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# *In-vivo* Anti-Cancer activity of Polysaccharide Conjugate on DLA Cell lines

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**KEYWORDS**

Anti-Cancer activity, 5 FU, Chitosan, Conjugate, DLA

**ABSTRACT:**

Cancer is a complex disease with various forms that affect individuals globally. Projections for 2040 suggest a concerning surge, with the global burden of cancer expected to reach 28.4 million cases, representing a substantial 47% increase compared to 2020. The main limitations to the use of 5-FU in cancer therapy are based on its high water content and low oral bioavailability. The conjugation with chitosan can overcome the limitations of the pure 5-FU, which include insufficient bio distribution and less systemic circulation.Therefore, the current research aimed to assess the in-vivo anti-cancer efficacy of the 5-Fluorouracil conjugate. When compare to the cancer control, the results showed a significant decrease WBC and an increase in RBC count and haemoglobin levels after treatment with the conjugate.Moreover, treatment with the 5-FU combination resulted in a significant increase in the levels of anti-oxidant parameters like glutathione, nitric oxide, catalase, and superoxide dismutase.

## 1. Introduction

Cancer is a complex disease with various forms that affect individuals globally. Understanding the epidemiology and occurrence rates of different types of cancer is crucial for effective prevention, diagnosis, and treatment strategies. Greenlee et al. [1] provided comprehensive statistics on cancer frequency, incidence, mortality, and survival rates in the year 2000, highlighting the prevalence of cancers such as rectal, breast, lung, prostate, and colonic neoplasms in the United States population.

Different types of cancer exhibit varying patterns and characteristics. Yang et al. [2] identified lung cancer, liver cancer, stomach cancer, leukemia, and esophageal cancer as among the top five types of cancer. In a study focusing on cancer patients in Minia Governorate, Egypt, childhood tumors were predominantly leukemia and CNS tumors[3], aligning with global trends where leukemia and CNS tumors are common among children.

Endometrial cancer, a significant malignancy in women, is traditionally classified into type 1 and type 2[4]. Type 1 endometrial cancer is estrogen-dependent with a favorable prognosis, while type 2 is estrogen-independent and associated with poorer outcomes[5]. Factors like body mass index (BMI) have been linked to endometrial cancer stage, with lower BMI associated with advanced stages[6].

Gastric cancer can be categorized into intestinal and diffuse types based on histology[7], with these subtypes differing in microscopic features, pathogenesis, and prognosis. Additionally, the incidence of type I endometrial cancer has been correlated with obesity-related parameters such as weight, BMI, and cholesterol levels[8].

Breast cancer, a common cancer among women, is influenced by various factors. Studies have explored the association between blood types and breast cancer, with no specific blood group consistently linked to the disease[9]. Luminal-type breast cancer has been shown to have a better prognosis compared to other subtypes, with different Ki-67 expression levels impacting relapse risk and survival[10].

In conclusion, cancer comprises a diverse group of diseases with distinct characteristics and epidemiological patterns. Understanding the types of cancer, their occurrence rates, and associated risk

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factors is essential for developing targeted interventions and improving patient outcomes.

5-Fluorouracil (5FU) has been a cornerstone in cancer treatment for several decades. It exerts its cytotoxic effects through various mechanisms, including inhibiting thymidylate synthase, incorporating fluorinated nucleotides into DNA and RNA, and disrupting cellular processes (Longley et al., 2003; Murray et al., 2015). The understanding of 5FU's mechanisms has led to the development of strategies to enhance its anticancer activity (Longley et al., 2003). The drug has been widely used in the treatment of various malignancies, such as colorectal cancer and head and neck squamous cell carcinomas (Avallone et al., 2007). Despite the introduction of new agents and treatment strategies, 5FU remains a key component in many chemotherapy regimens (Gennaro et al., 2009).

The introduction of oral fluoropyrimidines like capecitabine as substitutes for infusional 5FU has gained attention, offering alternative administration routes with similar efficacy (Patiyil&Alberts, 2006; Altieri et al., 2017). Additionally, the combination of 5FU with other agents like oxaliplatin and leucovorin has shown synergistic effects in treating colorectal cancer (Emi et al., 2010; Avallone et al., 2007). The benefits of 5FU-based treatments have been demonstrated in various clinical trials, showing survival benefits and comparable outcomes to other adjuvant regimens (Figer et al., 2011; Cascinu et al., 2003).

Resistance to 5FU remains a challenge in cancer treatment, emphasizing the importance of understanding the determinants of resistance to optimize drug administration and develop novel treatment approaches (Tan et al., 2011). Research continues to explore new avenues for enhancing 5FU's effectiveness, such as combining it with other chemotherapeutic agents or targeted biotherapies (Avallone et al., 2007; Cacheux et al., 2011). The study of 5FU conversion pathways and mutations associated with chemoresistance provides valuable insights for improving treatment outcomes (Biagioni et al., 2020).

In conclusion, 5-Fluorouracil remains a vital component in cancer therapy, and ongoing research aims to optimize its use, overcome resistance, and enhance its efficacy through innovative strategies and combinations with other agents.

## 2. Materials & Methods

**Animals used:**

The Acharya & BM Reddy College of Pharmacy's institutional Animal Ethical Committee (IAEC) approved all animal experiments, which were carried out in compliance with the guidelines established by CPCSEA, New Delhi, India, and were referenced IAEC/ABMRCP/2018-2019/24.

**Study of Acute toxicity:**

The conjugate's acute toxicity investigation was conducted using the up-and-down methodology described in OECD standards 425. The study commenced at a dosage of 175mg/kg, subsequently increased to 550mg/kg, and concluded with administration of 2000mg/kg orally, adhering to the limit dose of 2000mg/kg.

A healthy set of female Swiss albino mice weighing 25 ± 3 g was obtained from of the Acharya & BM Reddy College of Pharmacy in Bengaluru. The mice were kept in regular environmental conditions in polypropylene cages with a standard feed and water provided in accordance with procedure. Committee for Control and Supervision of Experiments on Animals (CCSEA) established criteria for using and caring for laboratory animals, which were followed by animal maintenance.

**Daltons Lymphoma Ascites model**

**Cancer cell count and Induction of cancer**

The donor mice received 1ml of normal cell line intraperitonially,without delay from the peritoneal cavity collected 1 ml of ascites fluid and make up 10 ml with normal saline. 10 microliters (µL) of ascites fluid was spotted on modified Neubauer’s slide, and the amount of cells looked at in the chamber were calculated. To each mouse at the concentrations of $1\times10^6$ cells were injected intraperitoneally.

**Calculation**

The WBC chamber (A) has an area of 1 square millimetre, or 0.04 mm.

One square of the WBC chamber (D) has a depth of 0.1 mm.

The WBC chamber (V) has an area of 0.04 mm3 times a depth of 0.1 mm, or 0.004 mm3.

4 squares of the WBC chamber's total volume equals 4 x 0.004 mm3, or 0.016 mm3.

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The entire volume of WBC chambers consists four squares divided by the total number of cancer cells present plus the dilution correction factor yields the total number of cancer cells that will be induced.
Factor of dilution adjustment = 10.
There are 400 cancer cells altogether in the four squares of the WBC chamber.
Four chambers have a total volume of 0.4 mm3, or 400 cancer cells in 0.0004 ml.
The number of cancer cells in one millilitre (ml) is 400 × 10 = 0.0004 = $1 \times 10^7$ cells.
A volume of 0.1 millilitres contains 107 × 0.1 = $1 \times 10^6$ cancer cells.
Then, 0.1 ml of the aforementioned volume was given by intraperitoneal injection to each mouse.

**Transplantation of cancer cells**

Dalton's Lymphoma ascites (DLA) were purchased from the Amla Research Centre in Trissur.The DLA in vivo anti-cancer model was established through intraperitoneal transfer of $1\times10^6$DLA suspended in phosphate buffer in Swiss albino mice for 10 days. Ascitic fluid obtained from mice bearing DLA was administered to each experimental animal intraperitoneally at a volume of 0.1 ml, containing $1\times10^6$ cells of cancer cell suspension. The mice were divided into three groups, each comprising twelve animals.

**Treatment schedule**

Dalton's Lymphoma ascites (DLA) bearing cancer mice have been divided into three groups; each group consists of 12 animals, provided with food and water ad libitum.It was thought that Group I was the DLA control. 24 hours after the DLA transplant, group II received an oral dose of 5-FU conjugate (200 mg/kg), and group III received an oral dose of 5FU (20 mg/kg) for nine consecutive days. The animals were fasted for six hours following the final day of therapy in order to determine haematological parameters. Six mice in each group had their blood drawn using the retro-orbital plexus procedure in order to assess the 5-FU conjugate's in vivo anticancer efficacy. The average lifetime, percentage improvement in lifespan (%ILS), and body weight analysis were computed for the mice that survived.[24, 25, 26].

**Calculating the haematological parameters**

To ascertain the anti-cancer efficacy of 5 Fluorouracil novel Conjugate on haematological parameters in mice bearing Dalton's Lymphoma ascites, a evaluation was made between Group I (Cancer control), Group II (5 Fluorouracil Conjugate/Test), and Group III (5 Fluorouracil /standard). Blood was collected from each mouse using the retro-orbital plexus technique and collected into EDTA vacuum tubes for evaluation of hematological parameters. Red blood cells, white blood cells, and haemoglobin were counted in the blood samples using the animal blood counter.[27,28.]

**Analytical Statistics:**

The means ± standard errors of the means were used to express all values. Dunnett's test was used to monitor the analysis of variance for all the data, and a difference that was deemed statistically significant was defined as $P<0.05$.

**Estimation by using Tissue Homogenate:**

The liver was taken out of the animal after it was sacrificed, cleaned in cold salted water, let to cool, and then dried on paper. To create a 10% homogenate, 1 gram of tissue was combined by 10 ml of ice-cold PBS (pH 7) while spinning at Four thousand rpm. The integration process happens as fast as it can. After that, the mixture was centrifuged for 15 minutes at -4°C and 4000 rpm. Utilizing an Cary 60 UV-Vis spectrometer, the specialized instrument control program of ABMRCP was applied.

**1. Superoxide dismutase (SOD) estimation:** The anti-oxidative enzyme superoxide dismutase (SOD) is part of the defence mechanism against highly reactive oxygen species (ROS), which are produced when SOD converts xanthine and oxygen to uric acid and H2O2. Additionally, it catalyses the conversion of the superoxide radical (O2-) into molecular oxygen (O2) and hydrogen peroxide ($H_2O_2$), which serves as a crucial barrier against oxidative damage.In this assay, Superoxide dismutase functions by scavenging superoxide anions, which consequently reduces the rate of formazan dye formation. The presence of superoxide anions (ROS) leads to the reduction of a tetrazolium salt (NBT) to form a blue-coloredformazon product (NBT-disformazan) that absorbs light. This reduction reaction occurs in the presence of Phenazinemethosulphate and

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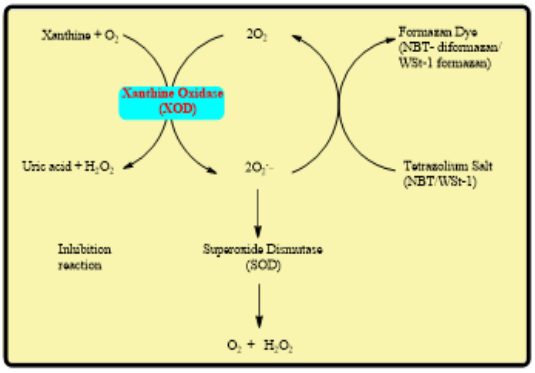
NADH (figure). The absorbance is measured at 560 nm.[30-32]




**Procedure:**

The method for assessing superoxide dismutase activity was developed by Kakkar et al. in 1984. During the procedure, 1.2 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3) and 0.1 ml of previously buffered medium were added. Additionally, 0.2 millilitres of 780 micrometers NADH solution, 0.1 ml of 186 µM phenazoniummethosulphate, and 0.3 millilitres of 300 micrometersnitrobluetetrazolium were added. To stop the reaction, at 30°C the resultant reaction combination was incubated for 90 seconds. Afterward, 4 ml of n-butanol and 0.1 ml of glacial acetic acid were added to the mixture, followed by centrifugation for 10 minutes at Four thousand rpm and 4°C. The organic portion of the layer was then analysed at 560 nm using the blank as a reference. The quantity of enzyme required to reduce 50% of the chromogen synthesis absorbance in the control sample under test conditions is defined as one unit of enzyme activity.[33]

$$\text{SOD (1U/mg)} = \frac{\text{Blank Abs. - Sample Abs)}}{\text{blank Abs/2}} \text{ x Total protein}$$

**2. Reduced glutathione (GSH) estimation:**

Most cells synthesise glutathione, also known as a-glutamylcysteinyl glycine, a non-protein sulfhydryl found in aerobic species. γ-glutamylcysteinesynthetase catalyses the ATP-dependent condensation of glutamic acid and cysteine to generate the ubiquitous tripeptide. Glutathione synthetase then adds glycine to create GSH. In this assay procedure, 5-thio-2-nitrobenzoic acid (TNB), which is yellow in colour and absorbs light at 412 nm, is created when the sulfhydryl group of glutathione (GSH) interacts with 5.5-dithiobis (2-nitrobenzoic acid) (DTNB/Ellmans reagent).The intensity of TNB's color is directly proportional to the quantity of glutathione present in the sample.[34]

The estimation of reduced glutathione was conducted using the method developed by Ellman in 1959.37 To begin, the supernatant 0.5 ml was combined with 1.5 ml of 0.2 M Tris buffer (pH 8) and 0.1 ml of 0.01 M DTNB. The combination was then raised to 5 ml using 2% SDS solution. In a similar way, blank reagent free of sample supernatant was generated. At 412 nm, the clear supernatant of the mixture's UV light absorption was measured.[35]

$$\text{GSH (mM)/g} = \frac{\text{Abs. at 412 nm X D.F X 1000}}{\epsilon}$$

In cases when $\epsilon \geq$ Coefficient of extinction = 13600 M-1 cm-1

**3. Catalase (CAT) estimation:**

Catalase is an antioxidant enzyme, found in both non-mammalian aerobic cells and mammalian aerobic cells having a cytochrome system. The hydrogen peroxide (H2O2) get degraded in presence of catalase enzyme into water and oxygen. The activity of this enzyme is quite high in kidney and liver but not in connective tissue. When the hydrogen peroxide (H2O2) get remove from the cell, oxidative damage against cell were prevented. This assay, the absorption were carried out with UV light at 240 nm in test solution containing hydrogen peroxide, determining the enzyme activity in the sample tissue. When the decomposition of enzyme get start the light absorption get decrease.[36-39]

$$2H_2O_2 + \text{catalase} \longrightarrow 2H_2O_2 + O_2$$

**Procedure:**

Aebi et al., 197438, measured the catalase activity.In one test tube, 0.1 ml of tissue homogenate supernatant, 1.9 ml of phosphate buffer (50 mM, pH 7), and 1.0 ml of freshly prepared H2O2 (30 mM) were added. Following the addition of H2O2, the absorbance at 240 nm was monitored and recorded at the 30-second and 3-minute time points. Reaction mixtures in which the same supernatant was not added served as blanks. One unit of enzyme activity is defined as the enzyme concentration required to induce a 50% change in absorbance in the control sample within one minute.[40]

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Formula:

$$\text{CAT (1U/mg)} = \frac{\text{Abs. ( Change in absorbance) x Volume of reaction mixture}}{\text{3.6 x Volume of enzyme added x Total protein}}$$

**4. Nitric Oxide (NO) Estimation:**

Nitric oxide (NO, a short-lived physiological messenger) exhibits characteristic properties in regulating physiological responses such as cell migration, vasodilation, immune response, and apoptosis. Being lipophilic and highly diffusible. The synthesis involves the catalysis of L-arginine, NADPH, and oxygen into NO, L-citrulline, and NADP+. Scavenging activity of nitric oxide is detected using sodium nitroprusside. The test is based on the enzymatic conversion of nitrate to nitrite in the presence of the enzyme nitrate reductase. It is a Griess reaction-based assay involving a two-step diazotization reaction, where acidified nitrite (NO2-) forms a nitrosating agent that reacts with sulfanilic acid, resulting in the formation of the diazonium ion. This ion couples with ethylenediamine to produce a chromophoreazo-derivative (azo dye), which is absorbed at 450 nm.[41, 42]

**Procedure:**

The technique was created by Marcocci et al. in 1994 and was based on the scavenging of nitric oxide. This method involved filling a dry, clean test tube with 1 ml of the incubated solution, 4 ml of the test sample (supernatant), and 1 ml of a 25 mM sodium nitroprusside solution. After thorough shaking of the test tube, 1.5 ml of the Griess reagent was added. Nitric acid was diazotized with sulphanilamide, followed by interaction with naphthylethylenediaminedihydrochloride, resulting in chromophoreproduction. On 540 nm absorbance was promptly measured. Without the test sample control sample was prepared.[43]

**Formula:**

The following formula was used to determine the test sample's percentage inhibition of radicals:

$$\mathbf{A_{control} - A_{sample} \times 100 / A_{control} = NO\ inhibitory\ ratio}$$

Where

Acontrol = In the absence of sample Absorbance of the control

Asample = Absorbance of sample

**Statistical analysis:**

Each data point is presented as Mean ± SEM (n = 8). Software called GraphPad Prism 7 was utilised to evaluate the data.One way ANOVA was used to analyse the parameters, and Dunnett's multiple comparison test was used to determine whether results were significant (P<0.001(***).

**3. Results**

**Determination of hematological parameters**

**Treatment impact on Hemoglobin (Hb%)**

The effects of 5 Fluorouracil novel Conjugate and regular 5 Fluorouracil on haemoglobin were studied in Dalton's Lymphoma ascites induced mice; the results are shown in Figure 1 and Table 1. The Hb level was found to be lower in the cancer control mice (DLA). In comparison to the cancer control group, treatment with 5-FU conjugate and standard 5FU resulted in a substantial rise in 5-FU Conjugate.

**Table 1:Treatment impact on Hgb, RBCs, and WBCs(7th Day)**

| Groups | Hgb (g/dl) | RBC 1x10$^6$ /mm$^3$ | WBC 1x10$^3$/mm$^3$ |
|---|---|---|---|
| **Cancer Control** | 6.67± 1.08 | 2.69 ± 0.88 | 19.87 ± 0.29 |
| **Conjugate (20mg/kg)** | 8.00 ± 0.95* | 4.70 ± 0.26* | 10.90 ± 1.09* |
| **5FU (20 mg/kg)** | 9.53 ± 0.63* | 5.8. ± 0.26* | 13.13 ± 0.54* |

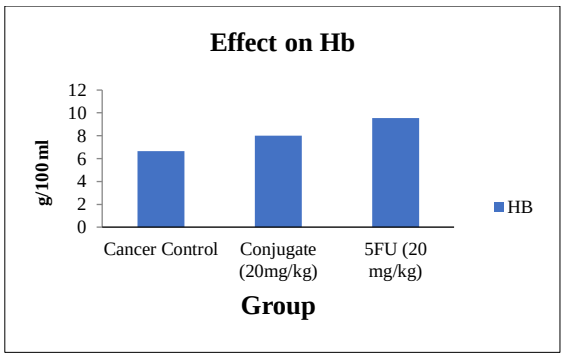


**Figure 1:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

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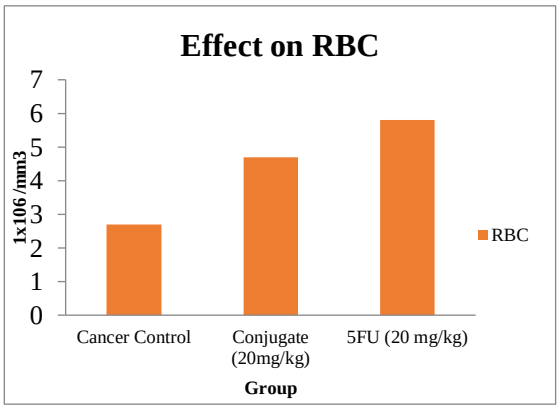


**Figure 2:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

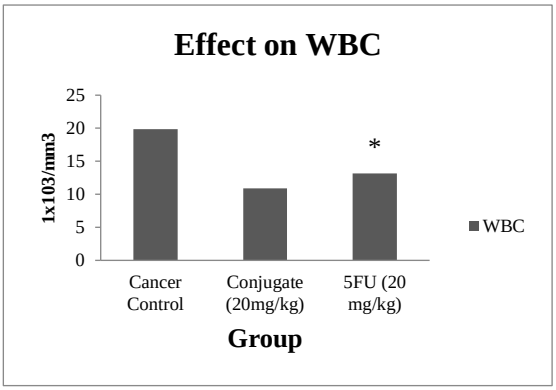


**Figure 3:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

**Table 2:The effect of drugs on Hgb, RBCs, and WBCs (14th Day)**

| Groups | Hgb (g/dl) | RBC 1x10$^6$ /mm$^3$ | WBC 1x10$^3$/mm$^3$ |
|---|---|---|---|
| **Cancer Control** | 3.63± 0.49 | 2.71 ± 0.59 | 34.63 ± 0.42 |
| **Conjugate (20mg/kg)** | 6.57 ± 0.14 ** | 4.70 ± 0.56** | 21.20 ± 1.36 ** |
| **5FU (20 mg/kg)** | 5.60 ± 0.3 ** | 3.07 ± 0.56** | 19.63± 1.25 ** |

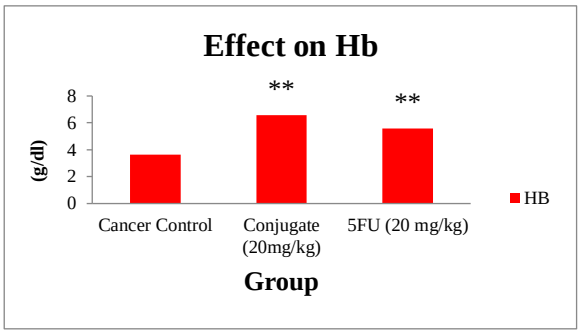


**Figure 4:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed byDunnet test.

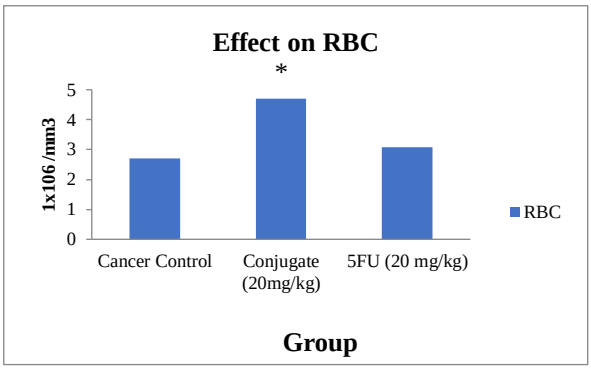


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**Figure 5:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

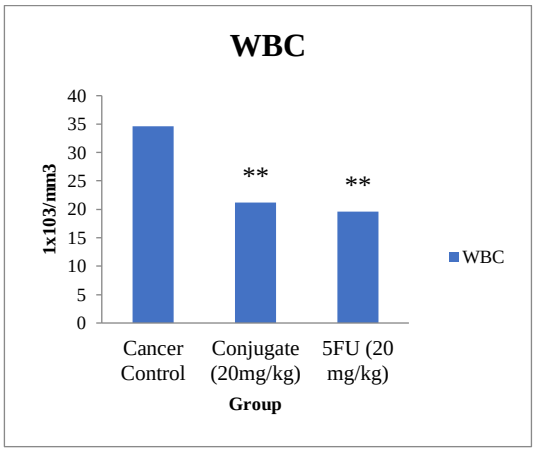


**Figure 6:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where,

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*P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

**Table 3:The effect of drugs on SOD, GSH, Catalase and NO-DLA**

| Groups | SOD (IU/mg protein) | GSH (mM/g) | Catalase (µM/mg protein) | NO (mM/ml) |
|---|---|---|---|---|
| **Cancer Control** | 0.301± 0.01 | 0.399 ± 0.02 | 0.615 ± 0.07 | 0.65 ± 0.04 |
| **Conjugate (20mg/kg)** | 0.794±0.06 ** | 0.653 ± 0.05* | 0.861 ± 0.09* | 0.75 ± 0.02 |
| **5FU (20 mg/kg)** | 0.711±0.02 * | 0.783 ± 0.02* | 0.881± 0.04* | 0.79± 0.09* |

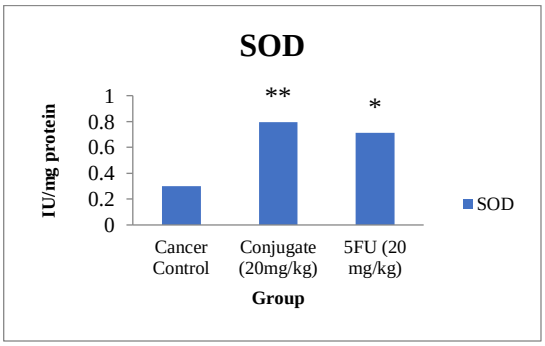


**Figure 7:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

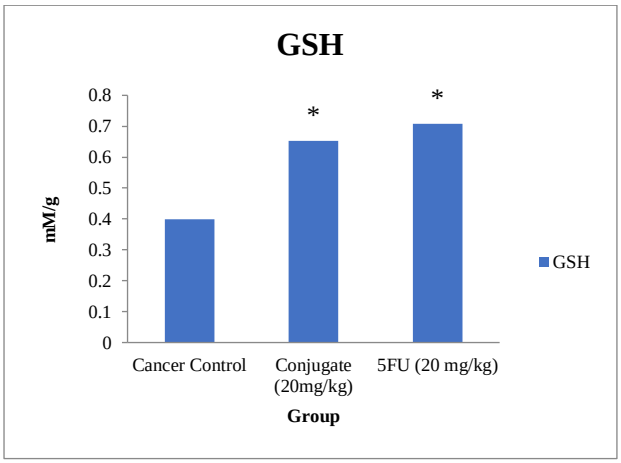


**Figure 8:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

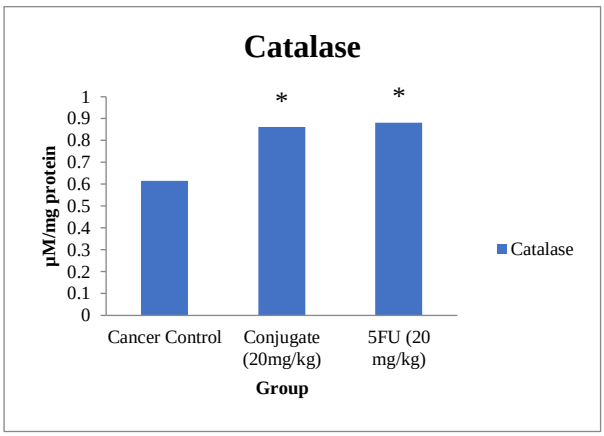


**Figure 9:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

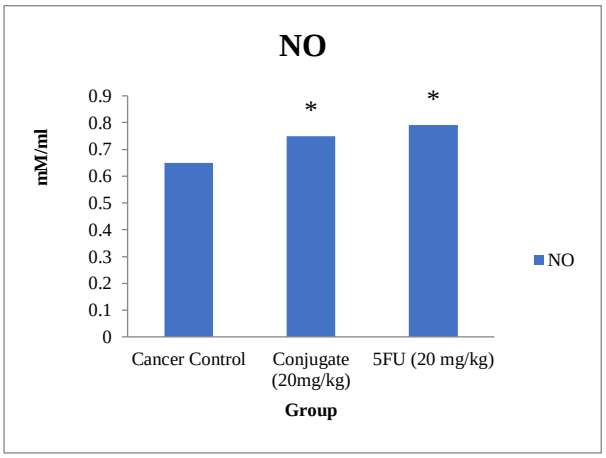


**Figure 10:** Each point represents the mean ± standard error of the mean (n=6 mice per group), Where Where, *P<0.05(*), P<0.01(**), P<0.001(***)*, where taken as significant when compared with control.Data were analyzed by one-way ANOVA followed by Dunnett's test.

## 4. Discussion and Conclusion

Three groups of mice were used, with Group I serving as the DLA control. Group II was administered a 200 mg/kg oral dosage of 5-FU conjugate, whereas Group III was given a 20 mg/kg oral dose of 5FU.

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When measuring the haematological parameters on the seventh day, the DLA control group had a high WBC count and a low RBC count, which suggests the presence of inflammatory mediators in the blood and tumours. Reduced red blood cell count indicates the presence of immature red blood cells in the blood as a result of altered hemolysis brought on by bone marrow rupture. The 5-FU Conjugate group has higher levels of Hb, RBC, and WBC in comparison to the 5-FU group.

On the fourteenth day, in contrast to the five-FU group, the 5-FU Conjugate group progressively raised its Hb, RBC, and WBC levels. This indicates a decline in malignant tumours as evidenced by a reduction in inflammatory radiations.

Antioxidant parameters such as SOD, GSH, Catalase, and NO have been documented, and their fall in the DLA control groups can be attributed to the activity of malignant tumours. The 5-FU conjugate group exhibits higher levels of CAT and SOD, indicating a major impact on the tumours. The number of malignancies is shown by the fall in levels. Therefore, it is evident from the increased levels of SOD, CAT, GSH, and NO that treating malignant tumours with medication is effective.

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**Conflict of interest**

The authors declare no conflict of interest

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