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Nano encapsulated polymeric Scopoletin suppresses the progression of colorectal cancer by regulating cytokines and inflammatory mediators in AOM/DSS murine model

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ABSTRACT

Scopoletin (6-methoxy-7-hydroxycoumarin) belongs to the family of coumarins with numerous pharmacological benefits. The present study deals with examining the efficacy of Nanoencapsulated polymeric Scopoletin (NEP-Sc) in murine colon cancer model. Male Balb/c mice were supplemented with NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-fluorouracil (25 mg/kg b.w.) for 10 days consecutively post-induction of colon cancer. Colon polyps and morphology were assessed using a macroscopical inspection, and their score establishes the ameliorative effect of NEP-Sc. Body weight, diarrhoea score, and spleen weight were also measured. The antioxidant status of the mucosal levels of glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (LPO) and nitric oxide (NO) were evaluated. The β-catenin and Ki-67 levels were analyzed through immunohistochemistry analysis to assess the inflammatory response. ELISA-based analysis was used to measure IL-4, IL-6, IL-10, TNF-alpha, IFN-gamma, and VEGF levels. All the aforementioned parameters were mitigated in AOM/DSS-triggered colon cancer in mice treated with NEP-Sc. Nanoencapsulated polymeric Scopoletin offered protection against AOM/DSS-induced colon cancer in mice. To sum up, our research findings suggest that NEP-Sc may act as a promising candidate for treating colon-associated cancer.

1. Introduction

Colon cancer is the fourth prevalent cancer that results in fatalities from cancer globally [1]. The median life expectancy of colon cancer patients has remained unchanged for the past years, despite advances in medical technology and research. There are currently very few therapy choices that pose a greater risk of negative side effects than they do of being effective. Consequently, there is an increasing need to find innovative medicines with no adverse effects [2]. CRC almost always originates from polyps which are noncancerous growths that develop in the mucosal layer (inner lining) of the colon or rectum. These polyps are common, found in approximately half of average-risk individuals aged 50 and their prevalence increases with age and is higher in men than in women. However, fewer than 10 % of polyps are estimated to progress to invasive cancer, a transformation that typically occurs gradually over 10–20 years, with the risk increasing as polyps grow in size [3]. A growing body of research observed that people with inflammatory bowel disease (IBD) and colitis have a higher chance of getting the illness than people without IBD. Most frequently, 10–15 % of IBD patients pass away from the illness that causes colitis-associated cancer (CAC). CAC prevention is currently far from being accomplished [4].

Azoxymethane (AOM) and Dextran sodium sulphate (DSS) can cause CAC in mice. The histological characteristics demonstrated in this model is comparable to individuals with IBD-linked malignancy [5]. The single infusion of the pro-carcinogen AOM, which the liver converts to the active agent Methylazoxymethanol (MAM), and intake of DSS in drinking water, makes it a best studied chemically inducible model with chronic colitis, weight loss, and bloody diarrhoea, which is followed by

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the growth of many colon tumors [6]. This cancer's growth closely resembles the pattern found in humans. Rodents injected intraperitoneally with AOM repeatedly develop colon tumors, a valuable model for CAC because it is simple to do and consistently effective, especially in vulnerable rats and mice [7]. Following AOM therapy, epithelial cells go through a pathogenesis process that progresses from a benign tumor to adenocarcinoma. In mice or rats, colon cancer caused by AOM develops over the course of 14 weeks [8].

Different drugs on the nanoscale ranges are produced by nanotechnology for cell specific targeting. Understanding biosystems using nanotechnology leverages nanoscale ideas and methodologies [9]. Due to their special qualities, such as their bioavailability, a higher surface-to-mass ratio, and capacity to transport other substances like probes, proteins, and medications, nanoparticles are used in medical applications [10]. Scopoletin (Sc) (7-hydroxy-6-methoxy coumarin), a phenolic coumarin belonging to phytoalexin group and appears to be yellow crystalline. Sc is reported to play a role in several important biological function's. Enhancing the properties of Scopoletin using nanotechnology can be a very promising therapeutic candidate in drug delivery. The goal of the current work is to explore the potentiality of Nanoencapsulated polymeric Scopoletin as potent anti-inflammatory and antioxidant agent. The study examines the protective impact of nanoencapsulated scopoletin in a DSS and AOM generated mice model of colon cancer.

2. Materials and methods

2.1. Drugs and chemicals

Scopoletin, Dextran Sodium Sulphate (40,000 MW), 5-Fluorouracil and Azoxymethane was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Rabbit Polyclonal Ki-67 and β-catenin antibodies were purchased from Invitrogen (Waltham, MA, USA), Tumor necrosis factor (TNF-α), Vascular Endothelial Growth factor (VEGF) kits were obtained from USCN Life Science Inc., USA. INF-Gamma was purchased from COSMO Bio, USA. Interleukins IL-4, IL-6 and IL-10 were purchased from KOMA Biotech (Seoul, Korea). All other chemicals used were of analytical grade.

2.2. Animals

Male BALB/c mice of 30 numbers weighing 15–20g were procured from Mass Biotech (Chennai, Tamil Nadu). The animals were housed for 14 days at PSG Institute of Medical Sciences and Research, Coimbatore, Tamil Nadu, India at 23 °C ± 2 °C with 12-h light/dark cycle, fed and watered *ad-libitum*. All treatments were adhered to guidelines as per Institutional Animal Ethics Committee (IAEC) - Committee for Control and Supervision of Experiments on Animals (CCSEA) (Ethical Clearance-Approval no:490/IAEC/2021 & SLIMS/38/IAEC/24–25).

2.3. Induction of colorectal cancer (CRC)

Experiments were conducted for 12 weeks with five groups with slight modifications as per protocol mentioned by Parang et al. and Seetha et al. [7,11]. The representation of treatment strategy followed for this study is depicted in Fig. 1. The Group 1 comprised the normal group, receiving normal drinking water and chow food, and were not treated with AOM/DSS and did not receive any intervention during the study period which served as normal control in this experimental study. Group 2–5 mice received Azoxymethane (10 mg/kg b.w.) intraperitoneally on experimental day 0, then DSS (2.5 % w/v) and tap water for every alternative week and till 12th week of total experimental period (i.e. 12 weeks). CRC was induced in Group 3 in the same way as in Group 2 along with administration of 5-Fluorouracil (5-FU) (25 mg/kg bw) intraperitonially for last 15 days of experimental period till end of the 3rd month. The Group 4 & 5 received the same quantity of azoxymethane/DSS and nanoencapsulated polymeric Scopoletin (NEP-Sc) (2.5 mg/kg bw & 5 mg/kg bw respectively) injected intraperitonially for last 15 days of experimental period till end of the 3rd month.

2.4. Preparation of NEP-Sc and characterization

Nanoencapsulated polymeric Scopoletin was prepared according to the method of [12], with slight modifications. The prepared NEP-Sc was characterized and confirmed by particle size analysis and FESEM analysis. Detailed method of preparation and results of the characterization has been reported in our previous study [13]. The prepared NEP-Sc was also assessed for their anticancer property in HT-29 human colorectal adenocarcinoma cells and showed significant findings. Hence the current study aimed to study the efficacy of the prepared NEP-Sc *in vivo* against colon cancer in mice models.

2.5. Disease activity index (DAI) score

Body weight, stool consistency and gross bleeding of the mice were documented twice a week to calculate DAI score to assess the severity of illness in colon [14,15]. The method of scoring is illustrated in Table 1. The DAI is calculated using the formula, DAI= Body weight + stool

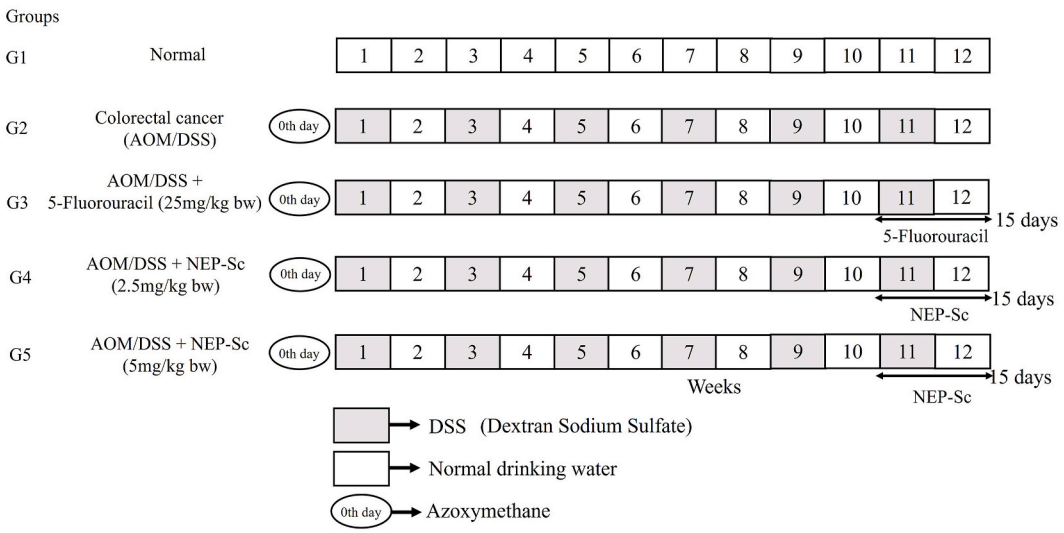

Fig. 1. Schematic representation of AOM/DSS induced Colo rectal cancer.

Table 1
Scoring system to assess the Disease Activity Index (DAI).

Score	Weight loss	Stool consistency	Bleeding
0	No loss	Normal	None
1	1–5 %	Normal	None
2	6–10 %	Loose	Slight bleeding
3	11–20 %	Loose	Slight bleeding
4	>20 %	Watery diarrhoea	Gross bleeding

consistency + gross bleeding/3.

2.6. Morphological analysis of the colon

After the treatment period, the animals were euthanized intraperitonially with a combination of ketamine (50 mg/mL) and xylazine (20 mg/mL) [16]. Cardiac blood was collected, centrifuged at 5000 rpm for 10 min, and serum was separated for further analysis. To get rid of RBCs and clots, the mice's colon was dissected, rinsed with phosphate buffered saline (PBS; pH 7.4) and measured the number of polyps, weight, and length of the colon.

2.7. Tissue preparation

All the collected tissues (colon) were utilized for histopathological analysis and homogenate preparation. At 4 °C, a polytron homogenizer was utilized to prepare 10 % of the homogenate using 0.05 M phosphate buffer (pH 7), centrifuged at 10,000 rpm for 20 min, and the supernatant was transferred to a new microfuge tube and stored at −80 °C.

2.8. Assessment of NEP-Sc's impact on oxidative markers

2.8.1. Reduced glutathione (GSH) assay

0.25 mL of homogenate was mixed with 0.05 mL of 25 % trichloroacetic acid (TCA) solution and chilled for few mins. Supernatant was collected by centrifuging at 3000g for 5 min. The sample contained 0.15 mL of homogenate, 0.4 mL of DTNB and 0.35 mL of 0.2 M sodium phosphate buffer (pH 8). Instead of homogenate, 0.15 mL of TCA is used in the reference cuvette. The reaction with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) produced yellow color which was measured at 412 nm and determined the amount of GSH in each gramme of tissue and was expressed as nmol/g of tissue [17].

2.8.2. Lipid peroxidase (LPO) assay

0.1 mL of homogenate, 0.38 mL of 0.8 % TBA, 0.38 mL of 20 % acetic acid and 0.15 mL distilled water made up the sample. The entire mixture was incubated for 1h at 95 °C. After incubation, 1.25 mL of butanol: pyridine mixture was added, centrifuged at 3000 rpm for 10 min, and the obtained supernatant was measured at 412 nm. LPO concentrations were measured in nmol/mg protein [18].

2.8.3. Superoxide dismutase (SOD) assay

The sample consists of 0.01 mL homogenate, 0.22 mL 0.1 M EDTA with 0.0015 % NaCN, 0.10 mL 1.5 mM NBT, and 2.3 mL 67 mM phosphate buffer at pH 7.8. 0.05 mL of 0.12 mM riboflavin was added, and absorbance was detected at 560 nm. After the initial reading, all tubes were lit evenly for 15 min. At 520 nm, the absorbance of the blue color was again measured. By comparing the absorbance of the sample to that of the control, the percent of inhibition was calculated and measured in U/mg protein [19].

2.8.4. Nitric oxide (NO) assay

The nitric oxide (NO) content was determined by reducing nitrate to nitrite with vanadium chloride (VCl_3) and then adding the Griess reagent [20]. Equal quantities of Griess reagent (0.1 g naphthyethylenediamine in 100 mL distilled water and 2 g sulphanilamide in 100 mL 5 % HCl) and VCl3 (400 mg in 50 mL of 1 M HCl) were added to homogenate. The absorbance at 540 nm was measured following 30 min of incubation at 37 °C. The results were compared to a reference nitrite curve, and the amount of nitrite present in each sample was determined and represented as mol NO/g tissue.

2.9. Histopathological study

Colonic tissue was embedded in paraffin and cut into 5 mm thick sections after being fixed with 10 % (v/v) neutral formalin. For microscopic examinations, sections were stained with hematoxylin and eosin [21]. The assessment system was used to score histological assessments is shown in Table 2.

2.10. Immunohistochemistry (IHC) analysis

Colon sections were paraffinized with a series of xylene reactions, followed by ethanol hydration series (100 %, 95 %, 70 %), and rinsed for 10 min with PBS. Histological sections were microwaved with sodium citrate at 650 W (7*3 min cycle) for the purpose of antigen recovery and treated with 2 % hydrogen peroxide for 15 min. It was then blocked with 10 % rabbit serum for 30 min at RT and incubating with primary antibodies (Ki-67 and β-catenin) at 4 °C overnight. After washing with PBS, the slides were incubated with anti-goat IgG-HRP secondary antibody for 20 min at RT before being rinsed with 1 % PBS for 15 min. The sections were stained with 3,3′-Diaminobenzidine (DAB) substrate and counterstained with hematoxylin and eosin for microscopic detection [22]. A Certified Pathologist examined the staining independently and graded it semi-quantitatively using the following criteria shown in Table 3 [23].

2.11. Effect of NEP-Sc on pro-inflammatory and anti-inflammatory cytokine levels

The collected serum was used to estimate mouse-specific IL-4, IL-6, IL-10, TNF-alpha, IFN-gamma, and VEGF employing appropriate ELISA kits and expressed as pg/mL as per the standard protocol mentioned in the respective kits [2].

2.12. Statistical analysis

The data are expressed as mean ± SD. Graphpad InStat 3.0 was used to conduct statistical analysis, including one-way analysis of variance (ANOVA) and Dunnett's test. Results from mice with AOM/DSS-induced colon cancer treated with NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-Fluorouracil (25 mg/kg b.w.) were considered statistically significant (* indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$) compared to those from the AOM/DSS-induced colon cancer group.

Table 2
Histopathological scoring system for analysis of morphology of colon tissue.

Score	Histopathological parameters
0	No evidence of leukocyte infiltration and inflammation
1	Some leukocyte infiltration and localized inflammation
2	Moderate leukocyte infiltration
3	High leukocyte infiltration and mild destruction of the crypt epithelium, moderate dilation of the mucosa, high vascular density and localized crypt loss
4	Severe diffuse inflammation, mass loss of goblet cells, severe destruction of the crypt epithelium

Table 3
Immunohistochemistry grading.

Score	Immunohistochemistry parameters
0	No overexpression of β-catenin and Ki-67 in comparison to normal colonic epithelium
1+	Mild overexpression
2+	Moderate overexpression
3+	Substantial, uniform overexpression of β-catenin and Ki-67

3. Results

3.1. Effect of NEP-Sc on macroscopic scoring and number of polyps during ulcerative colitis

After twelve weeks of the experiment, colon tumors were observed in the control group. In contrast, treatment with NEP-Sc significantly suppressed tumor development and reduced tumor size. The AOM/DSS-induced colon cancer model exhibited severe edematous inflammation and a marked reduction in colon length. However, administration of NEP-Sc (5 mg/kg body weight) alleviated inflammation and resulted in a noticeable increase in colon length. Various patterns and sizes of tumors shows that AOM/DSS administration resulted in a significantly larger lesion. Macroscopic scoring shows the effect of AOM/DSS induced colon cancer compared to 5-Fluorouracil and NEP-Sc (2.5 and 5 mg/kg b.w.) treated colon as shown in Fig. 2. The number of polyps were also reduced in treated groups compared to colon cancer control group.

3.2. Determination of the effect of NEP-Sc on body weight, bloody diarrhoea and disease activity index (DAI) score

Weight loss is an essential measure in mice with colon cancer; The mice with colon cancer had lower body weight than normal mice. After 3 weeks, treatment with 5-fluorouracil and NEP-Sc (2.5 and 5 mg/kg b.w.) ameliorated the effect of AOM/DSS in mice and resulted in an increase in body weight in the last 2 weeks of the experiment. All the mice during AOM/DSS treatment showed the prominent symptoms, with observed fecal blood throughout the experiment. Treatment with 5-fluorouracil and NEP-Sc (2.5 and 5 mg/kg b.w.) deduced blood in feces and rendered protective effect against AOM/DSS induced colon cancer group (Fig. 3). DAI score were observed after the administration of AOM/DSS. However, 5-Fluorouracil and NEP-Sc (2.5 and 5 mg/kg b.w.) injection relieved weight loss and diarrhoea in mice, that decreased the DAI score in comparison to the control group as depicted in Fig. 3.

3.3. Effect of NEP-Sc on antioxidants level

AOM/DSS induced colon cancer in mice significantly reduced GSH levels (685.29 ± 65.32 nmol/g tissue) in comparison to the normal group (1533.11 ± 75.55 nmol/g tissue). Treatment with NEP-Sc (2.5 mg/kg b.w.), NEP-Sc (5 mg/kg b.w.), and 5-fluorouracil administration showed marked level of GSH (965.32 ± 100.65, 994.52 ± 112.52, and 1056.42 ± 150.66 nmol/g of tissue), respectively. A significant increase in LPO levels was observed (8.23 ± 0.58 nmol/mg protein), whereas NEP-Sc (2.5 mg/kg b.w.), NEP-Sc (5 mg/kg b.w.), and 5-fluorouracil treated animals had a significant decrease (4.01 ± 0.85, 3.99 ± 0.75, and 3.95 ± 0.76 nmol/mg protein, respectively). The normal group produced 3.56 ± 0.45 nmol/mg LPO protein.

Lower levels of enzymatic SOD (72.56 ± 15.32 U/mg protein) were measured in comparison to the normal group (165.32 ± 12.34 U/mg protein). SOD levels were increased in mice treated with NEP-Sc (2.5 mg/kg bw), NEP-Sc (5 mg/kg bw), and 5-fluorouracil (95.32 ± 11.57, 102.32 ± 11.57, and 105.23 ± 12.98 U/mg protein, respectively). Nitric oxide levels in control group were significantly higher (23.21 ± 2.59 μmol) than in the normal group (8.32 ± 1.98 μmol). NO levels in the NEP-Sc (2.5 mg/kg b.w.), NEP-Sc (5 mg/kg b.w.), and 5-fluorouracil groups were significantly lower (18.23 ± 2.52, 16.53 ± 2.21, and 15.63 ± 2.89 μmol), respectively. The effect of NEP-Sc on oxidant markers were depicted in Fig. 4.

3.4. Histopathological study

Histopathological analysis revealed that AOM/DSS caused intestinal epithelial structure destruction, massive infiltration of inflammatory cells, crypt loss, and large area edema, as well as increase in pathological score. Normal mice, on the other hand, displayed no signs of colonic inflammation, damage, or neoplasms. However, tissue sections from 5-fluorouracil, NEP-Sc (2.5 mg/kg b.w.), and NEP-Sc (5 mg/kg b.w.) treated mice had more improved colonic histology by reducing immune cell infiltrates, minimization of epithelial cell loss, and gentle crypt distortion and inverted the morphological changes as shown in Fig. 5 (A) & 7.

3.5. Immunohistochemistry (IHC) analysis

IHC analysis was performed to determine β-catenin subcellular localization in various colonic neoplasms involved in the study (Figure). Immunoreactivities of β-catenin were observed with dark brown granules with little discrepancy in strength and transport. Higher levels of β-catenin were witnessed in AOM/DSS induced colon cancer control to normal group. 5-fluorouracil and NEP-Sc (5 mg/kg b.w.) administration ameliorated the effect of β-catenin and reduced its levels. Ki-67 is cell proliferation marker protein thar are highly expressed in colon cancer. Ki-67 cells were found throughout the cancer, indicating that AOM/DSS supplementation may promote cancer cell proliferation. Treatment with

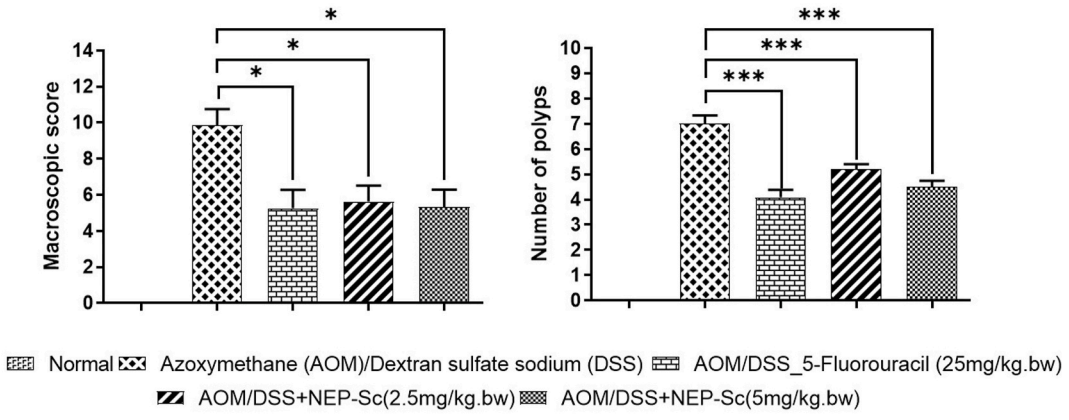

Fig. 2. Effects of NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-Fluorouracil (25 mg/kg b.w.) on macroscopic evaluation on mice colon weight and number of polyps. Values are expressed as mean ± SD. Values are significantly different from ulcerative colitis control group *(*p < 0.05, **p < 0.01 and ***p < 0.001)*.

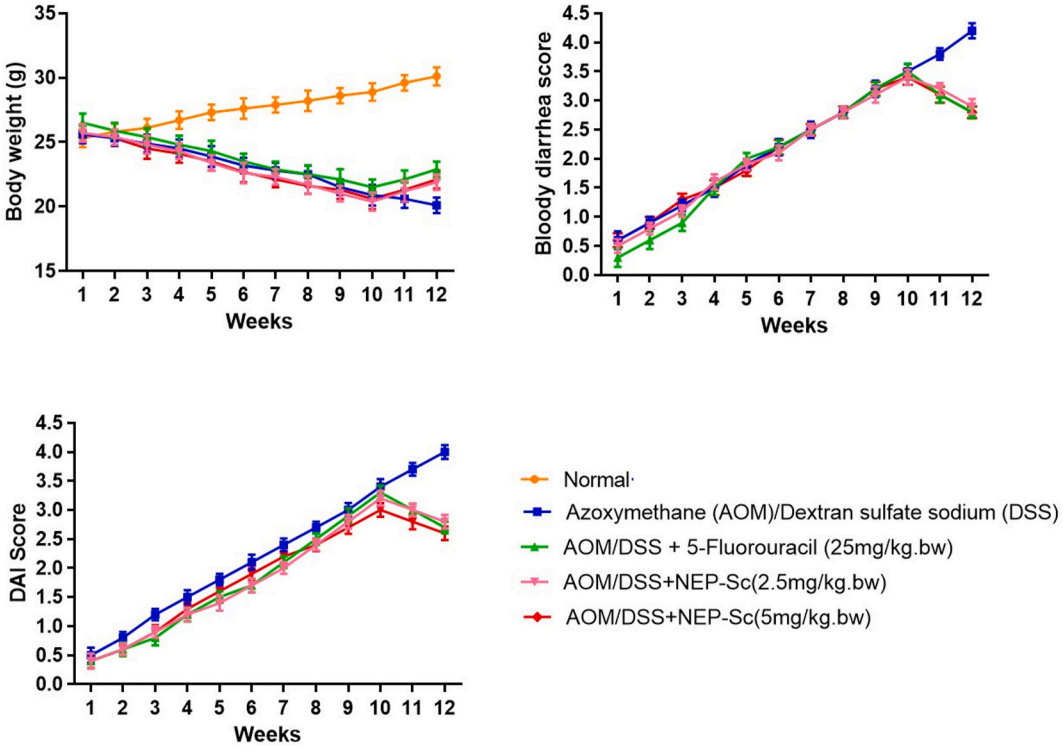

Fig. 3. Effects of NEP-Sc (2.5 & 5 mg/kg b.w.) and 5-fluorouracil (25 mg/kg b.w.) on body weight, bloody diarrhoea and DAI score. Values are expressed as mean ± SD.

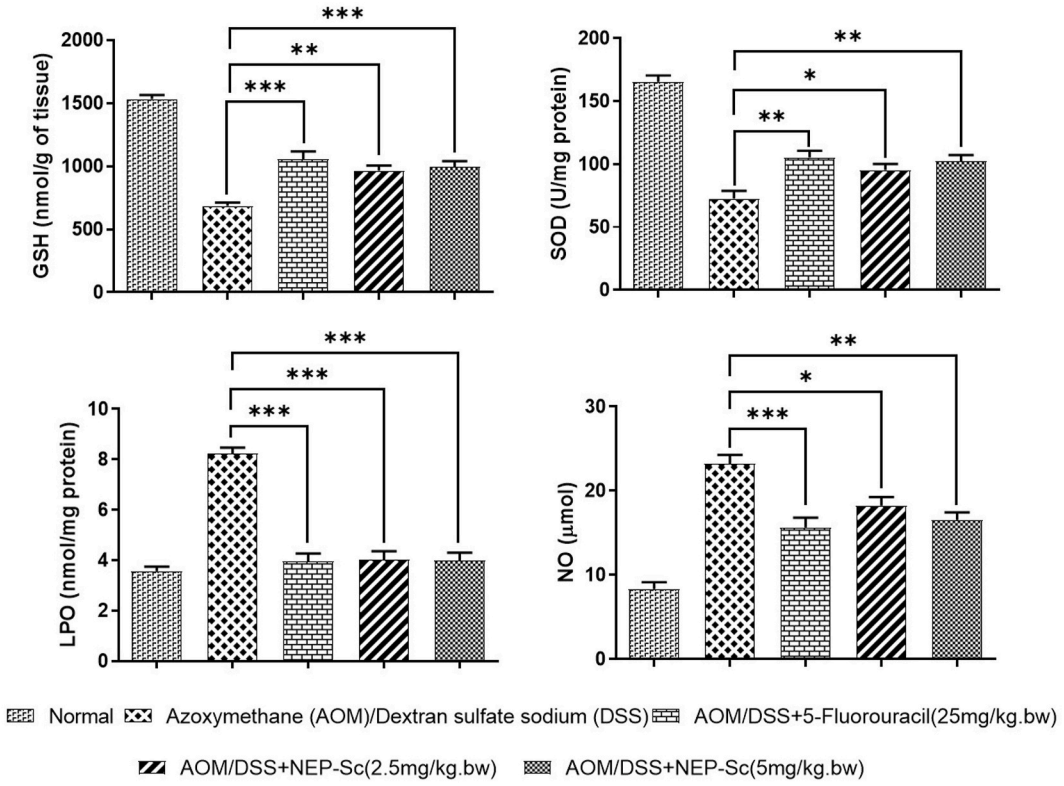

Fig. 4. Effects of NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-fluorouracil (25 mg/kg b.w.) on GSH, LPO, SOD and NO. Values are expressed as mean ± SD. Values are significantly different from control group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

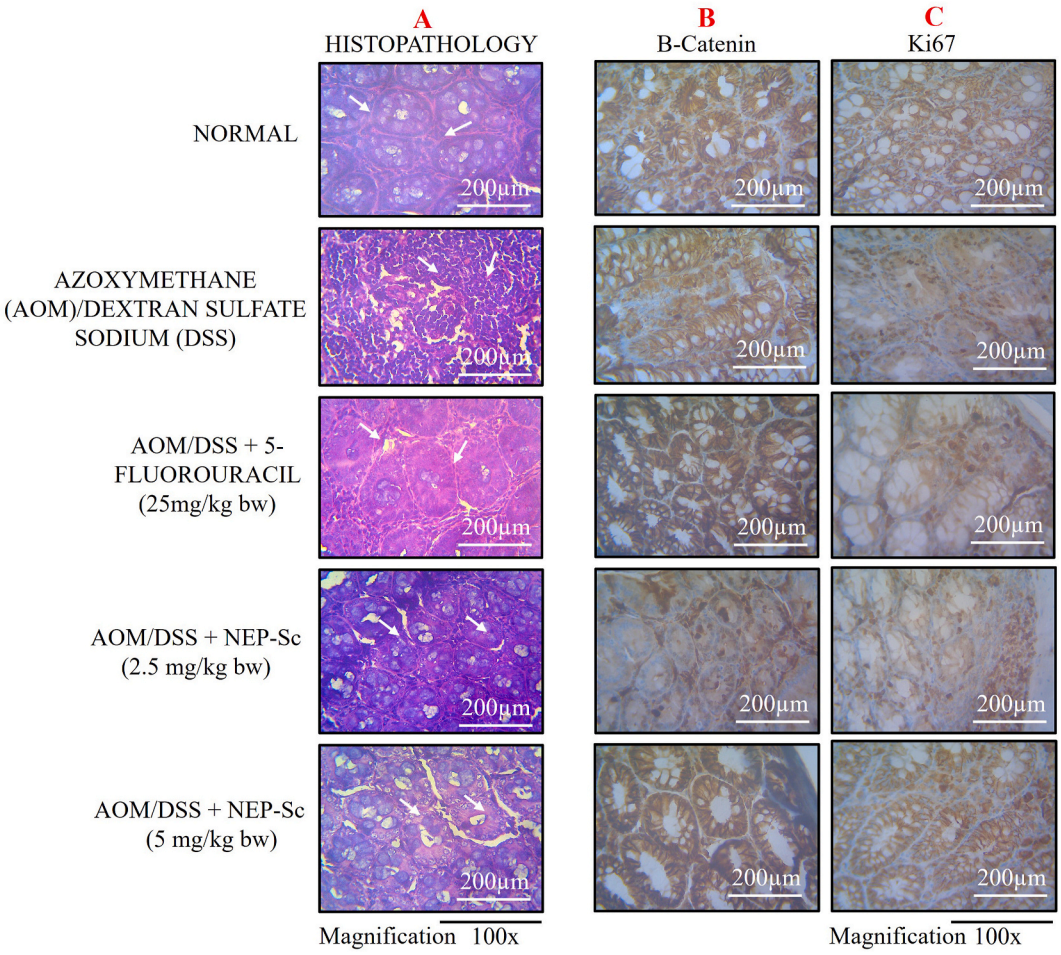

Fig. 5. A) Histopathological examination of the colon-NEP-Sc (5 mg/kg b.w.) revealed a decrease in inflammatory infiltrates, slight distortion in crypts and ameliorated the severity of colon damage. White arrows depict observable difference in pathology. B & C) Immunohistochemical expression of β-catenin and Ki-67. The colon immunohistochemical examination revealed that the β-catenin and Ki-67 level is increased in AOM/DSS induced Colo rectal cancer group. The β-catenin and Ki-67 expression observed in the epithelial surface and mononuclear cells of lamina and propria of mucosa. Treatment with NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-Fluorouracil (25 mg/kg b.w.) significantly decreased the expression level in the AOM/DSS induced Colo rectal cancer group [B) β-catenin C)-Ki-67].

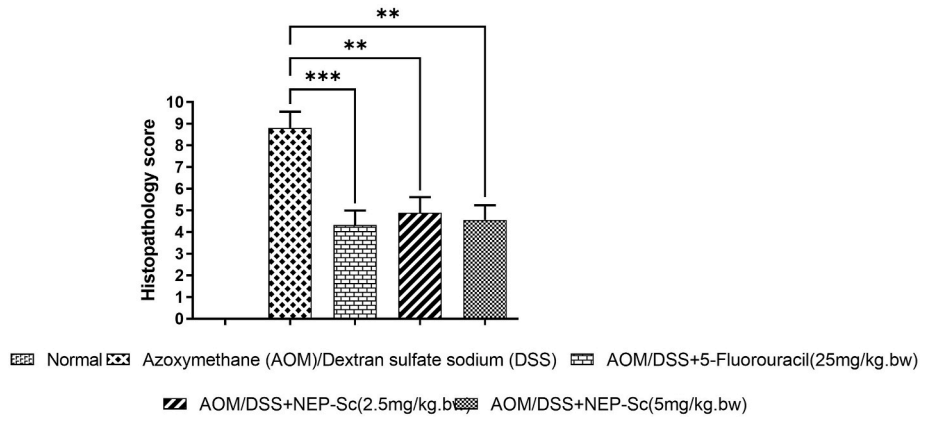

Fig. 6. Effects of NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-fluorouracil (25 mg/kg b.w.) on histopathology score. Values are expressed as mean ± SD. Values are significantly different from control group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

5-fluorouracil and NEP-Sc (2.5 and 5 mg/kg b.w.) declined Ki-67 expression level (Figs. 5B & 6C).

3.6. Determination of the effect of NEP-Sc on cytokines level

Determination of cytokine levels was carried out to study the dysregulated immune response mediated by pro and anti-inflammatory cytokines. Proinflammatory cytokines (IL-6, VEGF, IFN-Gamma and

TNF-alpha and anti-inflammatory cytokines (IL-4 and IL-10) was assessed to ascertain the effect on the control and the treatment groups by ELISA.

IL-6 levels were substantially higher in the AOM/DSS-induced control group (105.32 ± 2.90 pg/ml) than in the naive group (26.32 ± 3.20 pg/ml), as shown in Fig. 7. Treatment with 5-fluorouracil, NEP-Sc (2.5 mg/kg b.w.), and NEP-Sc (5 mg/kg b.w.) reduced the increase in IL-6 levels compared to the AOM/DSS-induced control group (75.32 ± 2.80, 79.23 ± 2.90, and 77.65 ± 2.84 pg/ml), respectively. VEGF levels in the control group were higher (115.32 ± 2.90 pg/ml) than in the normal group (23.25 ± 3.20 pg/ml). A marked decline in the groups treated with 5-fluorouracil, NEP-Sc (2.5 mg/kg b.w.), and NEP-Sc (5 mg/kg b.w.) (78.32 ± 2.80, 80.78 ± 2.90, and 79.65 ± 2.84 pg/ml), respectively were observed. Similarly increase in level of IFN-Gamma and TNF-Alpha was observed in treated and control group (160 ± 9.20 pg/ml and 15 ± 2.10 pg/ml) respectively. Notable decrease was observed in the 5-fluorouracil, NEP-Sc (2.5 mg/kg b.w.), and NEP-Sc (5 mg/kg b.w.) groups (Fig. 7). Anti-inflammatory cytokine levels (IL-4 and IL-10) was significantly lower in the AOM/DSS-induced control group (21 ± 4.30 pg/ml and 150 ± 24.30 pg/ml) respectively than in the treatment group as shown in Fig. 8. Administration of NEP-Sc (5 mg/kg b.w.) showed significant increase in these cytokine levels (46 ± 3.40 and 398 ± 32.10) as comparable to the standard drug, 5-fluorouracil treatment.

4. Discussion

Scopoletin has been reported to possess anticancer property in a variety of cancer both *in vitro* and *in vivo* [24–26]. Nano formulation has showed greater potential owing to improving drug efficacy, greater drug bioavailability, improved half-life of intravenous drugs [27]. NEP-Sc employed in the current study showed superior results in Azoxymethane-induced colon cancer models in mice. Colon weight to length ratio is used to determine the degree of chronic colitis and mucosal hyperplasia. Because DSS causes a shortening of the colon, this can be used as a visual index and has been used for the study of compounds with anti-inflammatory potential [28]. Microscopic observations by Arenesen et al., 2021 [29], showed that both C57BL/6J and A/J mice were detected with preneoplastic lesions with large number of colonic lesions due to administration of AOM/DSS. Treatment cycles demonstrated that the NEP-Sc ameliorated the effects of AOM/DSS in mice. A study by Ref. [29] observed that Evodiamine (EVO) decreased the weight loss, faecal blood and diarrhoea in mice. EVO has ameliorated the effects of AOM/DSS and decreased the DAI index.

NEP-Sc investigated in the present study was able to effectively counterbalance against oxidative stress, probably by scavenging of free radicals and singlet oxygen formation, which may be a prime contributing factor for protection against AOM/DSS induced colon cancer and reversal of damage and enhanced repair mechanism [4]. NEP-Sc possibly alleviated oxidative stress damage via regulation of endogenous oxidative enzyme systems like SOD, LPO, GSH and NO which are involved in colon defense systems. In the present study enzymatic antioxidants like GSH and SOD levels were found to be elevated in the AOM/DSS induced mice models supplemented with NEP-Sc however lipid peroxidation and nitric oxide levels were found to be reduced in the compared to the control (AOM/DSS alone). SOD is crucial for living cells since they catalyze the conversion of superoxide to oxygen and peroxide species. In contrast lipid peroxidation has shown to disrupt biological

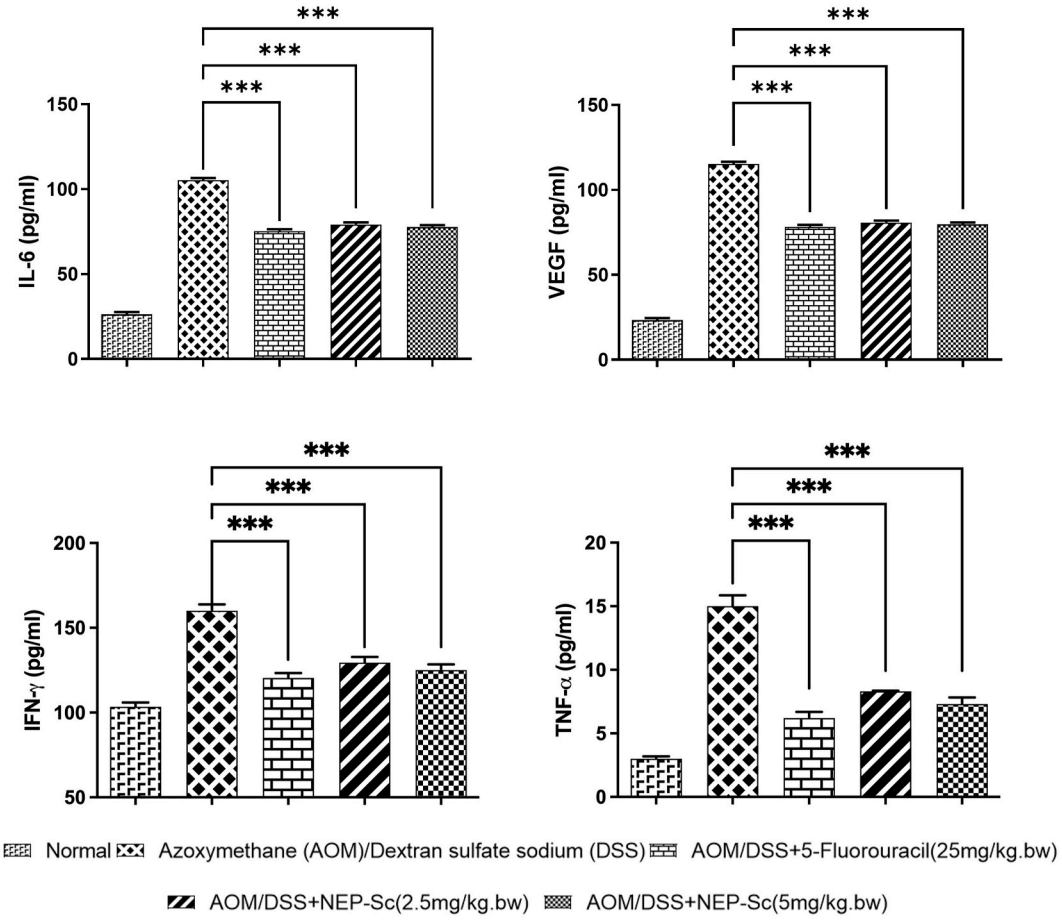

Fig. 7. Effects of NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-fluorouracil (25 mg/kg b.w.) on Pro-inflammatory cytokine ELISA levels (IL-6, VEGF, IFN-Gamma and TNF-Alpha). Values are expressed as mean ± SD. Values are significantly different from control group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

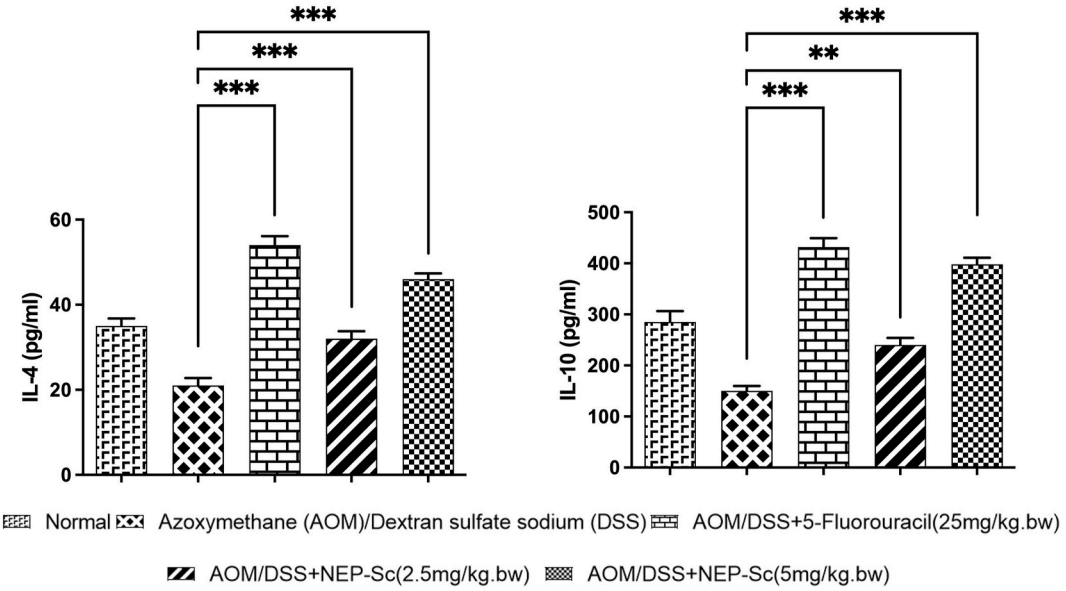

Fig. 8. Effects of NEP-Sc (2.5 and 5 mg/kg b.w.) and 5-fluorouracil (25 mg/kg b.w.) on Anti-inflammatory cytokine ELISA levels (IL-4 and IL-10). Values are expressed as mean ± SD. Values are significantly different from control group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

membranes possible leading to cell death via non-enzymatic and enzymatic auto oxidation [30].

Histopathological analysis is a critical clinical standard for detecting colon cancer. Classic histopathological morphology for colon cancer includes crypt loss, inflammation and pathological changes when stained with H&E. Additionally, it was discovered that analyzing colon tissues was a useful way to ascertain the protection against colon cancer caused by AOM/DSS [31]. Tissue samples were analyzed, 5-fluorourcail and NEP-Sc were found to have a protective effect against cancer model compared with the control group (AOM/DSS). Similar results were witnessed by Chen et al., 2014 [32], discussing that RS3, a resistant starch augmented preventive effect on colon cancer during histopathological examination.

Beta-catenin is an integral membrane protein which is a core component of E-cadherin complex facilitating cell to cell interactions by activating Wnt signaling. β-catenin upregulates urokinase plasminogen activator (uPA) expression in colorectal cancers promoting multistage carcinogenesis [33], Experimental evidence by Ref. [34], observed that β-catenin were higher in the cytoplasm and nucleus of adenocarcinoma cells [35], established that the administration of EPS1-1, a natural polysaccharide, was found with reduction of Ki-67 and PCNA cells. In the present study, high β-catenin protein accumulation was observed in the AOM/DSS induced colon cancer control group. However, both the standard (5-Fluorouracil) and NEP-Sc at both concentrations significantly ameliorated and decreased the β-catenin accumulation, which showed the protective effect of nano-modified Scopoletin. Similarly, Ki-67, a tumor proliferation marker, levels were found to be declined in AOM-induced colon cancer mice supplemented with NEP-Sc (2.5 and 5 mg/kg b.w.) [36].

The prime factors contributing to inflammation are cytokines, soluble factors and chemokines secreted by the tumor cells and inflammatory cells recruited to the tumor microenvironment like macrophages and mast cells [37]. Several studies reported elevated serum levels of cytokines like TNF-α, VEGF, IL-6 and IL-8 in Colorectal carcinoma patients and has important prognostic value. Researchers have employed TNFα antagonists are in phase I/II clinical trials and have been shown to be well tolerated in patients with solid tumors [38,39]. Pro-inflammatory cytokines (IL-6, VEGF, IFN-Gamma and TNF-α investigated in the present study has been shown to be elevated as like colon carcinoma conditions.

IL-6 has been connected to the etiology of spontaneous and inflammation-related colon cancer, according to several experimental studies. Higher levels of IL-6 are associated with the advanced stage of colon cancer and a decreased survival rate in patients [6]. VEGF (vascular permeability factor), an important angiogenesis regulator by initiating growth of blood vessel, inhibiting apoptosis in cells, and integrates precursor cells into capillaries development [40]. Therefore, inhibition or loss of VEGF had protective role in cancer mouse model linked with STAT-3 dependent tumor proliferation *in-vivo* [41]. TNF-α regulates oncogenic signaling pathways in epithelial cells, including NF-κB, and thereby promotes cancer cells growth and survival. Researchers have reported that in the azoxymethane (AOM)/dextran sodium sulphate (DSS) induced murine model of colitis-associated colon cancer, which lacks TNF-R p55, showed reduced mucosal damage upon AOM/DSS treatment. Accordingly, etanercept, which is a specific antagonist of TNFα, resulted in reduced number/size of tumors in the AOM/DSS model, thereby confirming the role of TNFα in inflammation-induced intestinal tumorigenesis [42]. In the present study NEP-Sc ameliorated and decreased pro-inflammatory cytokines, TNF-α, VEGF, IL-6 and IL-8 concentration significantly in the treatment group compared to the control. At the same time, deficiency of IL-10 and IL-4, an anti-inflammatory cytokine, seems to contribute to persistent intestinal inflammation (Ulcerative colitis) and Colitis associated Colon Cancer [43,44]. IL-10 is an important anti-inflammatory cytokine, which suppresses the macrophage/T-cell cytokine expression and inhibits the antigen presenting capacity [45–48]. Both Interleukin-4 and IL-10 are pleiotropic anti-inflammatory cytokines which function predominantly by suppressing the pro-inflammatory milieu. Activated T cells, mast cells, basophils, eosinophils, and NKT cells produce IL-4 [12]. Thereby reduced serum levels of cytokines like IL-4 and IL-10 observed in the control groups showed significant increase in NEP-Sc (5 mg/kg b.w.) treatment groups.

5. Conclusion

In the present study we have demonstrated that treatment with NEP-Sc has effectively ameliorated the effects of colon cancer by suppressing pro-inflammatory cytokines and improved the mucosal barrier functions. Further Nanoencapsulated polymeric Scopoletin promoted enzymatic antioxidant enzymes to counteract oxidative stress induced colon damage. Collectively, our results highlight that Scopoletin with nano-modification has a potential therapeutic role in targeting colon cancer.

CRediT authorship contribution statement

Kunnathur Murugesan Sakthivel: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rajan Radha Rasmi:** Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Loganathan Chandramani Priya Dharshini:** Writing – original draft, Investigation, Formal analysis. **Kalavathi Murugan Kumar:** Writing – original draft, Project administration, Methodology, Data curation, Conceptualization. **Venugopal Vinod Prabhu:** Resources, Methodology. **Balasubramanian Ramesh:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kunnathur Murugesan Sakthivel reports financial support was provided by India Ministry of Science & Technology Department of Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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