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Mitigation of 5-Flourouracil induced cardiotoxicity by Naringenin *via* targeting oxidative stress in *Wistar* rats

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Abstract
The purpose of this study was to assess the protective effects of Naringenin (NG) against cardiotoxicity induced by 5-Fluorouracil (5-FU) in *Wistar* rats. A total of 48 male rats, all of uniform size, were divided into four groups, each consisting of 12 animals. Group 1, designated as the control group, received normal saline (NS) orally (P/O). Group 2 served as the 5-FU toxic group and received intraperitoneal (IP) injections of 5-FU at a dose of 20 mg/kg body weight for the first 5 days. Group 3 received NG orally at a dose of 100 mg/kg body weight per day for 28 days, while Group 4 was injected IP with 5-FU and concurrently received NG orally for 28 days before being sacrificed on the 14th and 28th days of the experiment. The administration of NG significantly ameliorated lipid peroxidation (TBARS) and boostened increased levels of antioxidant concentration levels of glutathione (GSH) and superoxide (SOD) in group-4 rats along with mitigation of RBC abnormalities. In conclusion, the results of this study indicate that NG possesses significant potential in preventing 5-FU-induced cardiotoxicity through antioxidant properties.

Keywords: Anti-oxidant, Naringenin, erythrocytes, thymidylate synthase

Introduction
5-Fluorouracil (5-FU), a fluoropyrimidine, is employed as the third most prevalent chemotherapy medication for the treatment of various cancer types, including colorectal, skin and breast cancers. It converts into various metabolites and inhibit thymidylate synthase enzyme and incorporation into DNA and RNA, leads to oxidative stress and cause cell injury (Polk *et al.*, 2014) [1]. Further, 5-FU considered ranks as the second most commonly associated drug with cardiac toxicity (Sara *et al.*, 2018) [2]. Fluoropyrimidine-induced cardiac issues, like those seen with 5-FU, typically manifest as unusual chest pain, resembling angina during stress or rest and sometimes even as acute coronary syndrome (Mastubara *et al.*, 1980) [3]. Proposed mechanisms for 5-FU-induced cardiotoxicity include autoimmune-mediated myocardial injury, coronary artery spasms, endothelial damage, overall cardiac dysfunction, thrombogenic effects, metabolite repositioning and direct toxic effects causing myocardial necrosis (Eskandari *et al.*, 2015) [4]. There are several signaling pathways implicated in 5-FU-induced cardiac damage, with MAPK/ERK1/ROS being one of them (Arab *et al.*, 2018) [5]. Vasospasms, a known consequence of 5-FU treatment due to endothelial dysfunction, are associated with the COX-2 pathway. This pathway, either independently or influenced by ROS, can trigger vasospasms (Kosmas *et al.*, 2008) [6]. It's worth noting that oxygen-derived species have cytotoxic properties. Oxidative damage to cellular DNA, which includes mutations such as DNA sequence rearrangements and gene amplifications, can occur during the initial stages of cancer development (Sengul *et al.*, 2021) [7]. Various cancer tissues have been found to exhibit free radical-induced DNA damage (Rashid *et al.*, 2014 and Refaie *et al.*, 2022) [8, 9]. Oxidative stress plays a pivotal role in the pathogenesis of colorectal cancer and it is well-established that hydrogen peroxide and superoxide radicals are involved in the anticancer mechanism of 5-FU. These radicals are also responsible for various side effects associated with such therapies. Evidence of oxidative stress in cancer patients undergoing chemotherapy includes increased blood lipid peroxidation (LPO) and reduced plasma antioxidant levels (Arafat *et al.*, 2022) [10].

Plant-based diets have been linked to a lower risk of CVD due to rich source of phytochemicals, including polyphenols provide a pool of antioxidants to maintain ongoing health. Flavonoids are mainly natural pigments, primarily found in plants and comprise a basic phenolic structure (Tungmunnithum *et al.*, 2018) [11]. Among flavonoids, NG has many beneficial medicinal importance. This compound is naturally found in citrus fruits such as grapefruit and oranges, as well as in tomatoes. It is synthesized from the aromatic amino acid phenylalanine. NG exists in two primary forms: as the aglycone (NG) and in its glycosylated form, known as naringin (NIN) with very lipophilic in nature and absorbed directly through intestines (Rajadurai and Prince, 2006) [12].

Materials and Methods

All chemicals are procured from Qualigens Private limited, India (Mumbai) and SRL Private limited, India. Naringenin (CAS No: 10236-47) was obtained from Sigma (SAC-St Louis, MO, USA). The animal experimentation was assigned to conduct by ethical committee (No. 9/24/C.V.Sc., Hyd. IAEC-Rats/ 12.06.2021).

Experimental design

Twenty-four male *Wistar* rats (6–8 weeks, 180 ± 20 g) were obtained from Jeeva life Sciences. The Study rats were randomly assigned into four groups as follows:

a. Group-1, Control-received normal saline (NS) through oral gavage.
b. Group-2, served as toxic received 5-Fluorouracil @ 20 mg/kg b. wt - IP consecutively for initial 5 days.
c. Group-3, served as ameliorative group, received Naringenin @ 100 mg/kg b. wt for 28 days orally through oral gavage.
d. Group-4, combination received both NG for 28 days +5-FU- 20 mg/ kg b. wt for 5 days.

At the end of experimental period, blood was collected through retro-orbital plexus and rats were euthanised on 14th and 28th day. The cardiac tissues were removed, rinsed thoroughly with ice-cold saline solution, gently dried with blotting paper and subsequently stored in a frozen state until further analysis.

Blood smear preparation

Blood smear were prepared by using standard protocol given previously of leshmans stain (Sareen *et al.*, 2018) [13].

Analysis of oxidative stress indices

Small pieces of cardiac tissues were also collected and stored at -80 ºC to study the organ antioxidant profiles (GSH, SOD and TBARS). One gram of heart tissue was combined with 10 mL of Tris HCl buffer (pH 7.2), homogenised at 10% to determine all tissue oxidative stress parameters.

Estimation of lipid peroxidation (LPO) in heart homogenate

The extent of LPO was estimated by using standard protocol (Ellman, 1959) [14]. MDA is formed through the breakdown of polyunsaturated fatty acids, an index for lipid peroxidation estimation. MDA is a product of LPO and reacts with thiobarbituric acid (TBA) to get a product at 535 nm, widely used for the estimation of oxidative stress indices. Briefly, 200 µL homogenate, 10% trichloroacetic acid (TCA)-1mL and 1 mL TBA were taken and samples were heated at 96 ºC for 45 min and observed at 535 nm against blank.

Estimation of glutathione (GSH) in heart homogenate

To know antioxidant status, reduced GSH was estimated according to Ellman's method (Madesh and Balasubramanian, 1998) [15] by mixing tissue homogenate supernatant with 5, 5' dithiobis-2- nitrobenzoic acid and absorbs light at 412 nm.

Estimation of superoxide dismutase (SOD) in heart homogenate

For SOD estimation, tissue homogenate reaction involves the generation of superoxide by mixing pyrogallol autoxidation by scavenging superoxide anion radicals and expressed as the amount in mg of protein required to inhibit the MTT reduction (Luna, 1968) [16].

Statistical analysis

Data regarding the study was run to statistical analysis by using one-way Analysis of variance (ANOVA) using the statistical package for the social sciences (SPSS) version 15.0. Duncan's multiple comparison tests were done for the comparison among groups, and the significance level was set at $p<0.05$ (Snedecor and Cochran, 1994) [17].

Results and Discussion

While numerous studies have explored the protective properties of natural compounds against the adverse effects of chemotherapeutic drugs, our current research, as far as we are aware, represents the pioneering investigation into the potential protective role of NG against cardiac tissue damage induced by 5-FU. NG is considered relatively safe, with an LD50 of 5000 mg/kg (Ortiz-Andrade *et al.*, 2008) [18]. Naringin (NG 7-rhamnoglucoside) undergoes rapid conversion into NG through enzymatic processes involving rhamnosidase and Glucosidase enzymes. NG exhibits high lipophilicity and is readily distributed throughout the body and its metabolites have an affinity for binding with plasma proteins such as albumin (Moghaddam *et al.*, 2020) [19]. In addition to its direct antioxidant capabilities, NG can stimulate the endogenous antioxidant system by Upregulating the expression of Glutamyl Cysteine ligase, leading to an increase in glutathione levels. Its antioxidant effect primarily arises from the hydroxyl (OH^-) substituents it contains, which exhibit strong reactivity against reactive oxygen species (ROS) and reactive nitrogen species (RNS) by reducing NADPH activity (Reddy *et al.*, 2008) [20]. The hydroxyl radical (OH-) can readily donate its hydrogen to free radicals, thereby enhancing antioxidant capacity. Furthermore, NG contributes to an increase in vitamin C and vitamin E levels (Rajadurai and Prince, 2007) [21]. Its lipophilic properties enable it to bind to cell membranes, reducing the formation of free radicals and safeguarding the integrity of these membranes (Renugadevi and Prabu, 2009) [22]. Additionally, NG suppresses the elevation of MDA, thus providing protection against lipid peroxidation (Singh *et al.*, 2018) [23].

Soon after blood collection, smear was prepared and observed under microscope using oil immersion. 5-Fu causes lipid peroxidation of RBC membrane and changes in RBC abnormalities (Figure. 1) A- normal RBC, B-Acathocytes, C-Echinocytes, D-stomatocytes, E-Ovalocytes due to 5-FU, whereas G-is having mild RBC abnormalities The impact of 5-FU extends beyond lipid peroxidation, it also effects to oxygen carriers like RBCs. 5-FU causes changes in RBC morphology occur, linked to altered oxygen metabolism

(Nandhakumar *et al.*, 2013) [24]. Prior studies reveal 5-FU's impact on phosphate metabolism, causing O_2 consumption surge, reducing PO_2, increasing 2,3-BPG, shifting oxyhemoglobin to deoxyhemoglobin (Spasojevic *et al.*, 2009) [25]. 5-FU causes Echinocytosis, fluidity rise, potassium efflux, ion pump impairment follow, hampering RBC function, hindering oxygen transfer along with ATP decrease in treatment groups. In contrast, NG counters *via* antioxidant effects, mitigating RBC abnormalities and potentially restoring normal oxygen transport (Lone *et al.*, 2022) [26].

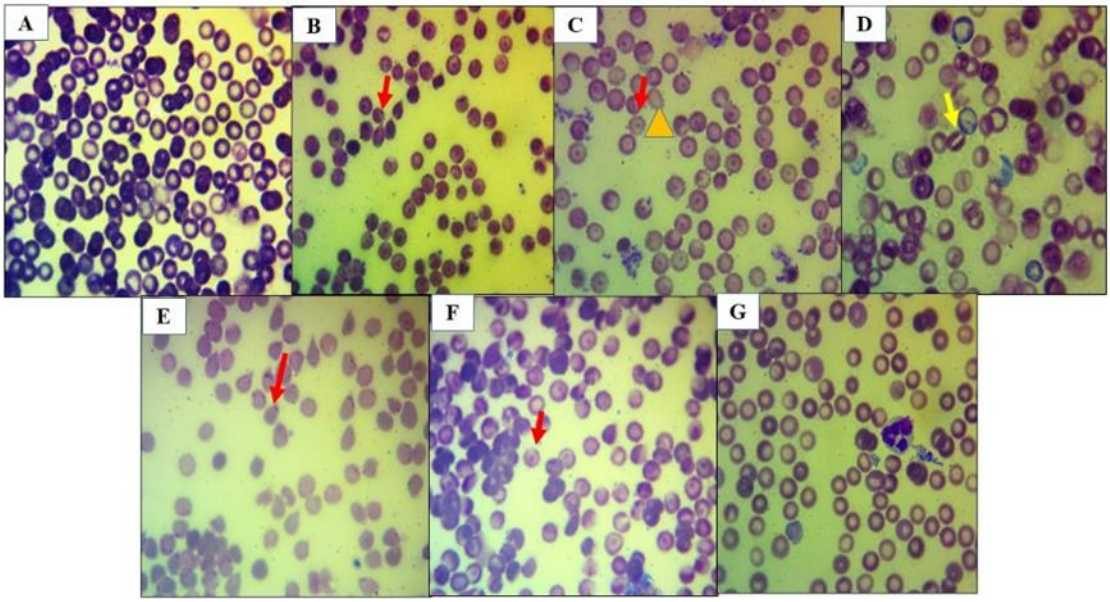
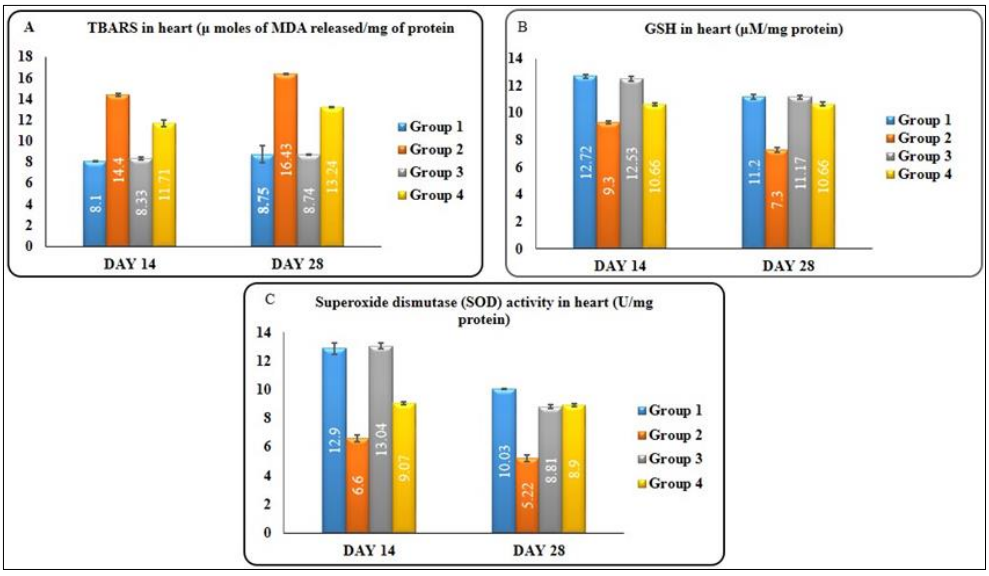


Fig 1: Effect of 5-FU on RBC morphology in blood smear. B-Echinocyte. C-Acanthocyte-traingle, Arrow-Echinocyte. D. Stomatocyte. E-Dacrocyte along with elliptocyte. F-Target cell. A,E-No RB Cabnormalities observed in group-1 and 3.

LPO, is an important one of the mechanisms involved in the production of ROS and determined by MDA measurement. Oxidative stress is an increased MDA level and diminished enzymatic and non-enzymatic activity (Famurewa *et al.*, 2019) [27]. When the imbalance in ROS production occurs, leading to the development of tissue injury. Due to the low concentration of free radical-detoxifying antioxidants like GSH and SOD in cardiac muscle, it is vulnerable to free radical damage (Elghareeb *et al.*, 2021) [28]. Moreover, Cardiac tissue has 150 times less amount of antioxidant compare to other organs like liver (Al-Asmari *et al.*, 2016) [29]. In our present experimental study, significant (P<0.05) increase in highly reactive MDA levels and a substantial decrease in GSH and SOD in 5-FU treated group compared with the control group on the 14th and 28th day of the experimental study which might be due to increased in oxidative stress caused by ROS production (Al-Hamdany and Al-Hubaity, 2014) [30]. While NG treated group 4 exhibits a significant decrease in TBARS concentration and a significant increase in GSH and SOD concentration, due to an antioxidant property by having free radicle scavenging property by increasing synthesis of endogenous glutathione synthesis (Rajadurai and Prince, 2006) [12]. The levels of these values in Group -3 (NG) treated were unaltered, indicating compound safety (Figure. 2).



Values are Mean ± SE (n=6) on day 14th and 28th; One-way ANOVA.
Means with different superscripts in a column differ significantly at p<0.05 (*).

Fig 2: Effect of NG on oxidative stress parameters

Conclusion

The results of present study provides more information on the oxidative stress caused by 5-FU–induced cardiotoxicity and NG verifies the antioxidant levels along with mitigation action of RBC morphology. As a result, NG eliminates oxidative stress due to anti-oxidant and anti-inflammatory properties. So, we conclude that NG treatment can used clinically by following confirmatory experimental studies at clinical levels.

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