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Research Article
Protective Effect of n-butylidenephthalide Against 1, 2-dimethylhydrazine Induced Colon Cancer in Mice

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Abstract

Background: Colon cancer is considered as the third most common cancer affecting both developed and developing countries. In the current study, n-butylidenephthalide (BP) a compound extracted from traditional Chinese herb Radix *Angelica sinensi* was assessed against 1,2-dimethylhydrazine (DMH)-induced colon cancer. **Materials and Methods:** To promote colon cancer, DMH (20 mg kg^{-1}, s.c.) was administered twice a week for two weeks. To evaluate the protective effect of BP, it was co-administered with DMH in 150 and 300 mg kg^{-1} (p.o.) to respective groups for a total period of seven weeks. At the end of treatment, colon tissue was excised for various biochemical and microscopic evaluation. **Results:** Administration of BP 150 and 300 mg kg^{-1} to respective groups revealed significant alteration in various biochemical parameters like MDA, GSH, SOD and catalase ($p<0.05$ and $p<0.01$), respectively. Microscopic studies conducted on colonic tissue revealed reduced crypt multiplicity following treatment with BP 300 mg kg^{-1}. Furthermore, histopathological and transmission electron microscopic studies showed remarkable reduction in dysplasia, normal cellular and sub cellular structures, respectively. **Conclusion:** In the present study, significant results were produced with the treatment of BP in graded manner and exhibited protective effect against DMH induced colon cancer in mice by virtue of its antioxidant and antitumor activity.

Key words: Colon cancer, 1,2-dimethylhydrazine, oxidative stress, aberrant crypt foci, n-butylidenephthalide

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colon cancer is the third most common cancer, which develops as non-cancerous benign tumor and eventually transforms into adenocarcinoma. According to Indian Council of Medical Research (ICMR), the incidence for colon cancer is higher in men than women in India[1]. Various predisposing factors like familial and medical history, sedentary life style involving lack of physical activities, smoking, alcohol, obesity and diet are responsible in the genesis of colon cancer[2]. Recent research revealed that intake of red meat significantly rises risk of colon cancer[3]. Cellular oxidative stress plays an important role in cancer development by causing DNA damage, which can be countered by endogenous free radical scavengers and antioxidants[4].

The DMH is a toxic environmental pollutant, which was reported as a specific colon procarcinogen. Animal studies showed that experimental colonic tumors induced by DMH were of epithelial origin with a similar histology, morphology and anatomy to human colonic neoplasms. This pro-carcinogen could thus provide an adequate model for studying colorectal cancer[5]. The DMH is believed to form active intermediates including azoxymethane and methylazoxymethanol in the liver, which are transported subsequently into the colon via bile and blood. Methylazoxymethanol is decomposed to form methyldiazonium ions, which methylate cellular components. The DMH also produces free radicals that induce oxidative DNA damage in the liver and colon. Damage to DNA from ROS is a consequence of oxidative stress and several oxidative DNA adducts, including 8-oxodG, have been implicated in the tumorigenic process[6]. The ACF system can be used as a short-term bioassay to screen potentially new chemopreventive agents and to evaluate the effect of protective factors at a very early stage of the carcinogenic process[7,8]. They are defined as crypts that have (a) altered luminal openings, (b) exhibit thickened epithelia, (c) are larger than adjacent normal crypts and (d) are microscopically elevated[8].

Radix *Angelica sinensis*, a traditional Chinese herb used in treatment of various ailments pertaining to blood circulation system, immune system, menstrual and gastro-intestinal disorders[9]. The n-butylidenephthalide is an active compound isolated from it, exhibiting wide range of pharmacological activities including cytotoxicity against prostate cancer[10], hepatic cancer[11], lung cancer[12]. The current study aims to assessing the protective effect of n-butylidenephthalide against 1, 2-dimethyle hydrazine (DMH) induced colon cancer in mice.

MATERIALS AND METHODS

Experimental animals: Thirty male Swiss albino mice weighing 20-22 gm were procured from National Institute of Nutrition (NIN), Hyderabad. Animals were caged in polypropylene cages, had free access to food and water with 12: 12 h dark/light cycle. Animals were acclimatized a week before the initiation of experimental protocol. All experimental procedures undertaken were in accordance with CPCSCEA guide lines, the study was reviewed and approved by Institutional Animal Ethical Committee, G. Pulla Reddy College of pharmacy, Hyderabad.

Chemicals: The 1,2-dimethylehydrazine, n-butylidenephthalide (BP), thiobarbituric acid, Griess reagent, vanadium trichloride, trichloroacetic acid, EDTA and Ellman's reagent were purchased from Sigma aldrich. All other chemicals used were of analytical standard.

Study design: After the completion of acclimatization period, animals were randomly selected and divide into five groups each containing five animals and following treatment was given:

- **Group 1:** Served as normal control and received saline twice a week for a period of two weeks and simultaneously was co-administered with tween 80 (p.o.) for a period of seven weeks
- **Group 2:** Served as disease control and received DMH 20 mg kg^{-1} (s.c.) twice a week for a period of two weeks and tween 80 (p.o.) for a period of seven weeks
- **Group 3:** Received DMH 20 mg kg^{-1} (s.c.) twice a week for two weeks and was co-administered with BP 150 mg kg^{-1} (p.o.) for a period of seven weeks
- **Group 4:** Received DMH 20 mg kg^{-1} (s.c.) twice a week for two weeks and was co-administered with BP 300 mg kg^{-1} (p.o.) for a period of seven weeks
- **Group 5:** Received saline (s.c.) twice a week for 2 weeks and BP (300 mg kg^{-1}, p.o.) for 7 weeks

During the treatment period, body weight was carefully noted with a weekly interval. After completion of the treatment, animals were sacrificed by cervical dislocation using diethyl ether as anesthesia. Excised colonic tissue was subjected to various microscopic and biochemical estimations.

Determination of Aberrant Crypt Foci (ACF): To determine Aberrant Crypt Foci (ACF) isolated, colonic tissues were fixed

using 10% formalin solution. Tissues were stained with 0.2% methylene blue for 5 min and observed under light microscope. The ACF were distinguished by their slit like opening, darkly stained, size and pericryptal zone[13].

Biochemical estimations

Determination of malondialdehyde (MDA) concentration: It was done by the method suggested by Ohkawa *et al.*[14] Tissue homogenate (2%) in 0.15 M KCl with 8.1% SDS (200 µL) was incubated at room temperature for 5 min. Acetic acid (20%, 1.5 mL) and equal volume of 0.8% thiobarbituric acid was added and reaction mixture was heated at 95°C for 90 min. Distilled water of 1 and 5 mL of butanol/pyridine (15:1) solution was added after cooling the mixture was centrifuged at 1000 rpm for 15 min and resultant colored layer was separated and measured at 532 nm using UV spectrophotometer[14].

Determination of Superoxide dismutase (SOD) concentration: It was performed by the method suggested by Misra and Fridovic[15]. Tissue homogenate supernatant (0.5 mL) was taken, to it 1.5 mL of carbonate buffer (pH 10.2), 0.5 mL of 0.1 mM EDTA was added, 0.4 mL of epinephrine was addedbefore measuring OD at 480 nm using UV spectrophotometer[15].

Determination of catalase activity: It was determined by the method described by Aebi[16]. Supernatant of 2% tissue homogenate (0.1 mL) was added to cuvette containing 1.9 mL of 50 Mm H_2O_2 absorbance was read at 240 nm for 15 and 30 sec using UV Spectrophotometer[16].

Determination of Glutathione (GSH) concentration: It was analyzed according to Ellman's method, where 10 mg of tissue was homogenized in 0.1 M phosphate buffer (pH 7.4). About 0.5 mL of tissue homogenate was added with equal volume of 20% trichloro acetic acid containing 1 mM EDTA and allowed to stand for 5 min and subjected to centrifugation at 200 rpm for 10 min. Supernatant (200 mL) was added with 1.8 mL of Ellman's reagent (0.1 mM) containing 0.3 M phosphate buffer with 1% sodium citrate. This mixture was read at 412 nm using UV spectrophotometer[17].

Determination of total nitrite concentration: It was estimated in accordance to griess reaction. Tissue homogenate (100 mL, 2%) prepared in normal saline, 100 mL of VCl_3 solution and griess reagent was added and incubated at 37°C for 3 min. The OD was measured at 540 nm[18].

Histo-pathological evaluation: Isolated colon sections were fixed in 10% buffered formalin for 24 h the specimens were dehydrated in ascending grades of ethanol and embedded in paraffin wax. About 5 mm thick sections were made and deparaffinized using xylene and ethanol. The slides were washed with PBS and permeabilized with 0.1 M citrate, 0.1% triton X-100; these sections were stained with hematoxylin and eosin and observed under light microscope.

Statistical analysis: Results are expressed as Mean±SEM and analysis was performed by one way ANOVA followed by Tukey's multiple comparison tests by using Graph Pad prism software (5.03 versions).

RESULTS

Body weight: Induction of colon cancer significantly decreased the gain in body weight in diseased control mice, when compared to normal control. Concurrent treatment with BP (150 and 300 mg kg^{-1}) significantly improved ($p<0.01$*) colon cancer induced loss in body weight, when compared to disease control mice and this effect was dose dependant manner (Table 1).

Aberrant crypt foci: No ACF were detected in the colon of any of the animals untreated with DMH. The BP, applied at low dose (150 mg kg^{-1}) and high dose (300 mg kg^{-1}) significantly decreased ($p<0.05$) the number of total aberrant crypts, ACF and crypt multiplicity in a dose-dependent manner (Fig. 1 and Table 2).

Biochemical estimations

Colonic MDA, nitrite, SOD, GSH and catalase: Administration of DMH in disease control group exhibited elevated levels of oxidative stress markers such as MDA, nitrite where as reduced endogenous antioxidants and free radical scavengers like SOD, catalase and GSH. Co-administration of BP (150 and 300 mg kg^{-1}) doses significantly reversed ($p<0.01$) the effect of DMH dose dependently as shown in Table 3.

Table 1: Initial and final body weights and body-weight gain in normal mice and DMH administered mice concurrently treated with vehicle and BP

Groups	Initial (g)	Final (g)	Weight gain (g)
Normal control	24.67±1.211	31±2.828	6.33±1.211
Disease control	25.33±1.211	28.67±0.816	4.167±0.752*
BP (150 mg kg^{-1})	24.83±1.941	30.00±1.414	5.33±1.366
BP (300 mg kg^{-1})	24.17±1.169	30.33±1.211	6.167±1.722
Standard control	24.83±1.472	31.33±1.862	6.5±1.871

Data is expressed as Mean±SEM, *$p<0.01$ when compared to normal contro

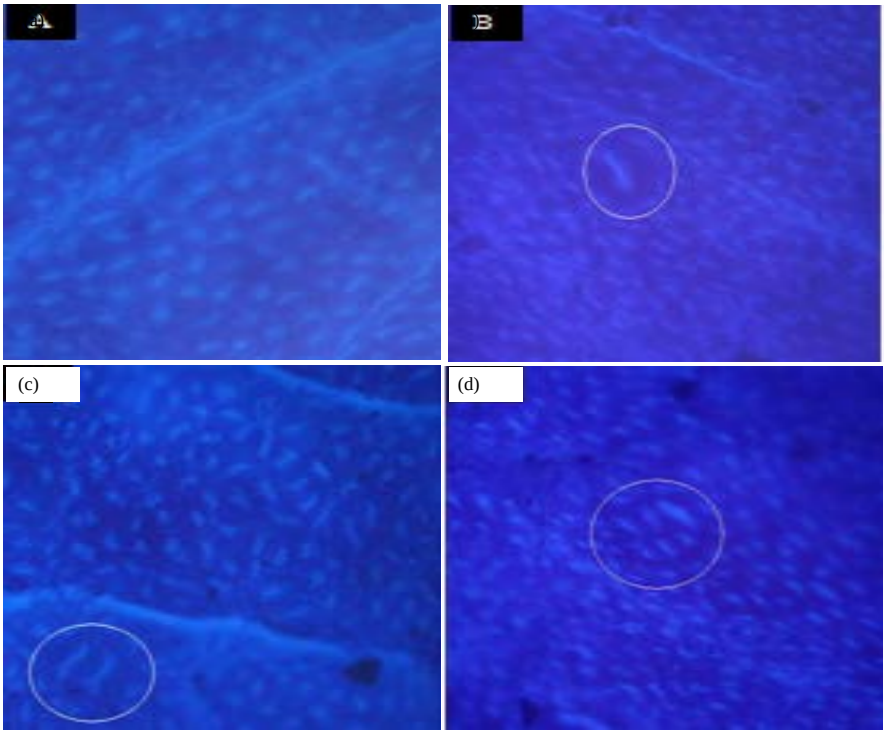

Fig. 1(a-d): (a) Normal colon, (b) Colonic ACF with 1 crypt, (c) Colonic ACF with 2 crypts and (d) Colonic ACF with ≥4 crypts

Table 2: Effect of piperine (25 and 50 mg kg^{-1}) on DMH-induced colonic ACF formation and crypt multiplicity in mice

	No. of ACF with						
	1	2	3	≥4	Aberrant	Aberrant	Crypt multiplicity
Groups	Crypts				crypts (AC)	crypt foci (ACF)	(AC/ACF)
Disease control	16.2±1.643	9.4±1.673	6.4±1.14	4.4±0.547	74.8±6.611	36.4±3.209	2.053±0.054
n-bp (150 mg kg^{-1})	13.60±1.140	7.2±0.837	4.4±1.140	2.4±0.547^{b}	55.2±5.762^{c}	29.8±2.168^{b}	1.918±0.0526
n-bp (300 mg kg^{-1})	10.40±1.140^{c}	6.3±0.837^{d}	2.2±0.837^{a}	1.4±0.547^{a}	38.00±3.872^{a}	21.40±1.517^{a}	1.736±0.0323^{c}

Data was expressed as Mean±SEM, ^{a}p<0.0001, ^{b}p<0.001, ^{c}p<0.01, ^{d}p<0.05 when compared to disease control

Table 3: Colonic levels of MDA, Nitrite, SOD, GSH and catalase in normal and DMH induced colon cancer mice concurrently treated with vehicle and BP

Group	MDA (nmol mg^{-1})	Nitrite (μmol g^{-1})	SOD (μmol g^{-1})	GSH (μmol g^{-1})	Catalase (K mL^{-1})
Normal control	19.02±3.755	8.896±1.269	5.168±0.754	4.830±0.627	0.588±0.093
Disease control	67.43±8.181$^{\alpha}$	31.87±3.834$^{\alpha}$	2.632±0.672$^{\alpha}$	2.655±0.345$^{\alpha}$	0.276±0.052$^{\alpha}$
BP 150 mg kg^{-1}	38.25±2.665$^{\alpha,a}$	16.97±2.123$^{\alpha,a}$	3.514±0.195$^{\beta}$	3.596±0.312$^{\alpha,a}$	0.382±0.021$^{\beta}$
BP 300 mg kg^{-1}	30.65±1.562$^{\beta,a}$	14.28±1.343$^{\beta,a}$	3.696±0.306$^{\beta,c}$	3.772±0.231$^{\beta,b}$	0.423±0.021$^{\beta,c}$
BP contol	16.63±2.739	8.733±0.732	5.240±0.608	4.814±0.228	0.589±0.107

Data was expressed as Mean±SEM, $^{\alpha}$p<0.0001, $^{\beta}$p<0.001 when compared to normal control, ^{a}p<0.0001, ^{b}p<0.001, ^{c}p<0.01 when compared to disease control

Histopathology: Histopathological findings of colon in normal mice revealed normal epithelium with regular crypts and goblet cells with no inflammation and dysplasia (Fig. 2a). The DMH treated mice had shown pathological findings like inflammatory cells and focal congestion in sub-mucosa and muscularis mucosa; colonic tissue also exhibited dysplasia (Fig. 2b). Treatment with BP (150 and 300 mg kg^{-1}) significantly ameliorated inflammation and focal congestion in muscularis mucosa and sub-mucosa layer. Moderate dysplasia is observed in mice treated with BP 150 mg kg^{-1} (Fig. 2c), whereas mild dysplasia in mice treated with BP 300 mg kg^{-1} (Fig. 2d).

Transmission electron microscopic studies: Administration of DMH caused notable changes in the cellular organelles and transformation of the normal cell, whereas the administration of BP in 150 and 300 mg kg^{-1} exhibited protective response by countering the devastating effect of DMH on cellular environment. As shown in Fig. 3, BP 300 mg kg^{-1} showed near to normal effect.

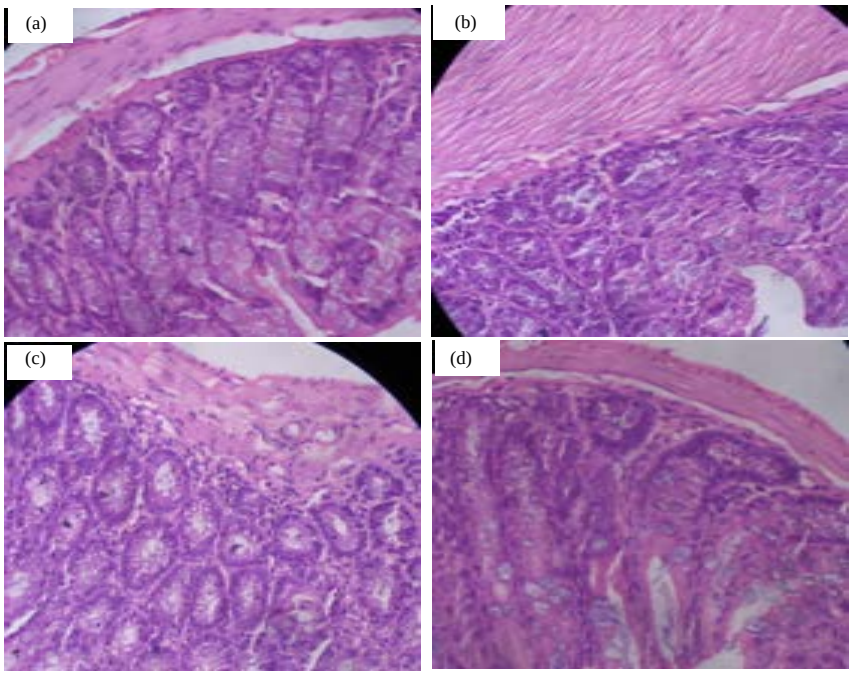

Fig. 2(a-d): Histopathological view of (a) Normal colon, (b) Diseased colon, (c) BP 150 mg kg^{-1} and (d) BP 300 mg kg^{-1}

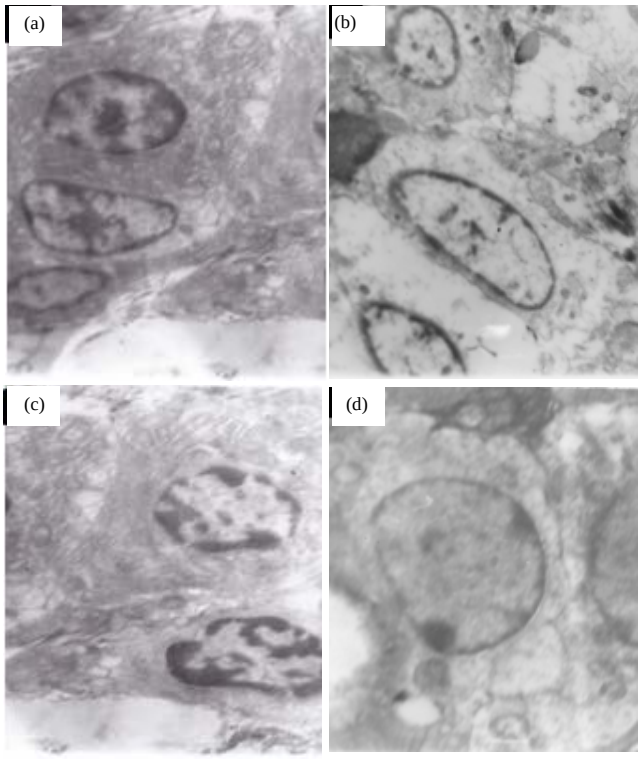

Fig. 3(a-d): Transmission election microscopic view of (a) Normal colon, (b) Diseased colon, (c) BP 150 mg kg^{-1} and (d) BP 300 mg kg^{-1}

DISCUSSION

Colon cancer accounts for about 8% of all cancer related mortalities, in recent times incidence of colon cancer is rapidly rising in Asian countries[19]. The devastating effect of any cancer can be estimated by survival rate of the effected individuals, in case of colon cancer despite attempts like early screening of susceptible individuals, could not produce a significant effect on survival rate. Colon cancer remains unnoticed unless spreading to the surrounding tissues, as survival and cure rate of it remains unaffected, China has shown highest survival rate and unfortunately India has the lowest[20]. A study conducted in Shanghai (China) produced a plausible relevance in curbing the cancer incidence by improving diet by incorporating fruits and vegetables[21]. The 1, 2-dimethyl hydrazine is a carcinogen, which specifically targets colon and produces mutation by causing DNA methylation, alteration in nucleic acid and histone proteins[22]. The sequence of colon cancer development in rodents is similar to one, which is exhibited by humans. In the present study, subcutaneous administration of DMH in mice produced aberrant crypt foci with higher crypt multiplicity, which is considered as a hallmark in colon cancer development, more over significantly reducing SOD, GSH and catalase. Clinical studies conducted on patients diagnosed with colon cancer show marked reduction of GSH and catalase due to the oxidative stess[23]. Similar studies reveal that higher concentration of MDA is associated with colon cancer[24]. In this study, remarkable increase in MDA levels were observed. Nitrite content was also found to be significantly elevated. Furthermore, signs of inflammation and changes in cellular environment were observed in histopthological and transmission electron microscopic studies, respectively.

The N-butylidenephthalide is a phthalide derivative, which has proven to be effective against various cancers including brain cancer; mechanistic studies revealed that it exhibits its action by causing cell cycle arrest in G_0/G_1 phase and inducing apoptosis by both p53 dependent and independent pathways[25]. In the current study, administration of BP exhibited protective effect against DMH induced colon damage, which was evident by modulation in various biochemical parameters in a dose dependent manner. Mice treated with only vehicle showed normal cell structure with normal mitochondria, endoplasmic reticulum and nucleus with centrally placed nucleolus. Mice treated with only DMH exhibited abnormal cellular environment with marginated chromatin, degenerated and shrunken mitochondria, endoplasmic reticulum and swollen nucleus with eccentric nucleolus. It is also evident by rapidly dividing nucleus and vacuolar degeneration. Thus, it shows that DMH treatment causes rapid cellular proliferation with abnormal cellular and sub-cellular organelles. Treatment with BP 150 mg kg^{-1} showed only a mild effect on intracellular organelles with condensed chromatin, slight vacuolar degeneration and thick and wide interstitium and indistinct nucleus. Treatment with BP 300 mg kg^{-1} showed better effect resulting in uniform cells and very mild vacuolar degeneration, distinct sub-cellular organelles like endoplasmic reticulum and mitochondrial matrix as shown in Fig. 3. Further investigation should be made to explore the pathways by which BP exhibits its protective action in colon cancer.

CONCLUSION

The n-butylidenephthalide treatment was effective in reducing the damage produced by 1, 2-dimethylhydrazine on colon in mice and exhibited modulatory effect on various biochemical parameters.

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