5-Fluorouracil And Curcumin With Pectin Coating As a Treatment Regimen For Titanium Dioxide Induced Colon Cancer Model

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Research Article

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Abstract

Objective: Induction of colorectal cancer in Wister rats using titanium dioxide and dimethylhydrazine and treatment using the physical conjugate of 5-Fluorouracil and Curcumin, with a synergistic approach.

Methods: Compatibility studies are evaluated by using FT-IR, Vero cell lines, and HCT-116 cell lines are used for evaluating the synergistic approach. This followed by induction using titanium dioxide and dimethylhydrazine in Wister rats and treatment using 5-Fluorouracil and Curcumin with pectin coating.

Result: The samples were found to be compatible. The synergistic effect was obtained at 1:1, 1:2, 1:4, and 2:1 ratio, where 1:4 ratio shows a CI_{50} value of 0.896, selected further for the animal studies when studied in HCT 116 cell lines and found to be safe with Vero cell lines. Colorectal cancer was shown to be induced within 70 days of administration of titanium dioxide and dimethylhydrazine. 1:4 ratio of 5-Fluorouracil and Curcumin (50:200) shows effective for the treatment of colorectal cancer within 28 days, proven using histopathology report, bodyweight analysis, and hematological reports.

Conclusion: 5-Fluorouracil and curcumin (1:4) ratio works with a synergistic approach and was proven effective for the treatment of colorectal cancer induced by the titanium dioxide and dimethylhydrazine.

1. Introduction

According to the International Agency for Cancer Research (IARC), colorectal cancer is the third most common cancer type worldwide during 2020, with nearly 2 million new cases [Cao et al. 2021]. In 2013, colorectal cancer killed nearly one million people worldwide, making it the second leading cause of cancer-related death. Although the cause is unknown, changes in eating habits can prevent 70% of sporadic cases of colorectal cancer. Recently studies reported that there is a correlation between food-grade titanium dioxide (TiO_2) with colorectal cancer. Because nanoparticles (NPs) such as food-grade TiO_2 have been shown in experimental models to have negative effects, there has been an increase in concern about the carcinogenic activity on human consumption of nanoparticle-containing foods. TiO_2 , a whitening agent also known as E171, can enter the human digestive system and bloodstream when consumed orally, according to new research [Bischoff et al. 2021].

In one study, human volunteers were given TiO_2 at a dose of 5 mg/kg. E171 has a daily intake of 1 mg/kg body weight for adults and 2 mg/kg body weight for children due to its widespread use in candies [Losso 2021]. Furthermore, the average adult in the UK consumes 5.4 mg of TiO_2 per day. As a result, numerous studies have been conducted to investigate the effects of taking TiO_2 orally. After a five-day dose of 8.6 mg/kg body weight, TiO_2 was found to reach the spleen, liver, and mesenteric lymph nodes, but tissue absorption was very low [Cao et al. 2020]. Rats with the adrenal gland developed necrotic cells in the cortex and pyknotic nuclei after being given 2 mg/kg body weight orally for five days. The amount of black pigment in both healthy and ulcerative colitis children's Peyer's patches increased with age, and it was discovered in both cases to be food-grade TiO_2 . Similar deposits, which the researchers believe to be

food-grade TiO_2 , were discovered in colon cancer, Crohn's disease, and Crohn's colitis without the disease [Cvetković et al. 2020, Brand et al. 2020]. TiO_2 was found in lymphoid tissue of patients with Crohn's disease, ulcerative colitis, and colonic carcinoma. In our research work we induced colorectal cancer in Wistar rats using TiO_2 -E171(with water) along with 1,2 Dimethyl Hydrazine (DMH) [Dudhipala et al. 2018] followed by its treatment using 5-Fluorouracil (5-FU), curcumin (CUR), and a combination of 5-FU and CUR [Anirudhan et al. 2017; Kumar et al. 2020; He et al. 2019].

Colorectal cancer is commonly treated with 5-fluorouracil (5-FU), a cancer drug that is chemically similar to uracil and thymine and used as a first-line treatment regimen [Crowder et al. 2018; Vodenkova et al, 2020; Jung et al, 2020; Sharma et al. 2019]. 5-FU is the first line regimen used for the treatment of colorectal cancer, more than 80% of it is catabolized by hepatic dihydropyrimidine dehydrogenase (DPD), while the remainder causes cell death by inhibiting RNA and DNA syntheses via fluorodeoxyuridine monophosphate (FdUMP) and fluouridine triphosphate (FdUMP) (FUTP). The most serious side effects of 5-FU are myelosuppression, diarrhea, cardiotoxicity, dermatitis, and mucositis and 5-FU is also possessing multidrug resistance (MDR). Hence, for overcoming this resistance part, the use of a natural chemosensitizer and chemotherapeutic agent- Curcumin is used. The combination of 5-FU and Curcumin can produce a synergistic effect and hence can overcome the MDR issues also. The use of this combination approach can reduce the dose of 5-FU and also the toxic effects. Since the absorption of curcumin takes place in the colorectal region and the safety profile of curcumin even at a high dose, this combination approach was taken for further studies.

Gingival mucositis developed in approximately 80% of cancer patients who received 5-FU treatment [Bachmeier et al. 2019]. Anticancer chemotherapeutics that targets the small intestine have been linked to gingival mucositis. Mucositis is a painful condition that can last months or years and has a negative impact on cancer patients' quality of life [Crowder et al. 2018]. It also increases their chances of developing hematemesis, which can lead to neutropenia and malnutrition. As a result of this link, mucositis is now recognized as a clinically significant disease. It also makes effective chemotherapy and radiotherapy impossible, putting a stumbling block in the fight against cancer. The anti-inflammatory drug 5-FU shortens gut villi, deepens the crypt, raises the apoptosis index, increases myeloperoxidase (MPO) activity, and lowers glutathione (GSH) levels in rats [Fonseca et al. 2021].

Cancer drugs cause apoptosis in both cancerous and healthy cells. Apoptosis is caused by cancer drugs in healthy intestinal cells, which can lead to complications such as mucositis [Fonseca et al. 2021]. The small intestine's mucosal replacement cycle is only 3–4 days long, making it especially vulnerable to anticancer drugs. GI mucositis can have a negative long-term prognosis because it increases the need for intravenous nutrition, decreases nutritional intake, and increases the risk of infection throughout the body. In Rat, this compound has been shown to inhibit skin, stomach, colon, and liver cancers. Curcumin, according to Goel et al., does not inhibit the expression of COX-1 mRNA or protein. Curcumin has been shown in humans to be a safe and effective colorectal cancer chemopreventive agent [Fonseca et al. 2020]. It has already been used in preclinical trials as a chemopreventive treatment, and the fact that these were successful suggests that curcumin's use will increase in the future. 5-FU and CUR was coated

with pectin for the specific delivery of the drug to the colorectal region since the pectin act as a pH sensitive polymer and helps in targeted drug release [Du et al. 2020]. With the coating using pectin the codelivery can be possible through oral route which is more convenient than injections. Because of its widespread use and numerous benefits as a chemopreventive agent for colon cancer, we hypothesize that 5-FU in combination with Curcumin can be effective for the treatment of colorectal cancer induced by chemical agents such as TiO_2 and DMH.

2. Materials And Methods

2.1. Materials used

TiO_2 was obtained as a gift sample from Bimal Pharma PVT, LTD Mumbai, DMH was purchased from Sigma-Aldrich, PVT, LTD, 5-FU and pectin was obtained from Avra synthesis PVT, LTD, Hyderabad, and Curcumin obtained as a gift sample from Himalaya PVT, LTD, Bangalore. All the samples were undergone with purity and confirmation before the initiation of the experiment.

2.2. Compatibility studies using FT-IR

During the combination, the compatibility of the ingredients used in the formulation is tested at the molecular level. Researchers used Fourier-transform infrared spectroscopy to learn more about molecular and chemical compatibility (FT-IR).

FT-IR (FT-IR Spectrometer, Shimadzu 8400-S, Japan) and potassium bromide (KBr) pellet method were used to investigate the interaction of 5-FU and CUR. In a 1:4 ratio, a physical mixture of 5-FU and CUR (1:1) was mixed with anhydrous KBr. The translucent pellet was created by compressing 100 mg of the finely powdered mixture using a hydraulic press at 15 tons of pressure. Individual pellets were scanned at a rate of 4 mm/s with a resolution of 2 cm. Any peak's presence or absence in the physical mixture was compared to the presence or absence of any peak in the pure drugs [Fonseca et al. 2019; Estácio et al. 2019].

2.3. *In Vitro* studies-synergistic activity in HCT 116 cell lines

The MTT assay was used to assess the cell toxicity of various formulations in Vero and HCT 116 cells. Cells were seeded at a density of 1×10^4 cells per well in 96-well culture plates and incubated for 24 hours. After that, the cells were reseeded with 5-FU, CUR, 5-FU: CUR (1:1, 1:2, 1:3, 1:4, 2:1, 3:1, & 3:2) at various ratios for 48 hours at 37°C and 5% CO_2 . As a starting point, the medium of culture was used. The cells were then incubated for 4 hours in each well in MTT solution (5 mg/mL). Following the removal of the unreduced MTT and medium, each well-received 200 μL of dimethyl sulfoxide (DMSO) to dissolve the MTT formazan crystals. A microplate reader was used to measure the absorbance (A) of the formazan product at 570 nm (Model 680, Bio-Rad, Hercules, CA, USA).

The half-maximum inhibitory concentration (IC_{50}) of samples was determined. To investigate the synergy between 5-FU and curcumin in the combination system, the combination index (CI) was calculated. The

following equation 1 was used to calculate CI:

$$CI_{50} = (D)_1 / (D_{50})_1 + D(2) / (D_{50})_2(1)$$

where CI_{50} is the combination index calculated using the IC_{50} values of the drugs, $(D)_1$ and $(D)_2$ are the concentrations of 5-FU and CUR in the combination system at the IC_{50} value, respectively, and $(D_{50})_1$ and $(D_{50})_2$ are the IC_{50} values of 5-FU and CUR alone, respectively. A CI_{50} of 1 indicates synergism, while a CI_{50} of >1 indicates antagonism [Acharya et al, 2021; Jiang et al. 2018; Hamdan et al. 2021].

2.4. Animals used

For this study, the JSS College of Pharmacy, Ooty, India provided 32 white 6-week-old Wister male rats. After one week of acclimation, the rat was divided into four groups of six rats each. All the procedures are followed by using the IAEC guidelines (JSSCP/OT/IAEC/36/2018-2019).

2.5. Preparation of E171

Solution of TiO_2 nanoparticles dispersion was prepared by ultra-sonification (Bandelin RK 100 H, Germany) method for 30 min in 1ml of distilled water (pH 7.0). The stock solution is prepared to contain a 1.0 mg/ml concentration. Before the administration of the injection, the solution was sonicated for 15 min once again for removing the agglomeration. The solution was further carried out for the zeta potential and particle size analysis [Hamdan et al, 2021; Vigneshwaran et al, 2021].

2.6. Particle size and Zeta potential

The mean particle/globule size and zeta potential (ZP) of TiO_2 nanoparticles were measured using a zetasizer ZS 90. (Malvern Instruments, UK). A photon correlation spectroscopy technique was used to determine the mean particle/globule size, which analyses fluctuations in dynamic light scattering caused by Brownian particle motion. The mean diameter of 10 mm diameter cells was measured at a 90° angle at $25^\circ C$. Because it reflects the electric charge on the particle surface, the ZP is an excellent tool for determining colloidal system physical stability. At $25^\circ C$, all size and ZP measurements were taken with disposable polystyrene cells and disposable plain folded capillary zeta cells diluted in the original dispersion medium [Ukalska-Jaruga. 2018; Je HJ et al.2017; Blevins et al. 2019].

2.7. Induction of colorectal cancer in animals

The Wister rats were administered with 5mg/kg body weight of TiO_2 for 5 days per week and 1mg/kg bodyweight of DMH 1 day per week for 70 days, bodyweight, hematological parameters, and histopathology reports were considered [Litschauer et al. 2020]. The bodyweight of the animals was analyzed on weekly basis. The pictorial representation of the induction procedure is given in figure 1.

2.8. Histopathology report

Before being processed for histopathology, each colon tissue sample was immersed in a 10% formaldehyde solution. The tissues were dipped in molten liquid paraffin before being solidified into

blocks, making slicing and staining much easier. Tissue-paraffin blocks were cut into 6 m thick sections using a rotary microtome (Leica, UK; Model No. RM2135). Tissue slices were stained with hematoxylin and eosin after being mounted on staining stands. Pathological examination of tissue slices was carried out using a digital microscope [Cisne et al. 2018].

2.9. Treatment

After the completion of 70 days, the treatment was started. Group one is taken as a control and administered with normal saline [Wolf et al. 2018]. In the second group, 5-FU with pectin coating was administered, the third group with CUR with pectin coating and the fourth group with the combination of 5-FU and CUR with pectin coating. The procedure for the treatment group is given in figure 2.

The experimental groups received oral dosage of group 1 with PBS, group 2 with 50 mg/kg 5-FU with pectin coating, group 3 with 200 mg/kg of CUR with pectin coating, group 4 with 50 mg/kg of 5-FU, and 200 mg/kg of CUR dissolved with pectin coating. The coated samples are dissolved in PBS for the ease of administration, while the control group, group 1 received phosphate-buffered saline (PBS) solution given through oral route. The Wister rats were in a room with a $24\pm 2^{\circ}\text{C}$ temperature, $60\pm 5\%$ relative humidity, and a 12-hour dark cycle. Beginning on the day of 5-FU administration, all Rat's weight and was recorded daily [Smith et al. 2020].

Preparation of the coating mixture with pectin: High ester pectin powder (6%w/v) was solubilized in distilled water at $20\text{--}22^{\circ}\text{C}$ and then for 30 minutes stirred mechanically at 500rpm, until full solubility was obtained. The coating was done by mixing 10ml of the pectin solution with 1g of the conjugated powder and stirred for 15 min at 500rpm [Li et al. 2020].

2.10. Hematological Parameters

All the animals were confined to blood collection at the time of sacrifice to measure hematological parameters. Blood was collected via a retro-orbital puncture. The above parameters were investigated to gain a better understanding of the curcumin and the 5-FU combination effect on specific blood components.

1. RBC- Red Blood Cell count
2. WBC- White Blood Cell Count
3. Hemoglobin content
4. Platelet count

After the retro-orbital puncture, blood was collected into pre-coated (Heparin 25 IU/mL) blood collection tubes. The concentrations of these various components were then measured with a cell counter [Avolio et al. 2018].

2.11 Statistical analysis

Graphpad prism 9 software was used for evaluating the statistical significance. An unpaired two-tailed students *t*-test was used for determining the comparison of the two groups statistically, followed by one-way ANOVA with a Bonferroni correction for the multiple comparisons followed with a bartlett's test. All the experiments were conducted at least three times ($n > 3$) (cell lines 3 times and for animal studies $n = 6$) and expressed as mean $\pm$ standard deviation ($\pm$ SD). The significance level was taken to be 95% ($p < 0.05$).

2.12 Ethics

Institutional Animal Ethical Committee (IAEC) approval was obtained from JSS College of Pharmacy, Ooty, TamilNadu, India (JSSCP/OT/IAEC/36/2018-2019). The work is carried out by following the IAEC guideline.

3. Results

3.1. Compatibility studies and purity studies using FT-IR

The 5-FU compatibility with CUR was tested using FTIR at the molecular and chemical levels, and the results are shown in figure 3 **and** Table 1. The FT-IR profile of 5-FU was shown in figure 1 (a), shows that the presence of peak N-H stretching: 3135.09 cm^{-1} , C=O Stretch: 1722.49 cm^{-1} , C-N (stretch): 1653.09 cm^{-1} , C-H (in plane): 1246.06 cm^{-1} , C-O: 1181.44 cm^{-1} , C-F: 1246.06 cm^{-1} . The FT-IR profile of CUR was reported as O-H Stretching: 3409 cm^{-1} , C-H Aromatic stretching: 3043 cm^{-1} , CH_3 asymmetric Stretching 2917 cm^{-1} , CH_2 asymmetric Stretching: 2917 cm^{-1} , $\text{C}\equiv\text{O}$ Stretching: 1627 cm^{-1} , C=C aromatic stretching: 1589 cm^{-1} , Benzene ring: 1516 cm^{-1} , CH_2 bending: 1430 cm^{-1} , CH_3 Bending: 1375 cm^{-1} , C-O Stretching: 1114 cm^{-1} and shown in figure 1 (b). The compatibility studies are carried out at 1:1 ratio, and peaks are depicted as N-H stretching : 3135.09 cm^{-1} , C=O Stretch: 1722.49 cm^{-1} , C-N(stretch): 1653.09 cm^{-1} , C-H (in plane): 1246.06 cm^{-1} , C-O: 1181.44 cm^{-1} , C-F: 1246.06 cm^{-1} O-H Stretching: 3409 cm^{-1} , C-H Aromatic stretching: 3043 cm^{-1} , CH_3 asymmetric Stretching 2917 cm^{-1} , CH_2 asymmetric Stretching: 2917 cm^{-1} , $\text{C}\equiv\text{O}$ Stretching: 1627 cm^{-1} , C=C aromatic stretching: 1589 cm^{-1} , Benzene ring: 1516 cm^{-1} , CH_2 bending: 1430 cm^{-1} , CH_3 Bending: 1375 cm^{-1} , C-O Stretching: 1114 cm^{-1} and shown in figure 1 (C). The physical mixture of all 5-FU and CUR showed no significant peak shift, indicating that the ingredients used for formulation are compatible.

Table 1
FT-IR studies data analysis.

S.NO	Compound	FT-IR data
1	5-Fluorouracil	N-H stretching :3135.09 cm ⁻¹ , C=O Stretch:1722.49 cm ⁻¹ , C-N(stretch):1653.09 cm ⁻¹ , C-H (in plane):1246.06 cm ⁻¹ , C-O:1181.44 cm ⁻¹ , C-F:1246.06 cm ⁻¹
2	Curcumin	O-H Stretching: 3409 cm ⁻¹ , C-H Aromatic stretching: 3043 cm ⁻¹ , CH ₃ asymatric Stretching 2917 cm ⁻¹ , CH ₂ asymmetric Stretching: 2917 cm ⁻¹ , C≡O Stretching: 1627 cm ⁻¹ , C=C aromatic stretching: 1589 cm ⁻¹ , Benzene ring: 1516 cm ⁻¹ , CH ₂ bending: 1430 cm ⁻¹ , CH ₃ Bending: 1375 cm ⁻¹ , C-O Stretching: 1114 cm ⁻¹ .
3	Curcumin and 5-Fluorouracil	N-H stretching :3135.09 cm ⁻¹ , C=O Stretch:1722.49 cm ⁻¹ , C-N(stretch):1653.09 cm ⁻¹ , C-H (in plane):1246.06 cm ⁻¹ , C-O:1181.44 cm ⁻¹ , C-F:1246.06 cm ⁻¹ O-H Stretching: 3409 cm ⁻¹ , C-H Aromatic stretching: 3043 cm ⁻¹ , CH ₃ asymatric Stretching 2917 cm ⁻¹ , CH ₂ asymmetric Stretching: 2917 cm ⁻¹ , C≡O Stretching: 1627 cm ⁻¹ , C=C aromatic stretching: 1589 cm ⁻¹ , Benzene ring: 1516 cm ⁻¹ , CH ₂ bending: 1430 cm ⁻¹ , CH ₃ Bending: 1375 cm ⁻¹ , C-O Stretching: 1114 cm ⁻¹ .

3.2. *In Vitro* studies-synergistic activity in HCT 116 cell lines.

The cytotoxic results were obtained for 5-FU, CUR alone, and also in combination. The ratio taken for the combination was 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, and 3:2 w/w. The cell line studies were conducted in Vero cells (adherent cell line that is cultured under conditions typical for many mammalian cell lines) and HCT-116 cell lines, which were the human colorectal cell lines. The IC₅₀ value of the samples was analyzed and with that CI₅₀ value was calculated using equation 1, where CI₅₀ >1, CI₅₀ =1, and CI₅₀ <1 shows antagonist, additive and synergistic effect. Furthermore, 1:1, 1:2, 1:4 and 2:1 ratio shows CI₅₀ value of 0.987, 0.985, 0.896 and 0.986 respectively. Hence, 1:1, 1:2, 1:4, and 2:1 was found to produce a synergistic activity on HCT-116 cell lines. IC₅₀ value of 5-FU and CUR was given in figure 4 (a) in Vero cell lines and figure 4 (b) HCT-116 cell lines. CI₅₀ value was given in Figure 5. The report of the cell line studies is given in Tables 2 (a) and 2 (b). The cytotoxic effect of the drugs and their combination was found to be safe when given to the normal cell lines. At a concentration of more than 48µg/ml, only the cytotoxic effect was found in the normal cells. Hence, the dose of the drug for producing synergistic activity was found to be safe for the normal cells.

Table 2
(a): In vitro studies using Vero cell lines.

Compound	5-FU ($\mu\text{g/ml}$)	CUR ($\mu\text{g/ml}$)
5-FU alone	72.011	NA
CUR alone	NA	87.85
5-FU:CUR (1:1)	68.25	82.75
5-FU:CUR (1:2)	66.26	72.32
5-FU:CUR (1:3)	64.25	68.25
5-FU:CUR (1:4)	62.48	66.53
5-FU:CUR (2:1)	54.25	68.26
5-FU:CUR (3:1)	50.42	65.42
5-FU:CUR (3:2)	48.26	62.56
All the reports were taken in triplicate n=3		

Table 2
(b): In vitro studies using HCT 116 cell lines.

Compound	5-FU ($\mu\text{g/ml}$)	CUR ($\mu\text{g/ml}$)	CI ₅₀
5-FU alone	45.29	NA	NA
CUR alone	NA	52.35	NA
5-FU:CUR (1:1)	43.24	50.6	0.987
5-FU:CUR (1:2)	39.26	45.83	0.985
5-FU:CUR (1:3)	35.62	41.27	1.00
5-FU:CUR (1:4)	27.32	35.26	0.896
5-FU:CUR (2:1)	25.28	29.65	0.986
5-FU:CUR (3:1)	32.64	33.2	1.136
5-FU:CUR (3:2)	26.26	28.6	1.061
All the reports were taken in triplicate n=3			

3.3. Particle size and Zeta potential

The TiO₂ size distribution showed a maximum peak of 269.7 nm, and a Z potential of -0.0218 V when titration at pH 7 distilled water. The transmittance was found to be 79.8%. The particle size and zeta potential results are given in figures 6 & 7 respectively.

3.4. Bodyweight analysis

From the beginning of the experiment till the sacrifice of the animals, the bodyweight of Wister rats was taken and recorded. Bodyweight was monitored constantly once a week on every seventh day from the induction of TiO₂ and DMH for 10 weeks (70 days). Moreover, the initial and final weights were measured, noted, and compared. The bodyweight of the rats was found to be changed from day 14 due to the induction of colorectal cancer followed by a reduction in the bodyweight, with the start of the treatment. The bodyweight of group 4 was found to reach the normal weight within 28 days, as compared to groups 1, 2, and 3 at the end of the study. The results show that when 5-FU and CUR combination strategy is followed, there show a reduction in weight and coming to the normal weight as compared to when 5-FU and CUR (with pectin coating) are given alone. The results are given in **figure 8** and Table 3.

Table 3
Bodyweight analysis report comparison.

SL. No	Group	Initial body weight (g $\pm$ SD)	Final body weight- After induction (10th week) (g $\pm$ SD)	Final body weight- After treatment (g $\pm$ SD)
1.	Group 1	228 $\pm$ 2.5	314 $\pm$ 3.5	355 $\pm$ 2.4
2.	Group 2	224 $\pm$ 3.6	314 $\pm$ 2.5	249 $\pm$ 2.5
3.	Group 3	235 $\pm$ 1.6	335 $\pm$ 2.8	251 $\pm$ 3.8
4.	Group 4	218 $\pm$ 3.3	315 $\pm$ 3.6	230 $\pm$ 2.5

The data is represented in the mean $\pm$ SD (n=6 per group). The significance value- p-value was found to be p=0.0124, (p<0.05) R² Value was found to be 0.6233. (one-way ANOVA with a Bonferroni correction for multiple groups and two-tailed Student's t-test for individual groups).

3.5. Histopathology report

The colon tissues were subjected to H&E staining for evaluating the tissue characteristics of each group after the induction and treatment. The histopathology reports of group 1, group 2, group 3, and group 4 are given in figure 9 (a, b, c, d). The reports depict that there shows a change in the tissue pattern of the colon cells when TiO₂ and DMH were administered for the induction of cancer. The cancer growth was observed where after the treatment with 5-FU and CUR combination, the architecture of the cells was returned to be normal.

3.6. Hematological Parameters

Hematological parameters were performed and noted to evaluate the changes in the concentration in blood content before and after the administration of the CUR, 5-FU, and its combination in the rats. The results are given in Table 4 and figure 10.

3.6.1. Red blood cells count

In group 1 shows a value of $5.52 \pm 1.20 \times 10^6/\text{mm}^3$ and there was a reduction in the red blood cells count compared with that of the normal value is $8.30 \pm 2.55 \times 10^6/\text{mm}^3$. Where the treatment group with Group 2 (5-FU alone) shows an RBC content of $5.52 \pm 1.20 \times 10^6/\text{mm}^3$. Group 3 (CUR alone) was reported with $6.89 \pm 1.05 \times 10^6/\text{mm}^3$ and Group 4 (5-FU: CUR) with $7.25 \pm 2.15 \times 10^6/\text{mm}^3$. There was a reduction in RBC count in Group 1 compared to that of the normal value and significantly there is an increase in the RBC count, which came to be normal when treated with the combination of 5-FU and CUR.

Table 4
Haematological parameters testing report

SL. No	Group	RBC Content ($10^6/\text{mm}^3 \pm \text{SD}$)	WBC Content ($10^3/\text{mm}^3 \pm \text{SD}$)	Hemoglobin content (g/dL $\pm \text{SD}$)	Platelet content ($10^4/\text{mm}^3 \pm \text{SD}$)
1.	Normal value	8.30 ± 2.55	8.20 ± 2.35	14.35 ± 1.50	25.15 ± 2.90
2.	Group 1	5.52 ± 1.20	17.86 ± 2.35	4.10 ± 1.25	39.42 ± 3.22
3.	Group 2	5.25 ± 1.22	15.25 ± 2.24	8.25 ± 2.16	35.22 ± 1.22
4.	Group 3	6.89 ± 1.05	12.75 ± 1.56	10.25 ± 2.25	32.26 ± 2.22
5.	Group 4	7.25 ± 2.15	10.85 ± 2.15	12.95 ± 1.20	28.42 ± 2.25

The data is represented in the mean $\pm \text{SD}$ (n=6 per group). The significance value- p-value was found to be $p < 0.0001$, (statistically significant) R^2 Value was found to be 0.8851. (one-way ANOVA with a Bonferroni correction for multiple groups and two-tailed Student's t-test for individual groups). Bartlett's statistic correlation was found to be 6.154 with a P-value of 0.1044, there is no SD's significant difference ($P < 0.05$) found.

3.6.2. White blood cells count

The WBC count was found to increase in group 1 ($17.86 \pm 2.35 \times 10^3/\text{mm}^3$) as compared to that of the normal value ($8.20 \pm 2.35 \times 10^3/\text{mm}^3$) and there is a decrease in the WBC count in group 2 ($15.25 \pm 2.24 \times 10^3/\text{mm}^3$), group 3 ($12.75 \pm 1.56 \times 10^3/\text{mm}^3$) and group 4 with ($10.85 \pm 2.15 \times 10^3/\text{mm}^3$). WBC content was found to be increased significantly in-group 1 where the cancer is induced and coming to the normal value in group 4 where the combination strategy is followed.

3.6.3. Hemoglobin count

To understand the role of 5-FU, CUR, and its combination in the level of hemoglobin, hemoglobin content estimation was carried out. Group 1 show a reduced Hb content ($4.10 \pm 1.25 \text{ g/dL}$) as compared to that of the normal value ($14.35 \pm 1.50 \text{ g/dL}$), and found to be increased after treatment in group 2 ($8.25 \pm 2.16 \text{ g/dL}$), group 3 ($10.25 \pm 2.25 \text{ g/dL}$), and group 4 ($12.95 \pm 1.20 \text{ g/dL}$).

3.6.4. Platelet content

The normal value for platelet content was found to be $25.15 \pm 2.90 \times 10^4/\text{mm}^3$, wherein in the case of group 1 it was increased to $39.42 \pm 3.22 \times 10^4/\text{mm}^3$ and decreased with the treatment with CUR and 5-FU, the platelet content of the groups was found in the order group 4 ($35.42 \pm 2.25 \times 10^4/\text{mm}^3$) < group 3 ($32.26 \pm 2.22 \times 10^4/\text{mm}^3$) < group 2 ($28.22 \pm 1.22 \times 10^4/\text{mm}^3$).

4. Discussion

Colorectal cancer is a major concern for people all over the world, regardless of socio-economic status. Despite having lower mortality rates than developed-country populations, this figure is expected to catch up with developed-country populations in the not-too-distant future. A growing body of research into the pathways of colorectal tumour pathogenesis, there emerge to have been no significant advances. For following the administration approach through oral route, 5-FU, CUR and Combination was coated with pectin. The use of pectin to create gastrointestinal-resistant emulsions is a promising strategy for delaying the digestion of tripropionin or other lipid-based materials and delivering tripropionin to the colon. Pectin is a pH sensitive polymer and can help in codelivery of the molecules to the targeted site which is colon.

5-Fluorouracil is the first-line chemotherapeutic agent for the treatment of colorectal cancer. But the combination strategy is mostly used, which is coming up with various side effects. Curcumin is a flavonoid which is used as a chemosensitizer and chemotherapeutic agent and proven with its ability for the treatment of colorectal cancer. The main aim of this study was to prove the efficacy of curcumin and 5-Fluorouracil in combination for the treatment of TiO_2 and DMH-induced colorectal cancer in Wister rats. Compatibility studies are analyzed by using an FT-IR report. Synergistic activity of the 5-Fluorouracil and curcumin was carried out by using MTT assay in HCT 116 cell lines. Followed by the induction of colorectal cancer in Wister rats by the chemical method using TiO_2 and DMH and after the induction of colorectal cancer, the rats are treated by using 5-Fluorouracil alone, curcumin alone, and the combination of both. Weight analysis, histopathology studies, and hematological parameters were reported for both induced and treated groups. The physical mixture of all 5-FU and CUR showed no significant peak shift, indicating that the ingredients used for formulation are compatible. The synergistic activity of the 5-Fluorouracil and Curcumin is found in the ratio of 1:1, 1:2, 1:4, and 2:1 in HCT-116 cell lines. In the ratio of 1:4 the Cl_{50} value was found to be 0.896. Induction of colon cancer was reported when using TiO_2 and DMH was given and proven through Weight analysis, histopathology studies, and hematological parameters. When the rats are given the treatment, the combination works better than the individual groups given alone. 5-Fluorouracil (50mg/kg) and Curcumin (200 mg/kg) was induced as a treatment regimen in colorectal cancer induced Wister rats and found to have a better result when 5-Fluorouracil (50mg/kg) and curcumin (200 mg/kg) was given. The combination strategy of 5-Fluorouracil and curcumin works effectively for the treatment of colorectal cancer induced in Wister rats using TiO_2 and DMH.

Histopathology reports show that, in group 1, cancer growth is observed which was substantially reduced when treated by using a combination strategy. The weight analysis also shows with the same report. The weight of the animals comes to be normal when treated with 5-FU and CUR combination. Hematological parameter's analysis also depicts that the combination works better when compared to the drugs used alone, where the blood contents were found to be normal value. In conclusion, the combination of 5-FU and CUR in the ratio of 1:1, 1:2, 1:4, and 2:1 produce a synergistic effect in HCT-116 cell lines. Among which, the 1:4 ratio was further carried out for the *in vivo* studies by inducing colorectal cancer using chemicals TiO₂ and DMH.

Conclusion

Food-grade titanium dioxide was proven as the cause of colorectal cancer in human beings. This chemical moiety is present in almost all coloured foodstuffs (like candies). Hence, the risk of colorectal cancer in humans is increasing day by day. The laboratory animals such as rats were administered with titanium dioxide and dimethyl hydrazine and found with the colorectal cancer induction within 70 days. Then, the treatment of these animals was carried out by using a physical conjugation of 5-FU and CUR. Which was found to be effective when taken in a 1:4 ratio. They produce a synergistic property and are found as an effective treatment regimen in Wister rats which was proven using weight variation, histopathology report, and also hematological parameter's evaluation.

Declarations

Author Contributions: CK: conceptualization, laboratory work, writing, original draft preparation; NDP: laboratory work investigation, RSK.: supervision, project administration, writing, Md. Habibur Rahman and Gulam Md Ashraf- manuscript review and editing.

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Figures

Figure 1

Induction procedure of colorectal cancer using TiO₂ and DMH.

Figure 2

Treatment procedure for groups 1 to 4. Group 1 was administered with PBS solution, group 2 with 5-FU coated with pectin, group 3 with Curcumin coated with pectin, group 4 with the physical mixture of 5-FU and CUR (1:4) coated with pectin. Six animals are taken in a group.

Figure 3

(a): FT-IR report of 5-FU (b): FT-IR report of Curcumin (C): Compatibility studies of 5-FU and CUR using FTIR

Figure 4

(a): IC 50 value of 5-FU and CUR in Vero cell lines (b): IC 50 value of 5-FU and CUR in HCT 116 cell lines

Figure 5

CI 50 value

Figure 6

Particle size report

Figure 7

Zeta potential report.

Figure 8

Bodyweight analysis report weekly. The data is represented in the mean $\pm$ SD (n=6 per group). The significance value- p-value was found to be $p=0.0124$, ($p<0.05$) R² Value was found to be 0.6233. (one-way ANOVA with a Bonferroni correction for multiple groups and two-tailed Student's t-test for individual groups).

Figure 9

(a)- Histopathology report group 1- induction of cancer with TiO₂ and DMH. (b)- Histopathology report group 2 with the treatment of 5-FU alone. (C): Histopathology report group 3 with the treatment of CUR alone. (d): Histopathology report of group 4 with the combination of 5FU and CUR.

Figure 10

Haematological parameters test report. The data is represented in the mean $\pm$ SD (n=6 per group). The significance value- p-value was found to be $p<0.0001$, (statistically significant) R² Value was found to be 0.8851. (one-way ANOVA with a Bonferroni correction for multiple groups and two-tailed Student's t-test for individual groups). Bartlett's statistic correlation was found to be 6.154 with a P-value of 0.1044, there is no SD's significant difference ($P<0.05$) found.