5-Methylcoumarin-4β-glucoside mitigated colon tumor progression in mice with AOM/DSS-induced colon carcinogenesis

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ABSTRACT

Context: The *in vitro* cytotoxicity profile of 5-Methylcoumarin-4β-glucoside (5-MC4βG) has been demonstrated to inhibit the proliferation of HT-29 adenocarcinoma cells. *Aim:* The need for newer and affordable chemotherapeutic agents is critical.

Main methods: We investigated the effect of 5-MC4βG on an azoxymethane /dextran sodium sulfate-induced colon carcinogenesis model in six groups of laboratory BALB/c mice for six weeks. While the first and second groups of mice served as vehicle and disease controls respectively, the third, fourth, and fifth groups were administered oral graded doses (25, 50, and 100 mg/kg) of 5-MC4βG. The sixth group was treated with 5-Flourouracil (15 mg/kg).

Key findings: The 100 mg/kg dose of 5-MC4βG upregulated *APC* mRNA expression by two-fold and downregulated *β-catenin* mRNA expression by two-fold compared to their respective disease controls. Furthermore, tumorigenic gene transcripts (*MDM2, BCL2, CDK1,* and *cyclin D1*) were downregulated by one-fold (except *cyclin D1* which was downregulated by two-fold), whereas pro-apoptotic genes (*p53, Bax,* and *Casp3*) were upregulated by two-fold following treatment with 100 mg/kg dose of 5-MC4βG. At the metabolic level, the bioactive compound lowered the expression of classical tumor markers; tissue polypeptide antigen, tumor-associated glycoprotein 72, and carcinoembryonic antigen by at least half. Histologically, 5-MC4βG intervention revealed a reduction in neoplastic cells associated with cellular necrosis.

Significance: 5-MC4βG reduced colon carcinogenesis in mice. Thus, this compound may be a promising candidate for colorectal cancer chemotherapy. Further development of 5-MC4βG will hopefully lead to the development of a potential anti-colon cancer drug candidate.

1. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-related mortality and the third most common type of cancer (in terms of new cases) in 2020 (Ferlay et al., 2020). With more than 930 000 deaths ascribed to CRC in 2020 and an expected mortality of over 1.6 million deaths per year by 2040 (Morgan et al., 2022), CRC carries an economic burden on healthcare systems worldwide. Moreover, the affordability and adverse effects of available agents used in CRC chemotherapy have deprived many patients of benefiting from the therapy. The average cost of CRC chemotherapy alone (without radiotherapy and surgical costs) ranges between $2000 and $18,000 (Bhimani et al., 2022). This relatively high cost is usually unbearable for patients in low- and middle-income countries, leading to noncompliance. Also, only few colorectal cancer chemotherapeutic agents (fruquitinib and encorafenib) have been developed to approval in the last decade.

Moreover, adverse drug reactions due to cancer chemotherapy are a global problem and constitute a major clinical problem in humans. The high incidence of toxicity and narrow therapeutic index of most colon cancer chemotherapeutic agents makes the search for newer, safer, and affordable agents imperative.

Natural products have served as important sources of chemotherapeutic agents, and because of their complexity in nature, these agents are capable of producing effective treatments against tumors (Uthman et al., 2021). A considerable number of anticancer agents available in clinical settings are of natural origin or derived from natural products (Huang et al., 2019) but only a few in clinical trials have been considered for CRC treatment; hence, there is a need for more effective agents from natural sources. 5-methylcoumarin-4β-glucoside (5-MC4βG) is a natural coumarin glycoside abundantly found in the leaves of *Vernonia glaberrima* (Welw. ex O.Hoffm.) H. Rob (Asteraceae). The *in vitro* cytotoxicity profile of the compound has been demonstrated to inhibit the proliferation of HT-29 colorectal adenocarcinoma cells (Alhassan et al., 2018). In our recent study, we reported a preclinical safety profile of 5-MC4βG with relative tolerability at 500 mg/kg body weight in laboratory mice (Abubakar et al., 2022). Therefore, we decided to study the anti-colon cancer effect of this bioactive compound using an animal model of colon carcinogenesis as a prelude to the development of a newer and better anticancer drug for effective therapy against colon cancer. In this study, we demonstrated the preclinical efficacy of 5-MC4βG in mitigating the progression of chemically induced colon carcinogenesis in laboratory mice.

2. Materials and methods

2.1. Drug preparation

5-MC4βG is a coumarin glycoside isolated and characterized from the leaves of *Vernonia glaberrima* as we reported elsewhere (Abubakar et al., 2022). A suspension of 5-MC4βG was prepared in 0.25 % carboxymethyl cellulose at concentrations of 25, 50, and 100 mg/kg. The bioactive compound was administered orally to all the tested groups. For the positive control group, 5-Fluorouracil (5-FU) was prepared in normal saline at a concentration of 15 mg/kg. 5-FU was administered intraperitoneally to the positive control group.

2.2. Animal handling

BALB/c mice (weighing 20–28 g) were purchased from the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja, and transferred to the animal house facility at the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, where they were maintained throughout the study period. The animals were housed in groups of three in transparent polycarbonate cages and kept at 25–30 °C in a well-ventilated room with a 12 h light/dark cycle. The University's ethical committee requirements for the use of animals, as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) were adhered to. The Animal research: reporting *in vivo* experiments (ARRIVE) guidelines was adhered to for the study. Before the experiment began, the animals were allowed *ad libitum* access to water and mouse chow for two weeks. The Usmanu Danfodiyo University animal use and care committee, University Research and Ethics Committee (animal), approved the research protocol and assigned reference number UDUS/UREC/2019/019. The services of an attending veterinarian were sought when necessary. Animals were handled as stipulated by the guidelines for the use of animals.

2.3. Azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colitis-associated colon tumorigenesis and treatment with 5-MC4βG

The Usmanu Danfodiyo University Research and Ethics Committee provided ethical approval for the *in vivo* experiments. The chemically induced colon carcinogenesis was performed as described by Crncec et al. (2015), with slight modifications. Briefly, female nulliparous mice (*n*= 90) were administered a single intraperitoneal injection of AOM (12 mg/kg body weight) at five weeks of age. One week after AOM injection, the mice received three cycles (5 days each) of 2 % DSS in drinking water (Fig. 1). A period of 14 days was allowed between each cycle. After the last cycle of DSS, the mice were allowed six weeks to ascertain the development of tumors based on our pilot study (data not presented).

The AOM/DSS-treated mice were divided into six groups (*n*= 15). The first group included mice that were not treated with AOM/DSS and did not receive any intervention during the study period (normal control). The second group consisted of mice treated with AOM/DSS but did not receive any subsequent treatment during the course of the experiment. The third, fourth, and fifth groups represented mice that were treated with AOM/DSS and further treated with 25, 50, and 100 mg/kg of 5-MC4βG, respectively. The doses were informed by an initial pilot study and a previous study of 5-MC4βG (Abubakar et al., 2022). The sixth group were treated with AOM/DSS and further treated with 15 mg/kg of 5-FU. All interventions lasted for six weeks (Fig. 1), and the weekly body weight of the mice was monitored, including daily observation of their health and well-being. To evenly disperse 5-MC4βG in solution before oral administration, 0.25 % carboxymethyl cellulose was added to solution of the compound. Equal treatment with the inert compound was effective in all groups.

2.4. Serum biochemical analysis

Six weeks after the commencement of the interventions, the mice were weighed, fasted for 8 h, and sacrificed by cervical dislocation under xylazine and ketamine anesthesia (10 mg/kg and 100 mg/kg, respectively). Blood samples were collected by cardiac puncture into plain blood sample tubes. Serum was separated and quantified for tissue polypeptide antigen (TPA), tumor-associated glycoprotein 72 (TAG-72), and carcinoembryonic antigen (CEA) using ELISA method (Hauptman and Glavač, 2017). The colons were harvested after sacrifice, rinsed with normal saline, and examined macroscopically before being placed in RCl2® for further analysis.

2.5. RT-qPCR analysis

The HiYield Total RNA Mini Kit (RBC Real Biotech, Taiwan) was used to extract total RNA from colon tissues, according to the manufacturer's protocol. Briefly, lysis buffer containing β-mercaptoethanol was added to the tissue and homogenized. The lysate was incubated at room temperature for 5 min and then filtered at $1000 \times g$. Then, absolute ethanol (70 %) was added to the filtrate, mixed, and the filtrate was transferred to the retention columns. After 2 min of centrifugation at $12,000 \times g$, the nucleic materials were washed with wash buffer and centrifuged at $12,000 \times g$. The column was spin-dried at $12,000 \times g$ and
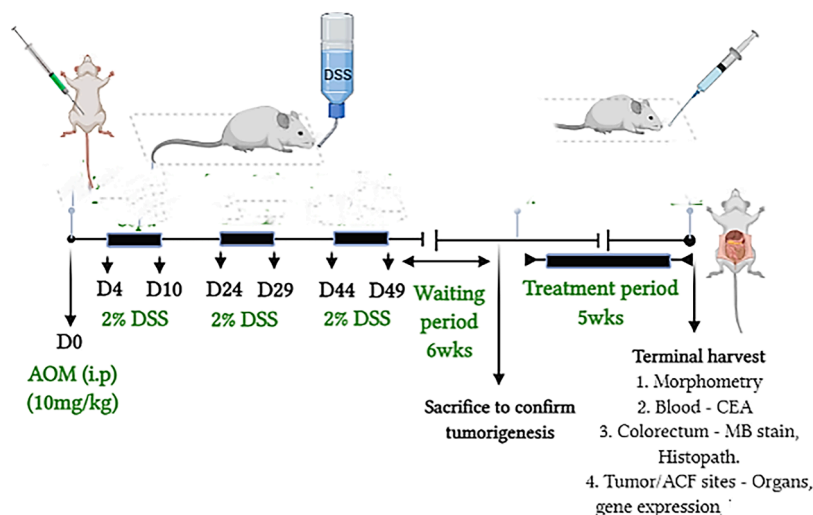
Fig. 1. Mice at 5 weeks of age were given an intraperitoneal injection of AOM at 12 mg/kg body weight (arrow head). One week after the AOM injection they were given 2 % DSS (MW 36,000–50,000) in their drinking water for one week (black box). Endoscopic observation was carried out at week 8 (open arrow). 5-FU was administrated intravenously at weeks 9, 10, 11, 13, 14 and 15 (arrows). All animals were sacrificed at week 16.

incubated for at least 3 min with 50 μL elution buffer. A purified RNA sample was eluted by brief centrifugation at $12,000 \times g$. The purity of the nucleic acid was analyzed using a NanoDrop spectrophotometer.

The purified RNA templates were directly used for RT-qPCR analysis (Nolan et al., 2006) using the One-step NZYSpeedy RT-qPCR Green kit (Nzytech, Lisbon, Portugal). One-step RT-qPCR was performed using a Rotor-Gene Q machine (QIAGEN, Düsseldorf, Germany). Briefly, a 10 μL reaction mixture containing $2 \times$ master mix, 400 nM forward and reverse primers, NZYRT mix, template, and nuclease-free water was prepared in a PCR tube (Nest Biotechnology, Wuxi, China). The reaction mixture was mixed and run on a real-time PCR machine. RNA fragments were amplified using the following thermal cycling conditions: reverse transcription at 50 °C for 20 min, polymerase activation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 55 °C for 30 s. The primer sequences used for the amplification of RNA fragments are presented in Table 1. The relative mRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, and *GAPDH* was used as the reference gene. Three independent biological replicates were analyzed.

Table 1 : The gene-specific sequences used for PCR amplification

2.6. Histopathological studies

Colons were fixed in 10 % v/v neutral-buffered formalin (NBF) and processed using the standard formalin fixed paraffin embedded (FFPE) protocol. They were sectioned using a microtome to produce thin sections (3–5 μm), stained with hematoxylin and eosin, and examined for histopathological features (Rolls, 2011). Bright-field photomicrographs of the colons were taken using a Retiga 2000R Fast CCD camera (Q-Imaging) attached to a Nikon E-600 microscope (Nikon, Tokyo, Japan).

2.7. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis of the data was performed using GraphPad Prism 5.0. One-way analysis of variance (ANOVA) or general regression analysis was used as appropriate. Tukey's test was used for *post hoc* analysis of differences detected using one-way ANOVA. The significance level was set at $P < 0.05$, 0.01 or 0.001. The values are presented as line graphs and bar charts.

3. Results

3.1. Effect of 5-Methylcoumarin-4β-glucoside on weight

Fig. 2 shows the mean weekly weight of the mice during intervention with vehicle, 5-MC4βG, or 5-FU. Except at week 4, when the average weight of the normal group differed from that of the diseased group and the 100 mg/kg 5-MC4βG group, the weights were not different from one another during the course of the intervention.

3.2. Effect of 5-Methylcoumarin-4β-glucoside on some tumor-related serum biochemical markers

In disease-control mice, a significant elevation in the serum levels of TPA was observed, but 5-MC4βG caused a significant reduction in serum levels of the marker. Compared with the disease control animals, the animals administered 5-MC4βG showed significant reduction of TPA levels at all concentrations (Fig. 3A). The bioactive compound also significantly serum TAG-72 levels in mice administered 5-MC4βG compared to those in mice that did not receive any intervention (disease control) (Fig. 3B). Comparing the levels of serum CEA, the levels of the serum marker in mice administered with 5-MC4βG were significantly lower as compared to a disease control group (Fig. 3C).

3.3. Effects of 5-MC4βG on the APC/β-catenin signaling

We assessed *APC* mRNA expression in diseased colon tissues normalized to that in normal tissues (Fig. 4A). We observed relatively weak expression of *APC* mRNA in colon tissues exposed to AOM/DSS. Colon tissues from mice treated with 5-MC4βG showed improved (from weak to strong) *APC* expression with increasing concentrations. Subsequently, mice treated with 100 mg/kg showed very strong upregulation in the expression of *APC* mRNA compared to the positive control mice. We also found aberrant *β-catenin* expression in disease control mice, which was subsequently weakly downregulated following treatment with 5-MC4βG or 5-FU. Furthermore, colon tissues from mice exposed to AOM/DSS showed upregulation in the expression *Cox-2* and *IL-6,* which was subsequently weakly downregulated following treatment with varying concentrations of 5-MC4βG.

3.4. Effects of 5-MC4βG on the MDM2/p53/p21 signaling

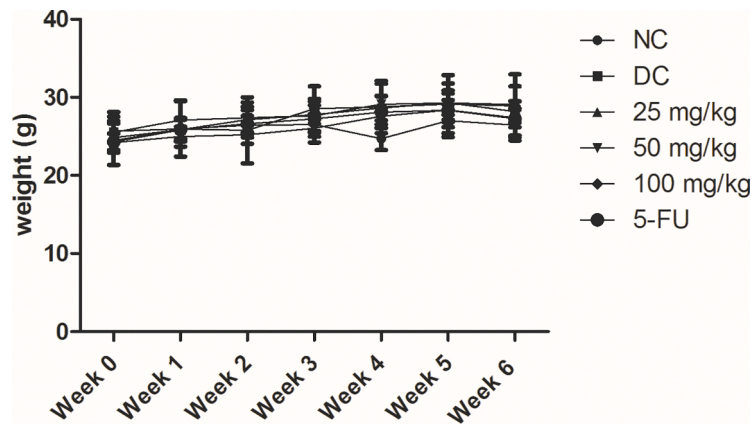As shown in Fig. 4B, the relative expression of *MDM2* was observed

Fig. 2. Effect of graded doses of 5-MC4βG on mean body weights of mice with AOM/DSS-induced colon carcinogenesis. Data are expressed as Mean ± SD. NC: Normal control mice; DC: Disease control mice; 25 mg/kg: 5-MC4βG at 25 mg/kg; 50 mg/kg: 5-MC4βG at 50 mg/kg; 100 mg/kg: 5-MC4βG at 100 mg/kg; 5-FU: 5-FU at 15 mg/kg.
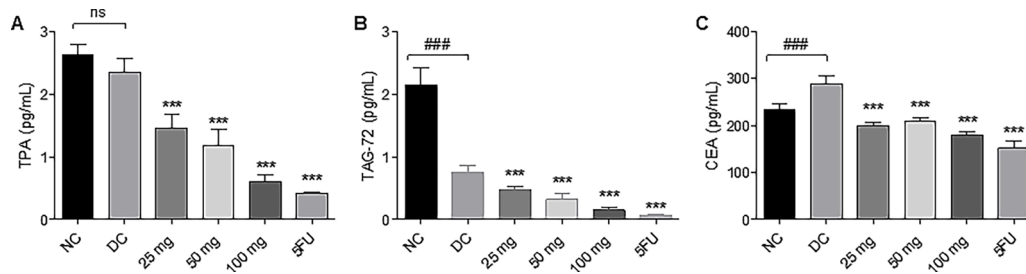

Fig. 3. Effect of graded doses of 5-methylcoumarin-4β-glucoside on some biochemical markers associated with colon cancer in mice. a= Tissue polypeptide antigen (TPA); b= Tumor-associated glycoprotein 72 (TAG-72); c= Carcinoembryonic Antigen (CEA). Data are expressed as Mean±SD; $\#\#\#p < 0.001$ as compared to normal control; $***p < 0.001$ as compared to disease control.
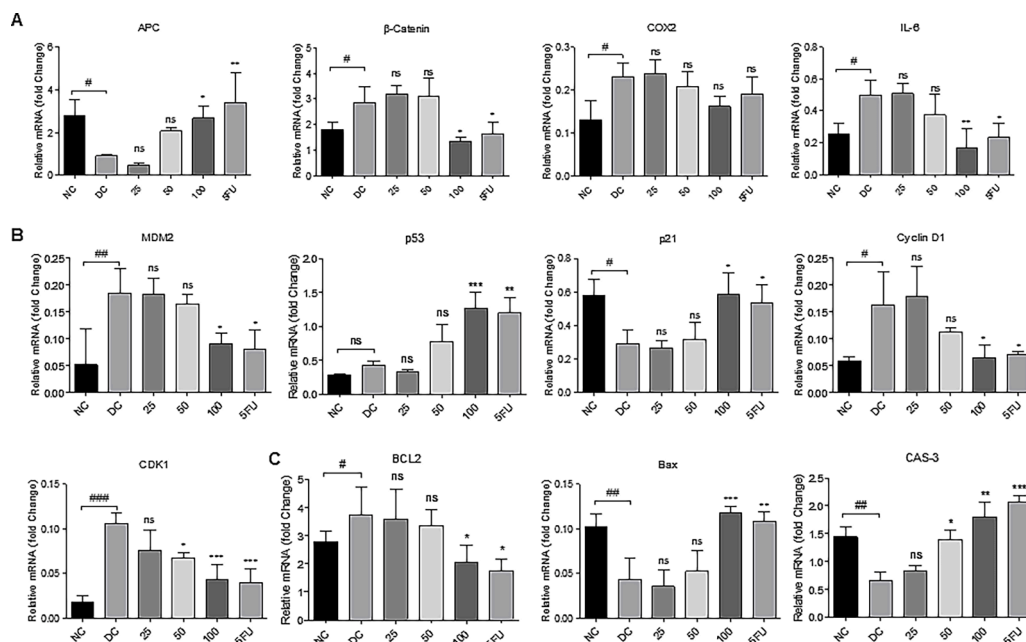

Fig. 4. Relative expression of mRNA in mice with AOM/DSS-induced colon carcinogenesis. The fold expression levels of (A) APC, β-catenin, COX2 and IL-6 genes; (B) MDM2, p53, p21, Cyclin D1 and CDK1 genes; (C) BCL2, Bax and CAS-3 genes. Data are expressed as Mean±SD; $\#p < 0.05$; $\#\#p < 0.01$, $\#\#\#p < 0.01$ as compared to NC; ns $p > 0.05$; $*p < 0.05$; $**p < 0.01$; $***p < 0.01$ as compared to DC.

in untreated colon tissues exposed to AOM/DSS, but was successively weakly downregulated in colon tissues after treatment with increasing concentrations of 5-MC4βG. Subsequently, we observed a very strong

$p53$ upregulation in all colon tissues treated with either 5-MC4βG or 5-FU; however, the expression of this gene was unremarkable in disease-control colon tissues. While the expression of $p21$ was unremarkable

in the colon tissues exposed to AOM/DSS even after treatment with 50 mg/kg of 5-MC4βG, its expression was subsequently weakly upregulated at the highest concentration (100 mg/kg) of the bioactive compound compared with the positive control. The relative expressions of *Cyclin D1* and *CDK1* were also weakly and strongly (respectively) upregulated in disease control tissues. Thereafter, the expression of these genes was downregulated in the mice treated with 50 and 100 mg/kg 5-MC4βG.

3.5. Effects of 5-MC4βG on cell death signaling

In disease control mice, the relative expression levels of *BCL2* mRNA was weakly upregulated, and the suppression of this gene was weakly downregulated after treatment with either 5-MC4βG or 5-FU (Fig. 4C). A very strong sequential upregulation of the proapoptotic genes *Bax* and *Casp3* was also seen in mice treated with 5-MC4βG compared to the positive control treatment, but the expression levels of *Casp3* in mice treated at 25 mg/kg did not differ from that in the disease control mice.

3.6. Effects of 5-MC4βG on the oxidative stress-induced cell death signaling

The expression levels of non-apoptosis-related genes were also assessed in the presence and absence of treatment. In disease control tissues, *GPX4* mRNA was aberrantly expressed in normal tissues (Fig. 5). Upon treatment, 5-MC4βG diminished *GPX4* expression at all concentrations with the effect being strongest at 100 mg/kg of the compound, but the expression of this gene was still upregulated, regardless of 5-FU treatment. On the other hand, *FSP1* mRNA was strongly upregulated in the disease-control colon and subsequently robustly decreased in the presence of 5-MC4βG treatment compared with 5-FU treatment. The relative expression of *GSR* mRNA was feebly lowered in the disease-control colon when compared to that in the normal colon. The expression levels of this gene did not differ regardless of treatment compared to the disease-control colon, but a slight increase was observed in mice treated with 5-FU (Fig. 5A). The expression levels of the oxidative stress-related genes *SOD1, CAT*, and *GPX3* were aberrantly downregulated in disease-control mice, and regardless of the different treatments, their expression levels did not differ from those in the disease-control tissues. However, strong aberrant expressions of *GPX3* mRNA were observed in

the positive control tissues as compared to the disease-control tissues (Fig. 5B).

3.7. Effect of 5-Methylcoumarin-4-β-glucoside on colon histology

Fig. 6a shows the normal histological structure of the colon from vehicle control mice, with distinctive mucosal epithelium (arrow), submucosa (SM), and muscularis mucosa (arrowheads). The colons of mice treated with AOM/DSS only (Fig. 6b) showed neoplastic cells with increased mitotic index and hyperchromatic pleomorphic nuclei (arrowheads). Cellular dysplasia is also observed. Fig. 6c shows the colons of mice treated with 25 mg/kg 5-methylcoumarin-4-β-glucoside after induction with AOM/DSS. Photomicrograph showing severe neoplastic growth in the epithelial lining. Neoplastic cells also showed an increased mitotic index (arrowheads). Fig. 6d shows that the colon of mice treated with 50 mg/kg of 5-methylcoumarin-4-β-glucoside after induction with AOM/DSS shows moderate nuclear pleomorphism in the epithelium (arrowheads). Cellular necrosis was also visible in these cells (arrow). Fig. 6e shows the colon of mice treated with 100 mg/kg of 5-methyl-coumarin-4β-glucoside after induction with AOM/DSS, showing neoplastic cells with mild nuclear pleomorphism (arrowhead) and cellular necrosis (arrow). Fig. 6f shows the colon of mice treated with 15 mg/kg of 5-FU after induction with AOM/DSS, showing epithelial cellular infiltration (arrow) and moderate neoplastic growth (arrowhead).

4. Discussion

In this study, we demonstrated the efficacy of 5-MC4βG in mitigating the progression of AOM/DSS-induced colon carcinogenesis in mice. At the transcriptional level, we performed gene expression studies to assess the array of tumor-related genes expressed in tissues exposed to AOM/DSS in the presence or absence of 5-MC4βG treatment. For comparison, we used 5-FU as a positive control because it remains a first-line treatment against CRC and acts through the p53-mediated activation of the p53/MDM2 ribosomal protein interaction (Sun et al., 2007), Wnt/β-catenin signaling (Cho et al., 2020), and ultimately involves cell cycle arrest and apoptosis induction (Yoshikawa et al., 2001). *APC* is a tumor suppressor gene that is substantially dysregulated in CRC,
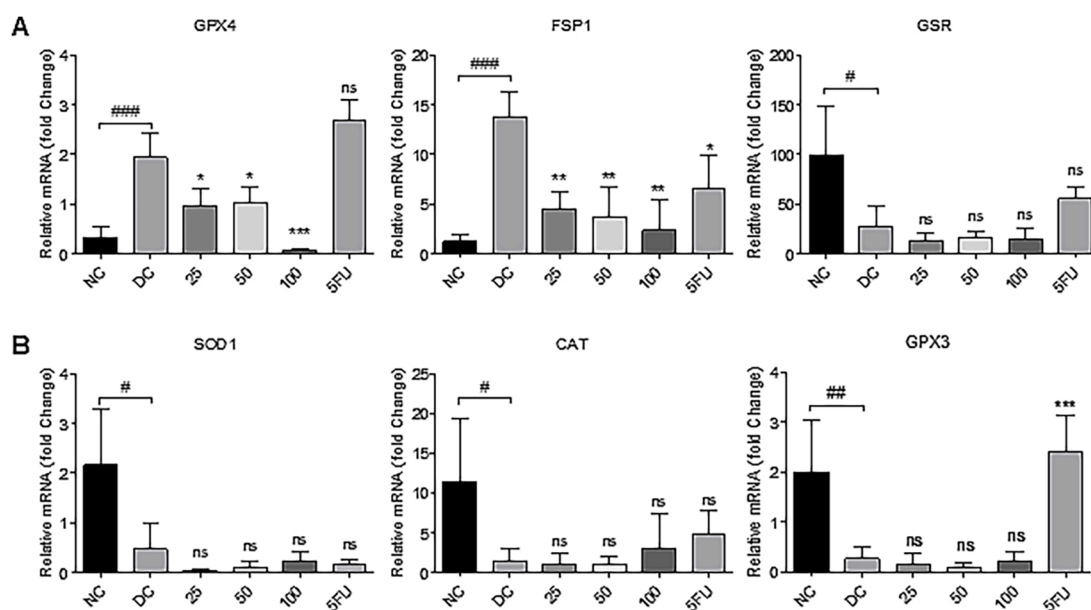

Fig. 5. Relative expression of mRNA in mice with AOM/DSS-induced colon carcinogenesis. (A) GPX4, FSP1, and GSR mRNA levels; (B) SOD1, CAT, and GPX3 mRNA levels. Data are expressed as Mean $\pm$ SD; #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ as compared to NC; ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.01$ as compared to DC.
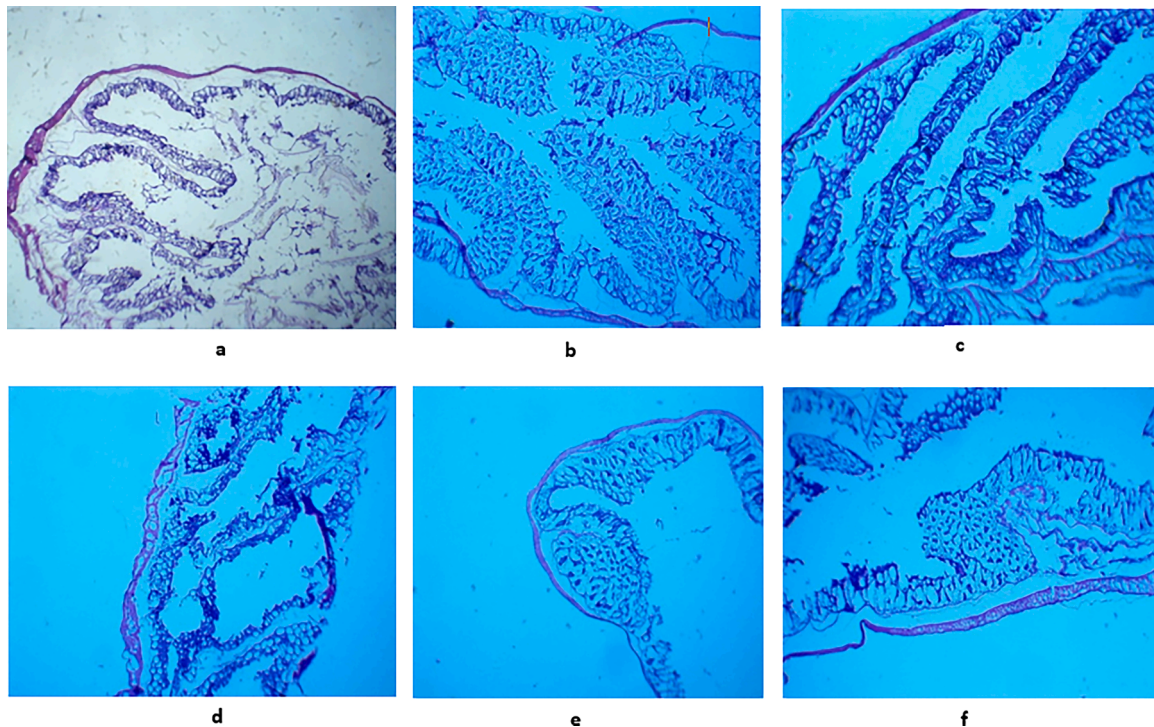
Fig. 6. Photomicrograph of sections of the colon of laboratory mice (H and E × 40). *a* = normal control; *b* = disease control; *c* = 5-MC4βG at 25 mg/kg; *d* = 5-MC4βG at 50 mg/kg; *e* = 5-MC4βG at 100 mg/kg; *f* = 5-FU at 15 mg/kg; SM= sub-mucosa.

resulting from mutational inactivation in the regions of the gene (Zhang and Shay, 2017). Conversely, APC mutations are not commonly observed in colon tumors from colitis-associated colon tumorigenesis induced by AOM/DSS, but the gene protects against the disease (Zeineldin et al., 2014). Our results suggest that 5-MC4βG may have a protective effect against colitis-associated colon tumorigenesis in mice by upregulating the expression of *APC* and subsequently downregulating the expression of *β-catenin* mRNA. Wnt/β-catenin signaling regulates intestinal homeostasis, and the pathway is aberrantly dysregulated in CRC through the inactivation of *APC* and the transcriptional activation of *APC* regulates β-catenin through the Wnt-induced β-catenin destruction complex (Parker and Neufeld, 2020). Recent data have shown that the traditional Chinese medicine Pai-Nong-San provides protection against AOM/DSS inflammation-induced colon tumorigenesis by downregulating β-catenin expression of *β-catenin* (M.M. Zhang et al., 2021), which supports our present findings. β-catenin has also been demonstrated to negatively regulate the expression of *Cox-2* and *IL-6* (Araki et al., 2003; Robinson et al., 2019) and the dysregulation of these inflammatory mediators has been implicated in the progression of CRC (Belluco et al., 2000; Roelofs et al., 2014). Our findings showed decreased expression of *Cox-2* and *IL-6*, suggesting a protective role of 5-MC4βG in mice with inflame`mation-induced colon tumorigenesis. The protective effects of the bioactive compound were similar to those reported in previous studies reported elsewhere (M.M. Zhang et al., 2021; Zhang et al., 2021b).

Dysregulation of *p53* expression is a notable biomarker that is most frequent in CRC, and this gene is activated in response to DNA damage, leading to G1 phase arrest of cell cycle progression through the transcriptional expression of the cyclin-dependent kinase inhibitor (*p21*) gene and apoptosis induction (Oh et al., 2019). Depletion in the expression levels of *p53* mRNA in disease control mice could be ascribed to the transcriptional upregulation of *MDM2*, which is known to destabilize p53 through MDM2-mediated p53 ubiquitination and degradation (Malami et al., 2017). Hence, suppression of *MDM2* expression by pharmacological agents stimulates p53 (Rigatti et al., 2012). In the present study, mice treated with varying concentrations of 5-MC4βG

showed increased expression levels of p53 by downregulating *MDM2* expression. The transcriptional upregulation of *p53* subsequently induces *p21* expression, given that p53 plays a key role in p21 stimulation in response to DNA damage. However, p21 can also be regulated irrespective of p53 expression (Macleod et al., 1995). Since p21 is necessary for p53-mediated G1 phase arrest of cell cycle progression (Waldman et al., 1995), we further assessed the relative expression levels of *Cyclin D1*. *Cyclin D1* promotes cell cycle progression by forming complexes with cyclin-dependent kinases (CDK4 or CDK6) and drives cells through G1 phase progression (Masclef et al., 2019) and which is downregulated by the activity of p21 (Palaiologos et al., 2019). The expression levels of *p21* mRNA were only found in mice treated with 100 mg/kg 5-MC4βG, which corresponds to the results observed for the expression levels of *Cyclin D1* and cyclin-dependent kinase 1 (CDK1) mRNA. The activation of p21 dependent cell cycle arrest in our study can be explained by a recent study involving mice with AOM/DSS-induced colitis-associated colon tumorigenesis (Sun et al., 2022).

Since apoptotic cell death signaling is mediated by p53 activation, downregulation of anti-apoptotic *BCL2* expression and sequential upregulation of pro-apoptotic genes are essential for inducing tumor apoptosis (Etti et al., 2017). As expected, the relative expression of the p53 downstream target genes, *Bax* and *Casp3* were upregulated in the colon tissues of treated mice, whereas *BCL2* mRNA was downregulated. Our data correspond to those of a study demonstrating the protective role of polysaccharides from Rhizopus nigricans in mice with AOM/DSS-induced colitis-associated colon tumorigenesis (Song et al., 2019). Kathiria et al. also reported an increase in the expression of p53-mediated proapoptotic genes and a decrease in anti-apoptotic genes in a study involving colitis-associated carcinogenesis (Kathiria et al., 2012).

The generation of reactive oxygen species (ROS) can play a vital role in alleviating colitis-associated colon tumorigenesis by increasing oxidative stress, which is the key to non-apoptotic cell death. In this process, glutathione (GSH) is depleted, leading to the inactivation of glutathione peroxidase 4 (*GPX4*), resulting in a high generation of ROS and ultimately cell death (Stockwell et al., 2017; Moon et al., 2022).

Glutathione reductase (GSR) plays a vital role in the generation of GSH, and its expression suggests downregulation of intracellular GSH. Although GPX4 is critical to cancer cell death, ferroptosis suppressor protein 1 (FSP1) has been shown to protect against ferroptosis induced by *GPX4* depletion (Doll et al., 2019). Looking at Fig. 5, 5-MC4βG could be mitigating colon tumorigenesis by inducing non-apoptotic oxidative cell death as one of its mechanisms of action. 5-MC4βG treatment caused a decrease in endogenous antioxidant enzymes suggesting an overwhelming increase in oxidative stress. This result is in agreement with a report published on a Chinese herbal formulation that ameliorated AOM/DSS-induced colitis-associated colon cancer (Wang et al., 2022). Moreso, our data from oxidative-related gene expression suggest the amelioration of colitis-associated colon tumorigenesis by 5-MC4βG.

At the metabolite level, we further investigated whether graded doses of 5-MC4βG did affect the levels of TPA, TAG-72, and CEA; to give insight into the compound's anti-colorectal cancer properties. These are classical tumor markers used in routine clinical practice (Swiderska et al., 2014). The graded reduction of TPA levels by 5-MC4βG suggests that a further increase in the dose of 5-MC4βG could further reduce the levels of the tumor marker, whose decrease during early stages of cancers corresponds with the nonproliferation of tumor cells and patients with high survival rates (Jelski and Mroczko, 2020). Our previous toxicity study on 5-MC4βG showed its safety in multiple doses employed in this study (Abubakar et al., 2022). This could mean that an increase in the dose of the compound beyond the maximum dose employed in this study could result in further lowering of TPA *vis-à-vis* reduction in progression of tumor proliferation without a complimentary increase in its toxicity. Serum expression of TAG-72 was similar to that of TPA. The serum levels of both tumor markers (TPS and TAG-72) were significantly reduced at our maximum utilized dose of 500 mg/kg of 5-MC4βG, demonstrating the anti-colorectal cancer activity of the compound. This is in agreement with the histological features of the colon of mice that were administered the maximum dose of the compound (Fig. 6e). Histological changes demonstrated a drastic reduction in neoplastic cells associated with cellular necrosis, which is synonymous with a near-peak effect of the compound. Compared with the group that received the maximum dose of the compound, the positive control group had a similar effect on the histology and serum levels of the tumor markers (TPS and TAG-72). Even after oral administration, 5-MC4βG demonstrated a similar efficacy to 5-FU in terms of histological features (Figs. 6e and 6f) and serum tumor markers. Further development of 5-MC4βG will hopefully lead to the development of a promising anti-colorectal cancer drug candidate. Due to variability and non-specificity in serum expression, the use of a single classical tumor marker, such as TPA, CEA, or TAG-72, to monitor the development, progression, and prognosis of an active tumor is highly discouraged in clinical settings (Stiksma et al., 2014; Guadagni et al., 1993). The three tumor-associated serum biochemical markers analyzed, TPA, CEA, and TAG-72, correlated positively with tumor regression properties, resorbing neoplastic cells, and arrest of further neoplastic cellular proliferation, suggesting potent anti-colorectal cancer activity.

5. Conclusion

Overall, 5-MC4βG significantly mitigated mRNA expression, tumor-related serum biochemical markers, and colon histology features, which are markers for the progression of colon carcinogenesis in laboratory mice. This compound demonstrated efficacy at the transcriptional, metabolic, and histological levels. Thus, this compound may be a promising candidate for CRC chemotherapy. Further development of 5-MC4βG will hopefully lead to the development of a potential anti-colon cancer drug candidate.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process.

He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs

Ethical approval

I am pleased to convey to you that the ethical approval for your project titled: In-vivo anti-colon cancer investigation and mechanism of action studies of 5-methylcoumarin-4∼-glucoside isolated from Vernonia glaberrima (Welw. ex O.Hoffm.) H. Rob has been granted. Please note that you are required to adhere strictly to the protocol stated in your proposal. Should there be any substantial change in the protocol, a fresh application is required.

CRediT authorship contribution statement

Ibrahim Malami: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Alhassan Muhammad Alhassan:** Conceptualization, Data curation, Funding acquisition. **Qamar Uddin Ahmed:** Data curation, Resources. **Syed Adnan Ali Shah:** Data curation, Methodology, Resources. **Mohammed Umar:** Data curation, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Muhammad Salisu Abubakar:** Data curation, Investigation, Methodology, Resources. **Mustapha Umar Imam:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration. **Bilyaminu Abubakar:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mustapha umar imam reports financial support was provided by Tertiary Education Trust Fund. 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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2024.100568.

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