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ORIGINAL ARTICLE

Inhibitory Effects of Triterpenoid Betulin on Inflammatory Mediators Inducible Nitric Oxide Synthase, Cyclooxygenase-2, Tumor Necrosis Factor-Alpha, Interleukin-6, and Proliferating Cell Nuclear Antigen in 1,2-Dimethylhydrazine-Induced Rat Colon Carcinogenesis

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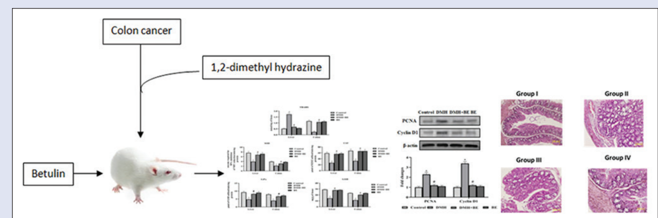
ABSTRACT

Background: Colon carcinogenesis is the third most commonly occurring malignant neoplasm and the second most common cause of cancer-related deaths globally. Betulin (BE) is a pentacyclic lupane-type triterpenoid naturally dispersed in many plants. It is also recognized as betuline, betulinol, or betulinic alcohol. **Objectives:** In this study, we explored the anticancer, antiproliferative, and anti-inflammatory effects of BE on 1,2-dimethylhydrazine (DMH)-treated rat model of colon cancer. **Materials and Methods:** Colon cancer was induced by a subcutaneous injection of DMH (20 mg/kg bwt) once a week for the initial 4 weeks of the experiment. We analyzed body weight, tumor incidence, tumor volume, total number of tumors, thiobarbituric acid reactive substances (TBARS), and levels of antioxidants (glutathione peroxidase, glutathione, catalase, and superoxide dismutase), bacterial enzymes (β-glucuronidase and mucinase), Phase I (cytochrome P450 and cytochrome b_5) and Phase II (GST and Glutathione reductase (GR)) detoxification enzymes, and inflammatory (cyclooxygenase-2, interleukin-1 beta [IL-1β], inducible nitric oxide synthase, tumor necrosis factor-alpha, and IL-6) and cell proliferative (cyclin D1 and proliferating cell nuclear antigen) markers. We also assessed the histopathological alterations found in experimental and control rats. **Results:** We observed decreased body weight and levels of antioxidants and Phase II enzymes; augmented tumor incidence, tumor volume, total number of tumors, Phase I enzymes, TBARS, and levels of bacterial enzymes; and irregular histopathological changes in DMH-treated rats. Moreover, the Western blotting analysis of colon tissues revealed upregulation of inflammatory and cell proliferative markers in DMH-treated rats. Oral supplementation of 20 mg/kg bwt BE led to inhibition of tumor formation and inflammation, regulation of cell proliferation, and restoration of biochemical parameters. Our findings were supported by histopathological analysis. **Conclusion:** Our results suggested that BE exhibited anticancer, anti-inflammatory, and antiproliferative effects against DMH-induced colon cancer in rats.

Key words: 1,2-dimethylhydrazine, antioxidant, betulin, cell proliferation, colon cancer, inflammation

SUMMARY

- Colon carcinogenesis is the third most commonly occurring malignant neoplasm and the second most common cause of cancer-associated deaths globally
- Betulin (BE) is a pentacyclic lupane range of triterpenoid naturally dispersed in many plants
- In this study, BE effectively alleviated 1,2-dimethylhydrazine-induced colon carcinogenesis in rats.



Abbreviations used: BE: Betulin; DMH: 1,2-dimethylhydrazine; TBARS: Thiobarbituric acid reactive substances; GPx: Glutathione peroxidase; GSH: Glutathione; CAT: Catalase; SOD: Superoxide dismutase; CYP_{450}: Cytochrome P450; Cyt-b_5: Cytochrome b_5; COX-2: Cyclooxygenase-2; IL-1β: Interleukin-1 beta; iNOS: Inducible nitric oxide synthase; TNF-α: Tumor necrosis factor-alpha; IL-6: Interleukin-6; PCNA: Proliferating cell nuclear antigen; ROS: Reactive oxygen species; NF-κB: Nuclear factor-kappa B.

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INTRODUCTION

Cancer is described by uncontrolled cell development, metastasis, and diminished apoptosis.[1] The development of carcinogenesis involves multiple steps, including cell proliferation and inflammation at the genetic and cellular levels that eventually reprogram a cell to suffer from unrestrained cell proliferation, followed by tumor development.[2] Colon carcinogenesis is the third most frequently detected malignant neoplasm and the second most important cause of cancer-associated deaths globally.[3] Its incidence had previously decreased in Asian countries, particularly in India; however, in recent years, it has increased severely in Asia.[4]

Antioxidant enzymes constitute an important defense mechanism against different exogenous and endogenous toxic molecules such as chemical carcinogens and reactive oxygen species (ROS). However, excessive amounts of ROS cause damage to cellular lipids, DNA, and proteins, resulting in oxidative stress.[5] Inflammatory permeation into the tumors is regulated by the release of cytokine mediators such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and IL-6.[6] TNF is the central regulator in the cytokine network system, which controls other cytokines, including IL-6 and IL-1β. TNF-α stimulates the phosphorylation of IκB, which induces ubiquitination. Following IκB degradation, finally, it translocates nuclear factor kappa B (NF-κB) into the nucleus where it attaches to DNA sequences and enhances several genes, thereby causing cell proliferation.[7] Increased level of ROS results in cell proliferation, which leads to oxidative stress and eventually molecular and cellular injuries. Cell proliferation is an important step in tumor development, and therefore, it is considered an essential target for improving anticancer agents.[8]

Natural products with different biological activities mainly aim for the treatment and prevention of diverse diseases, including cancer.[9] Natural plant-derived substances are easily available due to their abundance in nature and are considered as a potential anticancer drug for the treatment of various cancers.[10] Betulin (BE), also known as betulinic alcohol or betulinol, is a pentacyclic lupane range of triterpenoid that is naturally available in many plants.[11] Several studies have revealed that BE exhibits a wide variety of pharmacological and biological effects, including antibacterial, anti-inflammatory, antifungal, antimalarial, and antiviral activities.[12-15] Conversely, there are no scientific proofs for the anticancer benefits of BE against colon cancer. Therefore, the current investigation aimed to explore the anticarcinogenic potential of BE against 1,2-dimethylhydrazine (DMH)-induced colon cancer in rats.

MATERIALS AND METHODS

Chemicals

DMH and BE were obtained from Sigma Chemical Company, MO, USA. The primary antibodies against IL-6, proliferating cell nuclear antigen (PCNA), TNF-α, cyclooxygenase-2 (COX-2), cyclin D1, IL-1β, inducible nitric oxide synthase (iNOS), and β-actin were procured from Santa Cruz Biotechnology. All experiments conducted in this research work have been approved by the Institutional Ethical Committee (2019-28).

Animal model

Adult male Wistar animals (160–180 g) were maintained under conditions of constant temperature (25°C ± 2°C), humidity, and light (12 h light/dark). The rats were fed with ordinary animal pellet diet and water *ad libitum*.

Induction of carcinogenesis

DMH was mixed with EDTA (1 mM), and the pH was adjusted to 6.5 using NaOH (1 mM) to ensure the strength of the carcinogen. Then, the rats were subcutaneously injected with the solution at 20 mg/kg bwt once a week for the first 4 weeks of the experiment.

Experimental design

Twenty-four rats were arbitrarily separated into four investigation groups with six animals per group. Group I rats served as untreated controls. Groups II and III rats received DMH (20 mg/kg bwt) via subcutaneous injections once a week for the first 4 weeks. In addition, Group III animals received BE (20 mg/kg bwt) orally all day. Group IV rats received BE (20 mg/kg bwt) orally all day during the 16 experimental weeks. All rats received a modified pellet diet throughout the investigation period.

After the 16th week, all the rats were sacrificed. Liver, colon, and fecal samples were homogenized in proper buffer and centrifuged at 3000×g. The supernatant was collected for further biochemical assessment. Colon tissue was preserved in formalin (10%) and stored at −80°C for histological evaluations. The colonic mucosa and fecal pellets were homogenized in PBS, centrifuged at 2000×g for 10 min at 4°C, and the supernatant was used for the assays of fecal and colonic mucosal bacterial enzymes.

Body weight

During the investigation period, body weights of the experimental and control animals were evaluated. The animals were weighed at the start of experiments. Subsequently, they were weighed once a week and finally before the sacrifice.

Biochemical analysis

Thiobarbituric acid reactive substances (TBARS) in the colon and liver tissues were performed as described by Ohkawa *et al*.[16] Superoxide dismutase (SOD) levels were analyzed in the colon and liver tissues as described by Kakkar *et al*.[17] Catalase (CAT) levels were determined in both colon and liver tissues as described by Sinha.[18] The glutathione peroxidase (GPx) and glutathione (GSH) levels in the colon and liver tissues were analyzed as described by Rotruck *et al*.[19] and Beutler and Kelly,[20] respectively. Cytochrome P450 (CYP_{450}) and cytochrome b_5 (Cyt-b_5) levels in the liver and colon tissues were analyzed as described by Omura and Sato.[21] GST levels were analyzed as described by Habig *et al*.[22] GR levels were analyzed as described by Carlberg and Mannervik.[23] β-glucuronidase activity was determined as described by Freeman.[24] Mucinase activity was analyzed as described by Shiau and Chang.[25]

Histopathological analysis

The colon tissue sample was sliced and fixed using 10% formalin solution, dehydrated with graded ethanol (50% to 100%), and embedded in paraffin. Then, sections of 3–5 μm in thickness were cut and stained with hematoxylin and eosin. The slides mounted with the stained sections were observed under the microscope.

Western blotting analysis

The colon tissue was homogenized in ice-cold RIPA buffer containing 1% protease and phosphatase inhibitor cocktail. The homogenate was centrifuged at 12000 rpm at 4°C for 15 min. After centrifugation, supernatant was collected, and protein concentrations were estimated as described by Lowry *et al*.[26] These proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto Polyvinylidene Fluoride (PVDF) membrane. The protein expression of the primary antibodies, including iNOS, IL-6, PCNA, TNF-α, COX-2, cyclin D1, IL-1β, and β-actin, were assessed. The bands were visualized using enhanced chemiluminescence reagent kits and quantified using the ImageJ software.

Statistical analysis

The data were characterized as mean ± standard deviation. Statistical analysis was conducted using GraphPad Prism 8 software (San Diego, CA, USA). ANOVA, followed by Tukey's test, was used as a *post hoc* analysis. The differences were defined as significant at $P < 0.05$.

RESULTS

Effect of betulin on body weight

Figure 1 demonstrates the changes in body weight of experimental and control rats. Primarily, no considerable alterations were identified. However, the body weight of DMH-induced rats declined significantly ($P < 0.05$) as a final point compared to the control animals. Furthermore, BE treatment markedly inhibited the reduction in the body weight of DMH-induced rats ($P < 0.05$). BE alone and control animals exhibited no changes in body weight.

Effect of betulin on tumor incidence, tumor number, and tumor volume

Table 1 demonstrates the tumor incidence, total number of tumors, and tumor volume of control and experimental animals. We observed tumor development in all DMH-treated rats. Oral administration of BE in DMH-treated rats significantly reduced tumor incidence (by 16.66%), reduced tumor volume, and decreased the total number of tumors ($P < 0.05$).

Effect of betulin on thiobarbituric acid reactive substances and antioxidant status

Figure 2 illustrates the level of TBARS and antioxidants (GSH, GPx, SOD, and CAT) in the colon and liver tissues. The level of TBARS was markedly elevated in liver ($P < 0.05$) and decreased in colon tissues of DMH-treated animals compared to control rats. The antioxidant activities of GPx, SOD, GSH, and CAT decreased significantly in the colon and liver tissues of DMH-treated rats ($P < 0.05$). On the other hand, BE administration significantly elevated the activities of these TBARS and antioxidants compared to the rats administered with only DMH. BE alone and control rats showed no differences.

Effect of betulin on activities of detoxification enzymes

Figure 3 demonstrates the level of Phase I (CYP_{450} and Cyt-b_5) and Phase II (GST and GR) detoxification enzymes in liver and colon tissues of experimental and control rats. The levels of Phase I enzymes were markedly increased, whereas the activities of Phase II enzymes were considerably lowered in DMH-treated rats compared with the control animals (both $P < 0.05$). Oral supplementation of BE led to a significant reduction in Phase I enzymes and improvement in Phase II enzymes when compared with cancer-bearing rats (both $P < 0.05$). BE alone and control rats showed no differences.

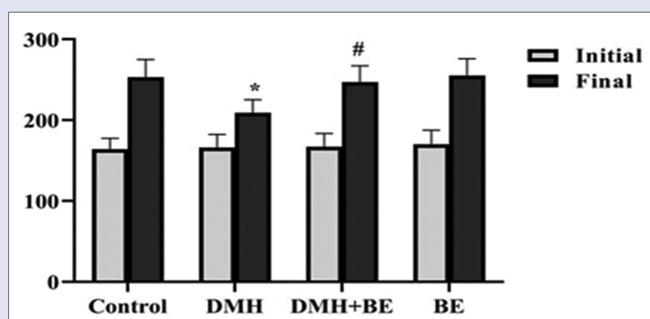

Figure 1: Initial and final body weight changes of control and experimental rats in each group. Values are given as mean ± standard deviation for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^{*}P < 0.05$ when compared with vehicle control group, $^{\#}P < 0.05$ when compared with 1,2-dimethylhydrazine-induced group

Effect of betulin on activities of bacterial enzyme

Figure 4 illustrates the activities of enzymes specific to the colonic mucosal and fecal bacteria, such as β-glucuronidase and mucinase, in the control and experimental rats. The bacterial enzyme activities were significantly increased in DMH-treated rats as compared to control rats ($P < 0.05$). BE administration significantly decreased the activities of these bacterial enzymes as compared to DMH-treated cancer-bearing animals ($P < 0.05$). BE alone and control rats showed no differences.

Effect of betulin on histopathological alteration of colon tissue

Figure 5a-d shows the colon tissues of experimental and control rats. The histological assessment of control (a) and BE alone (d)-treated rats demonstrated normal architecture of submucosal and mucosal layers. DMH alone (b)-treated animals illustrated proliferating mucosal glands with severe dysplastic alterations. The DMH-treated rats administrated with BE (20 mg/kg bwt) (c) showed glands within regular limits surrounded by lymphoid aggregates.

Effect of betulin on expression of inflammatory markers

Figure 6 illustrates the protein expression of inflammatory markers, including COX-2, IL-1β, IL-6, iNOS, and TNF-α, in colon tissues of experimental and control animals. The DMH-treated rats illustrated a markedly increased expression of these inflammatory markers compared to the control rats ($P < 0.05$). BE administration in DMH-treated rats markedly decreased the expression of inflammatory markers in their colon tissues ($P < 0.05$). BE alone and control rats showed no differences.

Effect of betulin on expression of cell proliferative markers

Figure 7 shows the protein expression of cell proliferative markers, including PCNA and cyclin D1, in colon tissues of experimental and control animals. The DMH-treated rats demonstrated markedly augmented levels of cyclin D1 and PCNA compared to the control

Table 1: Effect of betulin on total number of tumors, tumor incidence, and tumor volume of control and experimental animals

Groups	Total number of tumors (*n*)	Tumor incidence (%)	Tumor volume (mm^3)/rat
Control	0	0	0
DMH	(6)/6	100	13.27±0.69*
DMH+BE	(1)/6	16.66	5.37±0.07#
BE	0	0	0

Tumor volume was calculated using the formula V=4/3π (D1/2) (D2/2) (D3/2), where D1, D2, and D3 are the three diameters (in mm) of the tumor; ( ) indicates total number of rats bearing tumors. Values are given as mean±SD for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^{*}P<0.05$ when compared with vehicle control group, $^{\#}P<0.05$ when compared with DMH induced group. BE: Betulin; DMH: 1,2-dimethylhydrazine; SD: Standard deviation

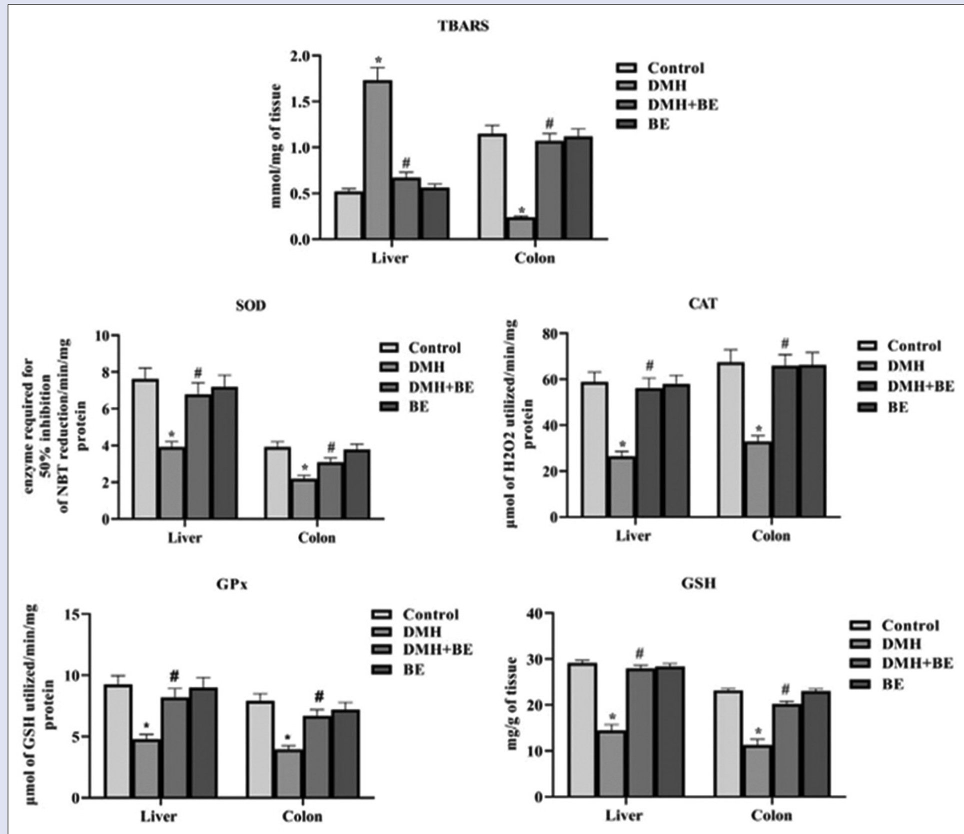

Figure 2: The level of thiobarbituric acid reactive substances and antioxidants were shown in the liver and colon tissues of control and experimental animals in each group. Values are given as mean ± standard deviation for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^{*}P < 0.05$ when compared with vehicle control group, $^{\#}P < 0.05$ when compared with 1,2-dimethylhydrazine-induced group

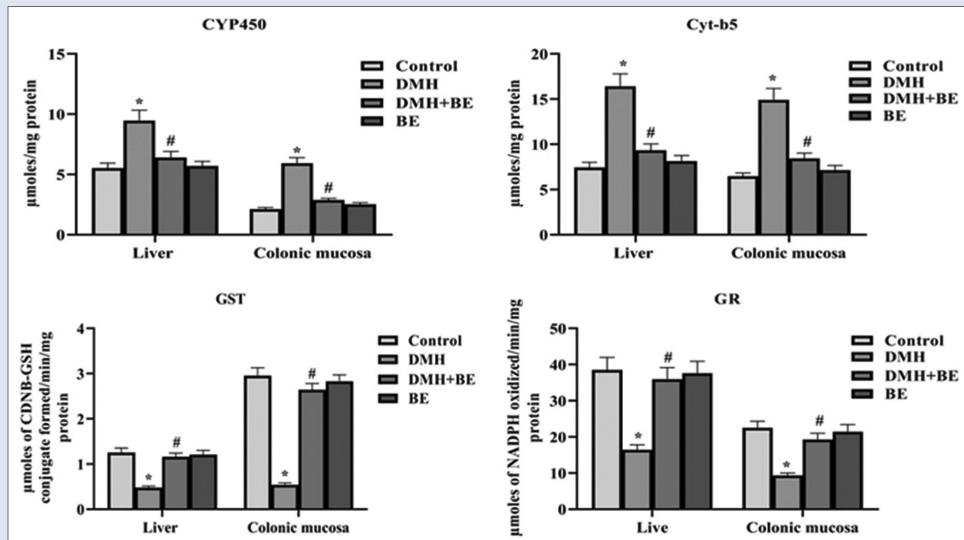

Figure 3: The level of Phase I and Phase II enzymes were shown in the liver and colonic tissues of control and experimental animals in each group. Values are given as mean ± standard deviation for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^{*}P < 0.05$ when compared with vehicle control group, $^{\#}P < 0.05$ when compared with 1,2-dimethylhydrazine-induced group

rats ($P < 0.05$). On the other hand, BE-treated cancer-bearing animals exhibited significantly reduced levels of PCNA and cyclin D1 in colon tissues ($P < 0.05$). BE alone and control rats showed no differences.

DISCUSSION

The rats administered with only DMH exhibited an increase in body weight at the start of the experiment that later reduced significantly; the

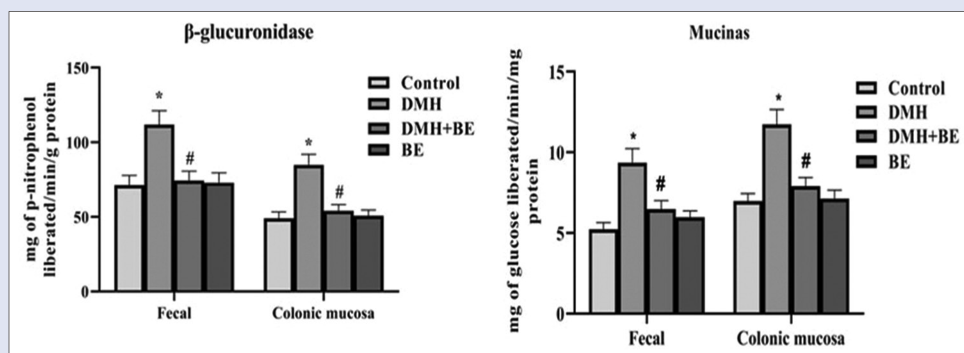

Figure 4: The level of bacterial enzymes was shown in the colonic mucosal and fecal tissues of control and experimental animals in each group. Values are given as mean ± standard deviation for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^{*}P < 0.05$ when compared with vehicle control group, $^{\#}P < 0.05$ when compared with 1,2-dimethylhydrazine-induced group

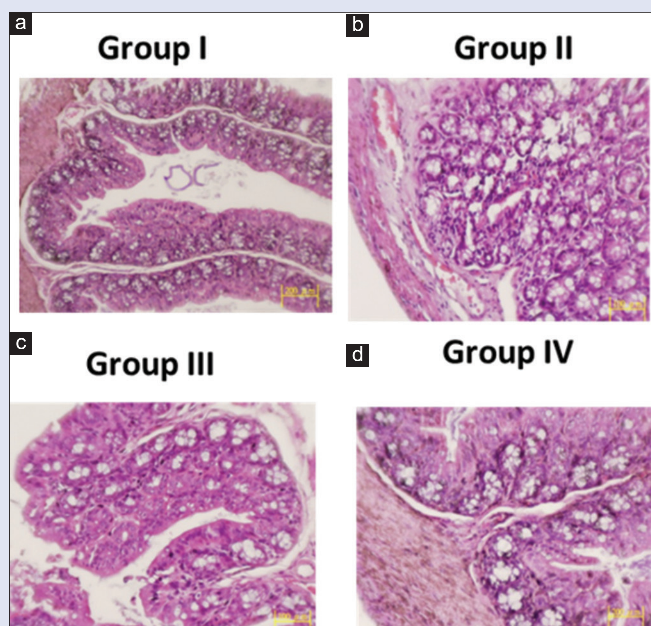

Figure 5: The histopathological changes of the cross section of the rat colon stained with hematoxylin and eosin. The colon of control (a) and betulin (d) alone-treated rat showing normal architecture with mucosal and submucosal layers. Whereas, colon of a 1,2-dimethylhydrazine (b) alone-treated rat was showed proliferating mucosal glands with severe dysplastic changes representing transformation to carcinoma. The colon of 1,2-dimethylhydrazine with betulin (c)-treated rat showing glands within normal limits

initial increase in the weight might be attributed to augmented polyp burden-driven cachexia and increased tumor formation.[27] Our results were in agreement with those of earlier reports in which DMH-treated rats showed decreased body weight and increased tumor growth.[28] Oral administration of BE to DMH-induced cancer-bearing rats led to an improvement in the body weight due to the inhibition of tumor growth, which subsequently inhibited cachexia. The histopathological analysis of the colon evidently revealed that BE prevented colon carcinogenesis primarily by altering the efficiency of DMH to activate neoplastic alterations. DMH-only supplemented rats exhibited proliferating mucosal glands with severe dysplastic alterations. BE alleviated histopathological alterations due to the anticarcinogenic effects. A previous study has also reported the antitumor potential of BE in colon cancer cells.[29]

Oxidative stress can induce cell damage due to mutagenesis, lipid peroxidation (LPO), and DNA damage, which have been related to different stages of tumor development.[30] LPO is a free radical arbitrated progression, which is implicated in the production of lipid radicals that lead to the formation of various degraded products, such as lipid hydroperoxides and malondialdehyde, and finally, injury to the cells.[31] Antioxidants have been shown to prevent the activation and development of carcinogenesis and neutralize cell damage.[32] Our current study has shown considerably decreased levels of antioxidants in DMH-treated animals, which could be due to their increased utilization, inactivation by hydrogen peroxide and glycation of the enzyme, and also decreased TBARS level. Earlier studies also reported augmented LPO and decreased levels of antioxidants in DMH-treated colon cancer in animals.[33] In the current study, the oral administration of BE considerably lowered the levels of TBARS as well as enhanced the antioxidants, which might be due to the strong antioxidant effects of BE. Previous reports have also demonstrated the antioxidant properties of BE on lipopolysaccharide-stimulated macrophages.[34]

Under normal conditions, Phase I metabolizing enzymes are the primary microsomal mixed-function oxidases and the polar groups of chemicals (e.g., hydroxyl group) that become substrates for conjugation by Phase II enzymes. Phase II metabolic enzymes are related to the formation and conjugation of glutathione conjugates, glucuronides, and sulfates.[35] β-glucuronidase is dependable for the hydrolysis of conjugated glucuronides in the lumen of the gut.[36] It might enhance the production of carcinogenic and toxic substances, which had earlier been detoxified by glucuronide conjugation in the liver, and consequently, penetrated the colon through bile or blood. Mucinase hydrolyzes the defensive mucin layer of the colonic wall, which exposes the underlying epithelial cells to the carcinogens cleaved by β-glucuronidase.[37] Therefore, in our study, the DMH-treated rats demonstrated improved activities of Phase I and bacterial enzymes and reduced Phase II enzymes similar to the previous studies.[35,38] Oral supplementation with BE significantly decreased the bacterial and Phase I enzyme activities and increased Phase II enzymes.

Chronic intestinal inflammatory process is an imperative risk factor in the etiology of colon cancer. During the inflammatory process, activated NF-κB stimulates several inflammatory genes such as IL-6, IL-1β, and TNF-α.[39] In the current study, DMH-treated rats demonstrated higher levels of inflammatory cytokines due to the stimulation of different types of stimuli. Karthikkumar *et al.*[40] also reported increased levels of pro-inflammatory cytokines in DMH-treated cancer-bearing animals. BE administration in DMH-treated rats decreased the levels of

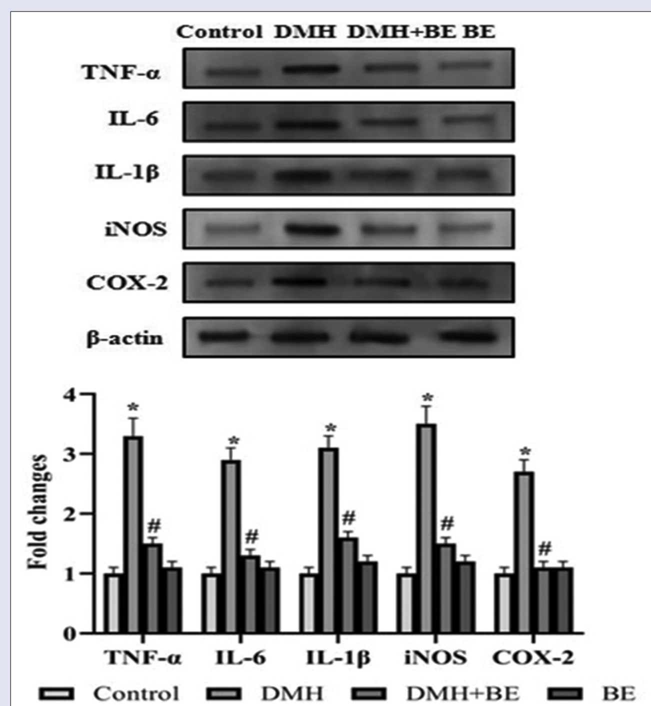

Figure 6: Representative images show the effect of betulin on the inflammatory markers such as tumor necrosis factor-alpha, interleukin-6, interleukin-1 beta, inducible nitric oxide synthase, and cyclooxygenase-2 of control and experimental rats. The band intensities were quantified by densitometry and normalized to respective β-actin loading control. Values are given as mean ± standard deviation for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^*P < 0.05$ when compared with vehicle control group, $^{\#}P < 0.05$ when compared with 1,2-dimethylhydrazine-induced group

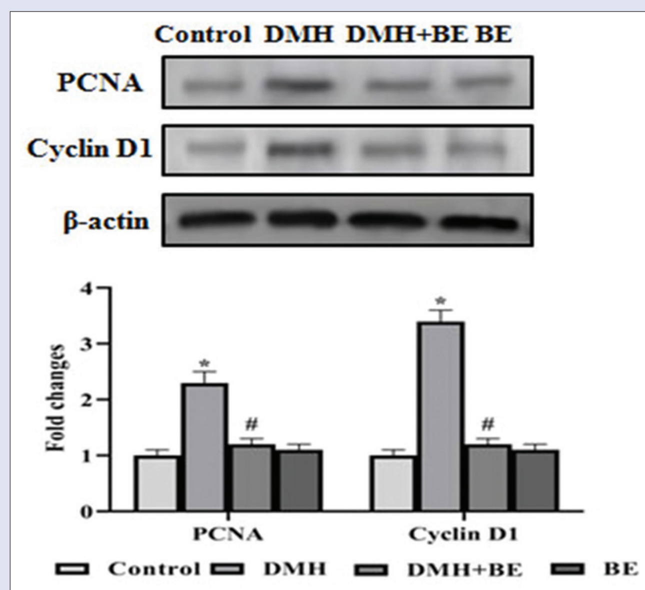

Figure 7: Representative images show the effect of betulin on the cell proliferative markers such as proliferating cell nuclear antigen and cyclin D1 of control and experimental rats. The band intensities were quantified by densitometry and normalized to respective β-actin loading control, and the representative graph shows the relative protein expression of fold changes. Values are given as mean ± standard deviation for groups of 6 rats in each. Statistical significance was determined by one-way ANOVA, followed by Tukey's *post hoc* test, where $^*P < 0.05$ when compared with vehicle control group, $^{\#}P < 0.05$ when compared with 1,2-dimethylhydrazine-induced group

inflammatory cytokines. The tumor-inducing property of iNOS in the colon might be associated with the capability of NO to enhance the levels of COX-2.[41] COX-2 is considered as an activator of carcinogenesis in several organs including the colon. COX-2 levels might be used as an indicator of cancer progression, and regulation of COX-2 expression has become one of the primary goals for the prevention and treatment of colon cancer.[42] Previous studies have reported that carcinogen-treated animals exhibited augmented expressions of COX-2 and iNOS due to colon tumorigenesis,[30] which corroborated our findings. Administration of BE decreased the levels of these inflammatory mediators owing to the anti-inflammatory properties of BE. A previous study also reported that BE inhibits the generation of inflammatory cytokines in LPS-stimulated macrophages.[34]

Cell proliferation plays a vital function in initiation, promotion, and progression of colon cancer.[43] Dysregulated cell proliferation is one of the major features of colon carcinogenesis, which involves different stages of tumor development. This dysregulation primarily occurs due to interrupted G1/S phase cell cycle transition during the growth of malignant neoplasm.[44] PCNA is a 36-kDa protein that acts as a central cell proliferative marker in various cancers, including colon cancer and cyclin D1, and is implicated in the G1/S phase transition of cell cycle. An augmented expression of these cell proliferative markers in DMH-treated cancer-bearing animals indicates upregulated proliferation of tumor cells. Earlier studies also reported increased expressions of these proliferative markers in colon cancer.[40,45] Oral supplementation of BE decreased the levels of these proliferative markers, which evidently showed antiproliferative activity of BE. Previously, BE has been shown to exhibit antiproliferative effects toward human colorectal adenocarcinoma HT-29 cells.[46]

CONCLUSION

BE administration inhibited tumor formation, protected the cells from oxidative damage, decreased the bacterial and Phase I enzymes, regulated cell proliferation, suppressed the inflammatory process via controlled release of pro-inflammatory cytokines, increased Phase II enzymes, and altered the histopathological changes in DMH-induced colon cancer model of rats. These results revealed the anticancer effects of BE and indicated that it could be used as a potential anticancer agent against colon cancer.

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Conflicts of interest

There are no conflicts of interest.

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