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Effect of Ethanol Extract of *Phyllanthus amarus* Leaf on 1,2- Dimethylhydrazine Induced Colon Carcinogenesis in Swiss Albino Mice

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ABSTRACT: *Phyllanthus amarus* is a highly medicinal plant with its therapeutic activities spanning a wide scope of diseases and ailments. Earlier literatures indicated the effectiveness of this plant in combating hepatitis, malaria, diabetes, and cancer. In the present study the effect of ethanol extract of *P. amarus* on colon cancer in Swiss albino mice induced by 1, 2 dimethylhydrazine (DMH) was investigated. 15 female Swiss mice of weight 25-35g were acclimatized for a week and randomized into 3 groups (5 per group). Group A (-DMH), Group B (DMH, 300mg/kg body weight of ethanol extract of *P. amarus*) and Group C (+DMH). The plant extract was administered daily with the aid of a gavage for 3 three weeks immediately after the administration of DMH. The investigation revealed that the carcinogenic effect of DMH-treated mice with increased CEA concentration was ameliorated by the administration of the ethanol plant extract at a dose of 300mg/kg bwt when compared to control. Caspase-3 activity was upregulated by the extract when compared to DMH-treated and control groups at $p<0.05$. Invariably, the activity of Caspase-3 in plant-treated group was not significantly different from control. TNF-α concentration in plant-treated mice was downregulated when compared to DMH-treated mice. The concentration of TNF-α of ethanol extract-treated group was not significantly different from the control group. Furthermore, histology of the liver and kidney revealed necrosis in the organ tissues of mice treated with DMH and gradual tissue repair presenting with moderate necrosis in the group treated with DMH and 300mg/kg bwt of the ethanol extract of *P. amarus*. In addition, there were no significant differences between the three groups ($p>0.05$) for haematological and oxidative stress indices. However, the *Phyllanthus* + DMH-treated group showed higher white blood cell count and haematocrit levels respectively when compared to the normal control (Group A). These results suggest that the ethanol extract of *P. amarus* may possess anticancer properties justifying their use in folklore medicine.

Keywords: *Phyllanthus amarus*, anticancer, liver and kidney, Carcinoembryonic antigen, TNF-a

Introduction

The *Phyllanthus* species known locally as “stone breaker” (believed to break up kidney stones), belong to Euphorbiaceae, one of the largest and commonest plant families in the world (326 genera, 7750 species). These plants are used traditionally to treat urolithiasis (eliminating the renal calculi), inflammatory bowel disease, diabetes and hepatitis B. One of the species of “stone breaker” (quebrapedra), *Phyllanthus amarus* is a small herb, usually under 30 cm tall, with numerous small oblong-elliptic or squarish leaves, glabrous, about 6-12 mm long; flowers very small, in cymules hidden under the leaves; cymules bisexual, of 1 male and 1 female flower; calyx-lobes 5, acute; pedicels 2 mm long; capsule small, depressed-globose; seeds 5-7-ribbed" (Isah and Ibrahim, 2014). This plant has a long history in medical herbalism system in the treatment of kidney and urinary bladder disturbances. Thus, substantial studies on *Phyllanthus* regarding their chemistry, pharmacological activity and clinical effectiveness have been carried out. The extracts of these plants have been reported to have pharmacological effects such as antiviral activity against hepatitis B and related hepatitis viruses, anti-bacterial activity, anti-hepatotoxic or liver-protecting activity, and hypoglycaemia properties (Etta, 2008; Mazumder *et*

al., 2006; Kloucek *et al.*, 2005). It occurs at low elevations (not collected above about 50 m) as a weed in villages, gardens, and cultivated fields, often abundantly naturalized" (Smith, 1981). It is common in disturbed places and croplands. It is a common weed found in cultivated and waste lands, propagated by seed (Joseph and Raj, 2011). The leaves of *P. amarus* have been shown to possess anti-carcinogenic, anti-tumor, antioxidant, antibacterial, antidiabetic, antifungal, and antiviral activities (Gupta and Vaghela, 2019). These medicinal properties in *P. amarus* can be attributed to the presence of its phytochemicals (Frank *et al.*, 2020).

Dimethylhydrazine (DMH), is a potent colon carcinogen, inducing colorectal tumors in experimental animals (Newell *et al.*, 2005; Saini *et al.*, 2009) and is the most widely used model of chemically induced colon carcinogenesis. DMH induced colon cancer is a multistep process involving a series of pathological alterations, such as formation of aberrant cryptic foci (discrete microscopic lesion) (Ionov *et al*, 1993) and it is metabolically activated in the liver by a series of reactions through intermediates (Azoxymethane) AOM and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite (Karthikumar *et al.*, 2020). The intermediates are highly reactive methyldiazonium ion which elicits oxidative stress by methylating biomolecules of the colonic epithelial cells, leading to inflammation and tumors in the colon (Swiderska *et al.*, 2014).

Cancer is a term used for a disease in which abnormal cells in the human body start dividing and growing without control and are capable of invading other tissues through the blood and lymphatic system, a phenomenon known as metastasis. Colorectal cancer (CRC) is a type of cancer that starts in the colon or rectum portion of large intestine in the gastrointestinal (GI) tract (Kopetz *et al.*, 2009). However, CRC is still an uncontrollable disease. In United States, CRC is the third leading cause of cancer-related deaths when men and women are considered separately, and second leading cause when both genders are combined. Most CRC starts with the polyps occurring on the epithelial lining of the colon or rectum. These polys may be benign (e.g. hyperplastic polyp), pre-malignant (e.g. tubular adenoma) or malignant (e.g. colorectal adenocarcinoma). It is estimated that about 20% cases of CRC have a family history of CRC. Some genetic syndromes are associated with greater risks of CRC, for example, hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) accounts for about 3% of people with CRC, and Gardner syndrome and familial adenomatous polyposis (FAP) are nearly always associated with CRC and are the causes of about 1% of all CRC cases. However, the majority of CRC cases are linked to environmental factors rather than heritable genetic changes (Rustgi, 2007). Occurrence of CRC may be dependent on various factors, namely diet, life style and other environmental parameters, including environmental and food-borne mutagens (Yang *et al.*, 2008).This research investigated the effect of ethanol extract of *P.amarus* leaf on 1, 2 dimethylhydrazine induced colon carcinogenesis in mice model.

Materials and methods

Collection and identification of Phyllanthus amarus plant: The leaves of *P. amarus* were collected from the Botanical Garden of University of Benin, Nigeria and was identified by an expert in the Department of Botany, University of Benin, Nigeria with voucher number 289BCM3425 assigned.

Plant extract preparation: The leaves of this plant were air-dried in the laboratory at the Department of Biochemistry, University of Benin, Benin City. The leaves were later pulverized to powdery form in Pharmacognosy laboratory at the Faculty of Pharmacy, University of Benin. 300 g of the powdered leaves of *P. amarus* was soaked in 2 L of absolute ethanol for 24 h with periodic stirring of the mixture. After 24 h, the mixture was filtered with fine cheese cloth, the residue was discarded and the filtrate was used to soak another 300 g of powdered leaves of same plant. This was allowed to stand for another 24 h with continuous stirring, thereafter, the mixture was again filtered. The residue was discarded and the filtrate was filtered with Whatman filter paper (No: 1) and subsequently concentrated with the aid of a vacuum concentrator at 30 °C. The concentrates was then weighed and used as experiment sample.

$$\%\ Yield\ = \frac{Mass\ of\ cude\ extract\ of\ plant}{Mass\ of\ \ dry\ weight\ \ of\ plant} \text{ X } 100$$

Animal study: Fifteen female Swiss albino mice of weight 25-35 g were purchased from *Kene-God Venture*, at the Department of AEB, University of Benin, Benin City, Edo State. They were maintained according to the Institutional Animal Guidelines (1912/PO/Re/S/16/CPCSEA) and acclimatized to diet and environment for 1 week after arrival. They were housed in a density of 5 animals per rack mounted plastic with detachable steel aerated covered cages and were given clean drinking water *ad libitum*. The temperature (20-22 °C) and lighting (12-h light/dark cycle) were constantly controlled. 1,2-dimethylhydrazine was dissolved in distilled water and was administered (25mg/kg body) orally. The volumes of gavage were from 0.25-0.35 mL. Oral administration of DMH lasted for 42 days (3 times a week). Upon completion of the doses of carcinogen, the DMH-treated

mice were randomized in 2 groups - Groups B and C. Group A served as negative control, Group B received 300 mg/kg body ethanol extract of *P. amarus* and Group C served as positive control. The plant extract was administered daily with the aid of a dolphin oral gavage for 21 days.

Animal sacrifice/sample collection: The animals were handled according to the guidelines for the treatment of laboratory animals. They were sacrificed at the end of the 21-day administration period (on the 22nd day) after an overnight fast. Each mouse was anaesthetized in a chloroform ($CHCl_3$)-saturated container and blood sample was collected via a heart puncture. The blood samples were collected in labeled *Lithium Heparin* and *Ethylene Diamine Tetra-Acetic Acid* (EDTA) tubes. The kidney and liver were collected in an organ bag with formalin for histopathology. Sections of the colon were placed in an organ bag containing 0.9% NaCl in distilled water which was later homogenized for antioxidant assay.

Carcinoembryonic antigen (CEA) assay: The level of carcinoembryonic antigen (CEA) was determined by enzyme-linked immunosorbent assay (ELISA) using rat carcinoembryonic antigen (CEA/CD66) kit.

TNF-α: The concentration of TNF-α was assayed using the Promokine Human TNF-alpha ELISA (Enzyme-Linked Immunosorbent Assay) kit. The kit uses an *in-vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human TNF-alpha in serum, plasma and cell culture supernatants.

Caspase-3 and 9: Caspase-3 and 9 Assay Kits (Colorimetric) ab39401 were used to assay for the activity of caspases that recognize the sequence DEVD

Antioxidant biomarkers: Superoxide dismutase (SOD) activity was assayed by the method (Misra *et al*., 1972). Catalase (CAT) activity was assayed as previously described (Cohen *et al*, 1970) and Malondialdehyde (MDA) level was estimated by the method Buege and Aust (1978).

Hematology assay: This was carried out using standard automatic hematology analyzers. ERMA haematology analyzer was used to perform the parameter of haematological analysis on whole blood (full blood cell count). This automated blood cell counter performs speedy and accurate analysis of 18 parameters in blood and it is used for *in-vitro* diagnostic use in clinical laboratories. The haematological analysis carried out by this haematology analyzer include direct measurement of the white blood cell (WBC) count, red blood cell (RBC) count, Haematology (Hgb), Haematocrit (HCT), Platelets (PLT), absolute lymphocyte (LY) count. The other parameters are derived which include mean cell volume (MCV), mean platelet volume (MPV), percentage lymphocyte (LY %), RBC distributor width (RDW) and platelet distribution width (PDW).

Statistical analysis: The results are presented as mean ± SEM. They were analyzed statistically via one-way Analysis of Variance (ANOVA) using Graph Pad Prism Package, version 10.3.0 and the significance of differences was determined using the Turkey-Kramer Multiple Comparisons Test as the post test for determination of significant difference between means. They were considered significant at $p<0.05$

Results

Results of the effect of ethanol extract of *P. amarus* on some oxidative stress markers of 1,2-dimethylhydrazine induced carcinogenesis in mice are presented in Table 1.

Table 1: Effect of ethanol extract of *P. amarus on* some oxidative stress markers of DMH-induced carcinogenesis in mice.

Groups	Treatment	MDA(x10^4)(mol/ wet tissue)	CAT (x10^{-2}(u/mg protein)	SOD (x10^3)(u/mg protein)
A	Normal Control (-DMH)	2.436±0.291^a	2.715±1.985^a	1.093±0.161^a
B	(DMH, 300 mg/kg bwt Et. *P. amarus*)	3.270±1.281^a	4.800±2.000^a	1.057±0.367^a
C	DMH Control (+DMH)	4.237±0.494^a	3.1850±27.850^a	0.966±0.203^a

All values are expressed as Mean ± SEM (n = 5). DMH = 1, 2 – Dimethylhydrazine; MDA = Malondialdehyde; CAT = Catalase; SOD = superoxide dismutase.

MDA concentration in control group was not significantly different from extract-treated group and DMH-treated. Invariably, DMH-treated group had the highest concentration of MDA indicating the effect of DMH in the colon. Similarly trend was observed in SOD and CAT activity. For CAT activity, DMH-treated mice had the least activity which again showed that DMH down regulated antioxidant activity in the colon of untreated mice.

Table 2: Effect of ethanolic extract of *P. amarus leaf* on some genetic biomarkers of DMH-induced carcinogenesis in mice

Groups	Treatment	CEA(ng/ml)	TNFα ($x10^2$)(ng/l)
A	Normal Control (-DMH)	1.850 ± 1.650^a	0.906 ± 0.024^a
B	(DMH, 300 mg/kg bwt Et. *P. amarus*)	1.000 ± 0.400^a	1.029 ± 0.003^a
C	DMH Control (+DMH)	4.2000 ± 0.300^b	2.970 ± 0.081^b

All values are expressed as Mean ± SEM. Values with different lowercase superscript represent significant difference at $p<0.05$. CEA = Carcinoembryonic antigen; TNFα = Tumor Necrosis Factor alpha.

Table 3: Effect of ethanolic extract of *P.amarus* leaf on caspase-3 activity of DMH induced carcinogenesis in mice

Group	Treatment	CASPASE-3 (rfu/mg protein) ($x10^{-1}$)
A	Normal Control (-DMH)	1.786 ± 0.108^a
B	(DMH, 300 mg/kgbwt Et. *P. amarus*)	1.276 ± 0.118^a
C	DMH Control (+DMH)	4.176 ± 0.230^b

All values are expressed as Mean ± SEM. Values with different lowercase superscript represent significant difference at $p<0.05$.

Table 4: Haematological parameters of ethanol extract of *P. amarus* leaf on DMH induced carcinogenesis in mice.

Groups	Treatment	WBC ($x10^9$/L)	HGB (g/L)	HCT (%)	LYMPH ($x10^9$/L)	RBC ($x10^{12}$/L)	GRAN (cfu/L)
A	**Normal Control (-DMH)**	8.23 ± 0.03^a	15.16 ± 0.16^a	47.40 ± 0.93^a	47.87 ± 2.22^a	7.94 ± 0.16^a	1.03 ± 0.08^a
B	**(DMH 300 mg /kgbwt Et. *P. amarus*)**	14.16 ± 0.32^b	16.36 ± 0.72^a	56.20 ± 1.87^b	68.36 ± 1.32^b	9.07 ± 0.86^a	1.6 ± 3.83^a
C	**DMH Control (+DMH)**	11.26 ± 0.31^b	16.26 ± 0.54^a	54.26 ± 0.32^b	70.9 ± 1.55^b	9.10 ± 0.44^a	1.1 ± 0.11^a

All values are expressed as Mean ± SEM (n = 3). Values with different superscript down the column represent significant difference at $p<0.05$. WBC: white blood cell count, RBC: red blood cell count, HGB: haemoglobin, HCT: haematocrit, LYMPH: lymphocytes, GRAN: granulocytes

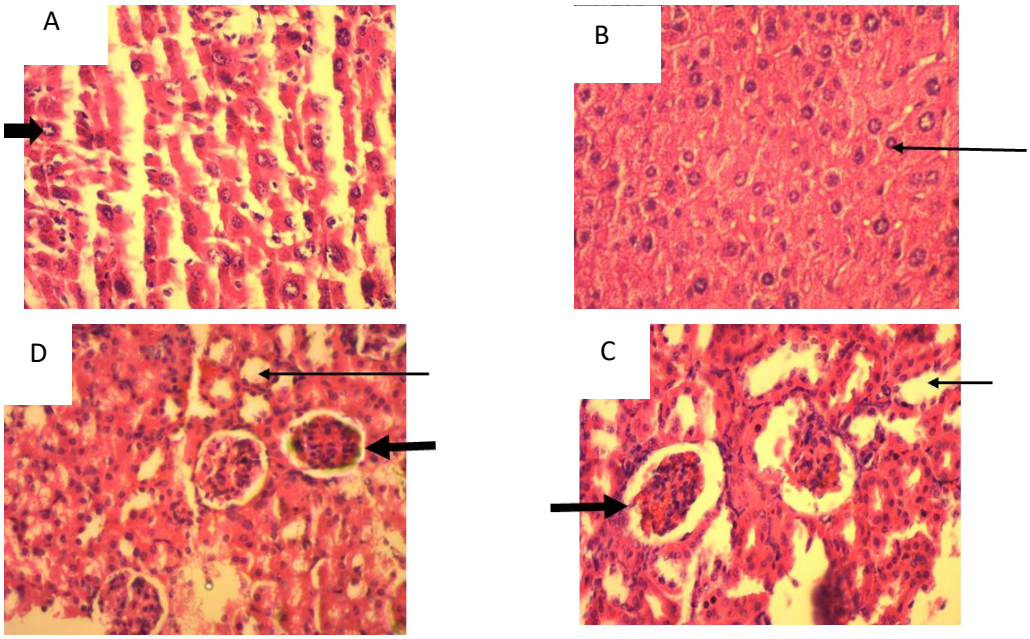

Plate A: (Control ×400 magnification): The histopathological examination of the liver of mice in the normal control shows normal hepatocytes (arrow). **Plate B: (DMH, 300 mg/kg bwt extract) ×400 magnification).** The histopathological examination of the liver of mice treated with DMH and 300mg/kg bwt shows normal hepatocytes (arrow). **Plate C: (+ DMH only) ×400 magnification).** The histopathological examination of the liver of mice treated with DMH shows necrosis of hepatocyte (thick arrow) & lobular lymphocytic infiltrates (thin arrow). **Plate D:** Control (Kidney) **(×400 magnification).** The histopathological examination of the kidney of mice in the normal control shows normal glomerulus (thick arrow) & normal tubules (thin arrow).

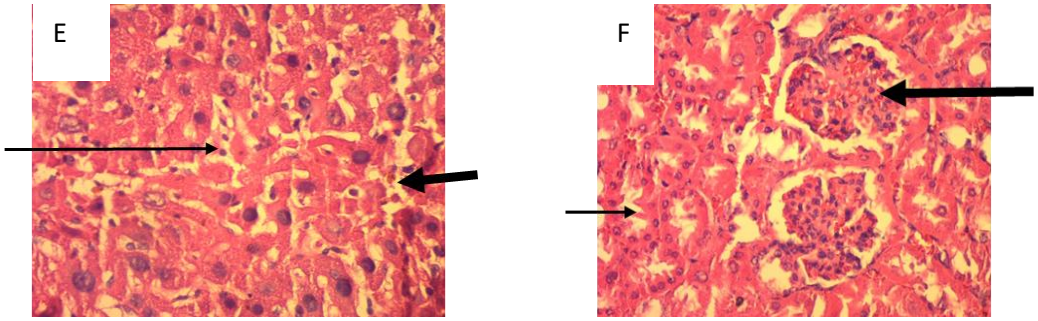

Plate E: (DMH, 300 mg/kg bwt extract) (×400 magnification). The histopathological examination of the kidney of mice treated with DMH and 300mg/kg bwt shows normal glomerulus (thick arrow) & normal tubules (thin arrow). **Plate F: (+DMH) (×400 magnification).** The histopathological examination of the kidney section of mice treated with DMH only shows normal glomerulus (thick arrow) & normal tubules (thin arrow).

Discussion

The bioactive components of plants have the potential to be major chemo-protective ingredients to prevent and/or manage cancer. Substantial evidence indicates that phytochemicals may play an essential role in colon cancer prevention and management (Gwyn and Sinicrope, 2002).

In this study, the preventive effects of ethanol extract of *Phyllanthus amarus* was investigated for their anti-colon cancer ability in Swiss albino mice. There is growing support for the concept that reactive oxygen species (ROS), which are known to be implicated in a range of diseases, may be important progenitors in carcinogenesis (Valko *et al.,* 2006). In the last decade, growing number of reports investigating association between ROS and carcinogenesis have been published. Researchers have proposed various consequences of oxidative stress that may be linked to carcinogenesis (Cejas *et al.,* 2004; Valko *et al.,* 2006; Mena *et al.,* 2009). From the results on antioxidant parameters (Table 4) there was no significant difference in the MDA level of mice treated with DMH when compared to the control and plant-treated group (300 mg/kgbwt.). Although, the concentration of MDA was significantly high in the diseased group. The plant extract was able to ameliorate the effect of DMH in the plant treated group. This report is in line with work done by Omoregie *et al.* (2017). Similar trend was observed in CAT and SOD activity. When control was compared to DMH and planted-treated, there was no significant difference across all the groups. Nonetheless, the diseased group had the least activity of SOD when compared to control and planted-treated groups. A possible reason could be attributed to the dose and the duration of administration of the ethanol extract of *P. amarus*. This finding is in accordance with the work of Skrzydlewska *et al.* (2005), who reported increased plasma and tissue catalase, MDA and SOD concentrations in colorectal patients which may be due to ROS production. These three tests are closely connected in their activity as superoxide dismutase functions to convert reactive oxygen species generated in biological systems to hydrogen peroxide, the hydrogen peroxide formed is toxic to biological systems and is converted to water by the activity of catalase. When there is a failure in the activity of these enzymes to convert reactive oxygen species to water, it would result in lipid peroxidation which would increase the concentration of malondialdehyde (MDA).

The carcinoembryonic antigen (CEA) of colorectal cancer is the most useful tumor marker to determine prognosis and to monitor patients with resettable colorectal cancer for early recurrence, and it is now widely tested (Gold and Freedman, 1965; Goldenberg *et al.,* 1981). The significant decrease ($p<0.05$) in the levels of CEA in mice administered 300 mg/kg bwt of ethanol extract of *P. amarus* when compared to the DMH-induced mice (group C) may suggest that the plant may contain anti-carcinogenic properties, as there was no significant difference between the *P. amarus*-treated mice and the normal control. The elevation of CEA concentration in DMH-treated group is in agreement with the work done by Lee *et al.* (2012). The increase of CEA in DMH-treated mice was as a result of the damage elicited by the effect of the carcinogen. Tumor necrosis factor alpha (TNF-α) concentration was also investigated. The concentration of TNF-α was significantly increased in the diseased group (+DMH) when compared to the control group and plant-treated groups. There was no significant difference between the groups treated with ethanol extract of *P. amarus* leaf and that of control at $p<0.05$, but DMH-treated group was significantly different from control. According to Waters *et al.* (2013) "Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine whose expression is increased in a variety of cancers" As mentioned earlier, TNFα is a major mediator of inflammation Sethi *et al.* (2012) with ambiguous effects.

In this study, Caspase-3 activity was down-regulated by DMH. The activity of Caspase-3 was significantly decreased in DMH-treated group when compared to control and plant-treated group, ethanol extract of *P. amarus* leaf upregulated the activity of caspase-3 in groups administered DMH in a manner similar to control

group. This again potentiates the anticancer potential of *P. amarus* leaf. This could only be possible as a result of the pharmacophores present in the plant extract.
Investigations on the haematological parameters revealed a significant increase ($p<0.05$) in the WBC of mice administered DMH when compared to the normal control. Treatment with 300 mg/kg bwt of ethanol extract of *P. amarus* did not produce a significant difference ($p>0.05$) in WBC count when compared to the DMH-treated group, as WBC in the *P. amarus*-treated group showed significant increase ($p<0.05$) in comparison to the normal control. A similar trend was seen in haematocrit and lymphocytes concentration. However, haemoglobin, red blood cells and granulocytes show no significance difference ($p>0.05$) across all the groups.
Histopathological examinations of the liver and kidney revealed necrosis of hepatocytes and globular lymphocytic infiltrates in mice treated with DMH indicating its hepato-renal toxicity. The effects were reversed upon the administration of 300 mg/kgbwt of *P.amarus* ethanol extract. This may be as a result of regeneration and repair of the liver and kidney, respectively, by the bioactive components in *P. amarus*. This is attributed to the flavonoids and phenolic contents of *P. amarus*. Also, the histological results is in tandem with the other findings.

Conclusion

The preliminary study was to investigate the effect of ethanol extract of *P .amarus* leaf on 1,2-dimethylhydrazine induced colon carcinogenesis in Swiss albino mice. The overall results suggest that ethanol extract of *P. amarus* leaf had ameliorating effects on DMH-induced colon carcinogenesis in Swiss albino mice. Noteworthy, 300 mg/kg body weight ethanol extract of *P. amarus* had ameliorative potential against the dysfunction triggered by DMH administration in the colon of Swiss albino mice.

Data availability statement

The data used to support the findings of this study are included in the article.

Ethical approval

Animal Ethics committee approval was given to carry out this study.

Competing interest

Authors declare no competing area of interest.

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