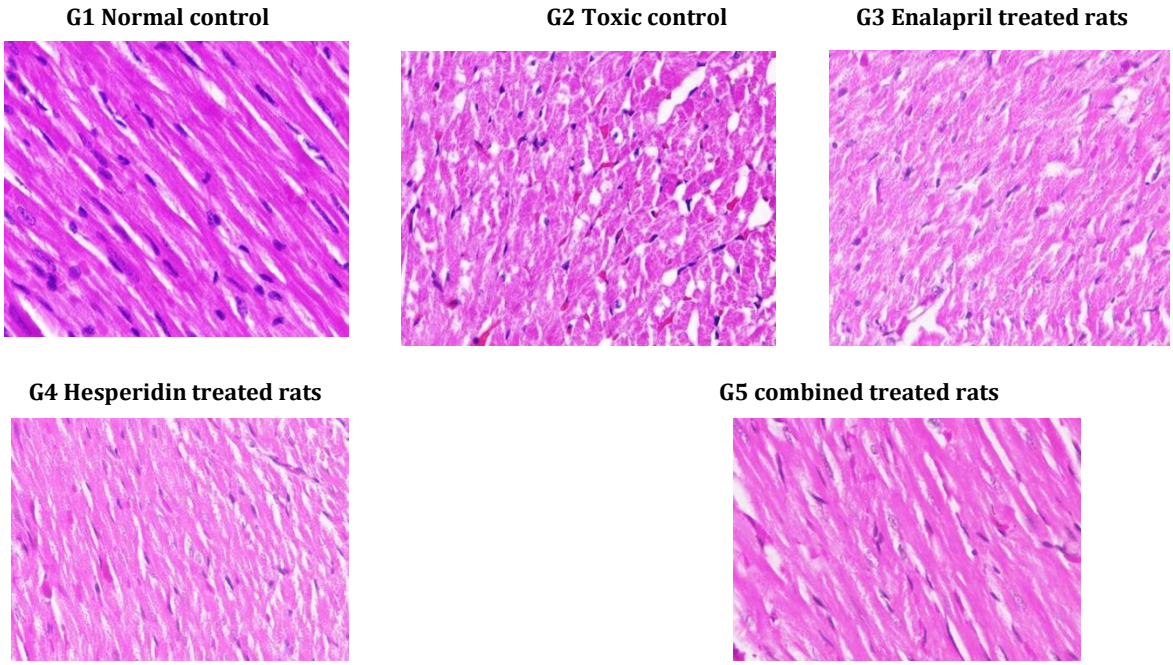

Fig:1 Cardiac histology of (**G1)** normal control showed the presence of cardiac myocytes with nucleus and intercalated disc.Cardiac histology of 5 Fluorouracil treated rats (**G2**) showed multiple necrosis and hyperemia, inflammation & marked fragmentation of myofibers. Cardiac histology of enalapril treated rats (**G3**) shows mild tissue damage, reduced necrotic area and fragmentation of myofibers. Histology of heart treated with hesperidin (**G4**) revealed the prevention of necrosis and hyperemia, reduced inflammation with small degree of myofibrillar damage nucleus. Histology of heart treated with both enalapril & hesperidin groups (**G5**) shows nearly regular arrangement of myofibrils, nucleus, intercalated disc with the presence of large vacuoles.

Table.1: Administration of 5-FU led to a dramatic (P<0.001), rise in the serum lipid levels particularly the triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) as well as very low-density lipoprotein (VLDL), but considerable decrease in the levels of the high-density lipoprotein (HDL). Pre-doses of enalapril, hesperidin, or a combination of the two prevented (P<0.001) these hypercholesterolemic and hypertriglyceridemic effects and at the same time aggravated concentrations of HDL**. Table.2** also shows that 5-FU therapy had a strong (P<0.001) response by raising the serum level of cardiac troponin I (Tnl), C-reactive protein (CRP), lactate dehydrogenase (LDH) and creatine kinase MB (CK-MB) and a decrease in nitric oxide (NO) and an increase in endothelin-1 (ET-1). The propensity to these cardiac biomarkers was significantly decreased with the co-treatment of enalapril, hesperidin, or even the both, thereby revealing cardioprotective activity.

As shown in **Table 2**, there are concentrations of cytokine in different experimental groups. In comparison to the control group, a serious (P 1001) increase in cardiac levels of IL-1, IL-8, IL-10 and TNFalpha was seen in rats treated with 5-fluorouracil (5-FU); the increase was reduced by pre-administration of enalapril, hesperidin or the combination.

The activity of the antioxidant enzymes as determined after exposure to regiments of interest was recorded in **table 3**, and there was also a significant reduction in superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) following 5-FU exposure in comparison with control rat. On the other hand, enalapril, hesperidin or their synergic use increased the concentration of SOD, CAT, GPX to the levels that were in line with the control animal, and reduced the concentration of malondialdehyde (MDA).

Histological examination (Fig:1) reveals that 5-FU administration causes vacuolar degeneration, multiple interstitial myocardial hemorrhages, valvulitis and pericarditis & vascular changes in ventricles of heart rat. Endothelial damage in myocardium result in myofiber necrosis and inflammatory reaction. Hesperidin prevent the occurrence of necrosis, reduce inflammation , myofibrillar damage and mild sarcoplasmic vacuolation, whereas co administration of Enalapril & hesperidin brought nearly normal appearance of the nucleus, intercalated disc with the presence of large vacuoles with regular arrangement of myofibrils.

Discussion

Cardiotoxicity is one of the major adverse effects of a number of anticancer agents and hence, the therapeutic advantage of chemotherapy is constrained. Confirmed incidence rates of heart failure, myocardial ischemia, arrhythmia, hypertension and thromboembolism related to chemotherapy treatment warrant consideration in strictness of observation [18,19]. The third-most widely used chemotherapeutic agent used in the treatment of solid cancer [20], 5-fluorouracil (5-FU) is used systemically in the treatment of breast, gastrointestinal, pancreatic and skin malignances. Modern evidence does, however, implicate the direct cardiotoxic effects of 5-FU on the basis of oxidative stress, angina-type spasm of coronary arteries mediated by the endothelium, and dysfunction of left ventricles[21]. Hyperlipidemia is an already known risk factor of atherosclerotic disease[22]. The current experimental model analyzed the changes in the serum lipid levels in rats under the effect of 5-FU administration, which has shown significant elevation of such parameters as the total cholesterol (TC), triglycerides (TAG) and VLDL-c and LDL-c fractions accompanied by a decrease in the HDL-c. The results obtained are aligned to increase endogenous lipid synthesis through cardiac cyclic adenosine monophosphate [23]. The atherogenic environment that ensued with raised TC:HDL-c and LDL-c:HDL-c reveals a considerable increase in the likelihood of cardiovascular morbidity [24][25]. The flavonoid hesperidin and angiotensin-converting enzyme inhibitor enalapril improved some of these serum lipid profiles, which associated with blocked cholesterol synthesis, increased LCAT activity and raised HDL-c levels and collectively, these have cardioprotective value [26].

Nitric oxide (NO) is a mandatory intracellular messenger the modulatory action of which is central in physiology as well as pathophysiology[27][28]. An emerging literature also shows NO production, in reaction to the alkylating agent 5-fluoro 2-deoxyuridine, or 5-Fu. The generation of NO in the mammalian tissues is based on the oxidation of arginine to citrulline using NADPH as an oxidizing source through nitric oxide synthase (NOS)[29]. An overproduction of NO may result in inflammatory sequelae by means of nitrosation, oxidative injury, and enhanced pro-inflammatory cytokines production. In a quest to couple the relationship between a 5-FU and NO and oxidative stress[30], the present study studies the forming of peroxynitrite by the reaction of NO with superoxide radicals[31]. Simultaneously, the experimental design observes the change of endothelin (ET-1) an endothelium derived vasoconstrictor, which, as indicated, stimulates proliferation in vessels. 5-Fluoro uracil administration increases the level of ET-1 in plasma and the flow of nitric oxide in plasma significantly and the combined dosage of hesperidin and enalapril reduces this effect. Validating clinical evidence shows that enalapril possesses cardioprotective effects, but hesperidin has vascular-specific protective effects, such as antiproliferative, lipid-lowering, and an antioxidant property. Cardiac troponin T (cTnT) and troponin I (cTnI) are exhaustive biomarkers of myocardial injury and the entry measure of the serum is studied to flow indicators of myocardial necrosis. The significant increase in enzymatic activities of CK-MB in the 5-FU treated rats reflects on membrane damage and permeability[32], and hemolytic activity is reflected in the parallel increase of LDH. All these results together indicate the inclusion of 5-fluoro uracil in the activation of NO-mediated inflammatory reactions that lead to structural myocardial damage. Also, the two interventions being tested, hesperidin and enalapril, show the successful effect of inhibiting these toxic pathways and reducing the release of cardiac biomarkers[33].

Interleukin-1 (IL-1) is a necessary pro-inflammatory cytokine so important in inflammatory conditions. Monocytes, macrophages, endothelial and fibroblasts produce IL-1 34. The current investigation showed that 5-fluorouracil [34] (FU) is a potent inducer of a number of cytokines, among which there are interleukins and tumour necrosis factor. High levels of IL-1, IL-2, IL-8, IL-10 and TNF-a were noted in cardiac homogenate obtained rats exposed to FU as compared to normal control (Table 4). There are remarkable decreases in these cytokines when rats receive hesperidin and enalapril monotherapy and their combination implies that this form of treatment can be effective in determining the severity of inflammatory infiltration and response of injured myocardium.

Several different pathways to cardiotoxicity caused by 5-FU exist, whereby the most common pathway in drug-induced cardiotoxicity is oxidative stress. Oxidative stress can be seen as a disproportion between excess generation of reactive oxygen species (ROS) and loss of endogenous antioxidant defense.[35] Oxidative stress induced by 5-FU treatment is a relationship with an excess ROS formation; namely, it elevates intracellular superoxide anion (O -2) levels.[36] Produced ROS trigger a cascade of apoptosis and guide cardiomyocyte damage.[37] Polyenoic fatty acids in the cell membrane are the main targets[38] [39], they The concentration of malondialdehyde (MDA) is an indicator of lipid peroxidation by ROS.[40] The high concentration of MDA means the raised oxidative damage. Moreover, 5-FU toxicity in arterial endothelium may be explained by the action of ROS.[41] Above results are coincident with the current study results which prove that 5-FU treatment generates certain level of oxidative stress and myocardial injury. So, it seems that the oxidative stress is involved in the cytotoxic action of the 5-FU. Hesperidin which is combined with enalapril has got antioxidant effect and the two agents have a great rise in activity of GPx as well as a lower level of the MDA in cardiac homogenates.[42] These results indicate that a combination of hesperidin and enalapril alters the release of SOD, CAT and MDA thus suppressing related free radicals to curb effects of heart disease complication. The antioxidant activity of this regimen will probably be implemented by inhibition of the

renin-angiotensin system and reducing the production of Ang II-induced ROS, which will eventually stimulate the antioxidant enzyme activities. As a result, there is a decrease in oxidative stress and compression of damages caused by MDA. Moreover, enough amount of nitrous oxide and endothelin production are preserved by the administration of hesperidin together with enalapril, thus sustaining vascular integrity. In association with anti-inflammatory, lipid-lowering and antioxidant activity, these functionalities manage to improve the cardiotoxic profile of 5-FU as a whole.

Conclusion

The administration of enalapril and hesperidin along with enalapril alone was protective against 5-FU-induced cardiotoxicity.Treatment with enalapril and hesperidin improved lipid profile, reduced proinflammatory cytokines & exert antioxidant property which indicate the combined effect exert therapeutic potential against 5-FU-induced cardiotoxicity. Histopathological reports also supported the study In conclusion, combined treatment of enalapril & hesperidin significantly reduces myocardial ischaemia & improves cardiac function.

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Conflicts of Interest

The authors declare no conflict of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval& consent to participate

This study was carried out & approved by the Institutional animal Ethical committee of K.M.College of Pharmacy, Uthangudi, Madurai. (IAEC/TNMGRU/KMCP /171/2022-23)

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