Evaluation of Anticancer Potential of *Mimosa diplotricha* Ethanolic Leaf Extract on N-Methyl-N-Nitroso Urea Induced Colorectal Carcinogenesis in Wistar Albino Rats

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ABSTRACT

Background: Colorectal Cancer, (CRC) ranks third among global cancer incidence statistics and occupies fourth position as far as cancer related death is concerned and the aim of the study is to evaluate the therapeutic potential of *Mimosa diplotricha* ethanolic leaf extract in chemically induced colorectal carcinogenesis in Wistar albino rats. **Materials and Methods:** CRC was induced by intrarectal instillation of N-Methyl-N-Nitrosourea (MNU), 2mg per rat in 0.5ml of distilled water three times a week for five weeks. Rats were grouped as control, induced, induced and standard drug treated (15mg/kg.bw), induced and plant extract treated (in two doses 200mg/kg.bw and 400mg/kg.bw) and normal rats with respective plant extract treatment (200mg/kg.bw and 400mg/kg.bw). Treatment were carried out for 60 days followed which animals were euthanized and serum markers, oxidative stress, microbial enzymatic, histological and immunohistochemical parameters were analysed. **Results:** *M. diplotricha* was found to be effective in the down regulation of serum inflammatory and tumour markers like tumour necrosis factor alpha, transforming growth factor beta, carcinoembryogenic antigen and colon cancer specific antigen-4 levels. Oxidative stress parameters and histological data also supported the therapeutic efficacy of *M. diplotricha*. Expression of anti-apoptotic protein Bcl-2 was also found be downregulated in induced animals treated with *M. diplotricha* as evident from immunohistochemical data. **Conclusion:** The results of present research suggest that *M. diplotricha* ethanolic leaf extract showed a significant therapeutic potential against chemically induced colorectal carcinogenesis.

Key words: CRC, Wistar rats, MNU, *Mimosa diplotricha*, Serum markers, Bcl-2.

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INTRODUCTION

Cancer constitutes to be the second leading cause of death globally next to cardiovascular disease and characterized by the expression of a normal cellular phenotype into a transformed one. The whole process of transformation is mediated by both extrinsic and intrinsic factors accompanied by a cascade of molecular events resulting in cellular proliferation, tumor formation and progression of malignancy. Colorectal cancer (CRC) is a frequently encountered gastrointestinal malignancy and as per estimates of American Cancer Society based on National Centre for Health Statistics data about 147,950 people would be diagnosed with colorectal cancer and 53,200 patients would die of the malignancy in 2020 of which 17,930 case registries and 3,649 individuals in both sexes would be below age 50.[1] Increase in CRC incidence is mainly due to change in dietary habits that contribute to elevated level of free radicals, changed life style and environmental conditions. CRC initiates as small out growths called polyps or aberrant crypt foci in inner lining of colonic mucosa which gradually progress into invasive adenoma and transforms into

malignant growth as a result of genetic and epigenetic alterations.[2] Colon carcinomas are usually associated with inflammations in intestinal mucosa. Frequently mutated genes in colon cancer includes Adenomatous Polyposis Coli (APC) gene leading to β-catenin stabilization and cellular proliferation mediated by Wnt/ β-catenin signalling. K-ras, is yet another proto-oncogene whose activation contributes to enhanced expression of Cyclooxygenase-2 (COX-2) leading to inflammation and associated carcinoma initiation and contributes as a risk factor in CRC progression. COX-2 expression also contributes to increased expression of inflammatory cytokine, tumour necrosis factor alpha (TNF-α) which further activates NF-kB signalling and tumorgenesis.[3,4] Transforming growth factor beta (TGF-β), signalling pathway is involved in biological process like cell proliferation, differentiation, migration and apoptosis. Elevated level of TGF- β for a prolonged time interval leads to transformation of normal cells to malignant cells. Elevated level of Carcinoembryogenic antigen (CEA) and Colon cancer specific antigen (CCSA-4) is a direct marker of colon carcinoma progression and metastasis.[4]

Apoptotic resistance is a unique characteristic of malignant cells. Apoptosis is a evolutionary preserved significant process involved in regulating cellular proliferation. Bcl-2 family of proteins play a key role in apoptotic regulation. Bcl-2 family harbours pro-apoptotic initiators, pro-apoptotic effectors and anti-apoptotic proteins of which Bcl-2 is an anti-apoptotic marker and overexpression of Bcl-2 in tissues indicate apoptotic inhibition.[5]

Genetic mechanism associated with carcinogenesis is highly heterogeneous and harbour varied molecular signalling pathways unique for each type of neoplasm and individual. This heterogeneity in tumour biology is the major challenge in oncology treatment strategies. Conventional and commonly practiced treatment options such as surgery, radiation and chemotherapy are beneficial, at the same time associated with high risk of side effects and toxicities. Hence, in medical oncology there is always a demand for alternative, complementary or supportive therapy to minimize the side effect. Natural products especially phytomedicine are widely explored due to enhanced curative potential with minimal side effects. Evidences substantiate that natural products harbouring bioactive ingredients are capable of modulating molecular signalling pathway involved in onset and progression of malignancy. Medicinal properties of plants are attributed to the presence of secondary metabolites like alkaloids, phenols, flavanoids, terpenoids, steroids etc. These secondary metabolites

are responsible for free radical scavenging and thereby reducing oxidative stress, a major risk factor for carcinoma onset. Dietary fibers, flavanoid rich vegetables and fruits, omega-3 fatty acids, Vitamin E and D are prescribed as dietary phytoconstituents to reduce the risk of CRC.[6] In this aspect exploring a phytochemical alternative for cancer therapy is an emerging strategy.

Mimosa diplotricha C. Wright ex Sauvalle is a perennial shrub species, belonging to subfamiliy Mimosoideae of the legume family (Fabaceae). The family comprises 40 genera and more than 2000 species and is pantropical in distribution.[7] In Formozan folk medicine roots of the plant is used as an antidote, analgesic, tranquilizer and haemostatic agent.[8] Plant is rich in polyphenols which are reported to have antimicrobial, antioxidant, anticancer and anti-inflammatory properties.[9] There are reports of isolation of bioactive compounds like pinoresinol, salicifoliol, quercetin, deoxyflavones, chalcones and diterpenoids from aerial parts and roots of *M.diplotricha* and their *in vitro* anti neoplastic activity on HT-29, A594, AGS, Hep G2 and PC3 cell lines.[9,10] These studies have documented a direct effect of the plant on various cancer cell growth directly, however to ascertain the efficacy of plant further research is needed using *in vivo* pre-clinical model system. Hence the aim of the present research was to evaluate the anti-colorectal cancer potential of *M. diplotricha* in MNU induced colorectal carcinoma in rat models that will aid in further exploring the species for anti CRC therapeutic in future.

MATERIALS AND METHODS

Chemicals: All chemicals including 5-flurouracil and N-Methyl-N-Nitrosourea (MNU) used for the study were of analytical grade and purchased from HiMedia laboratories (India) and Sigma-Aldrich (St. Louis, MO).

Plant collection, identification and preparation of extract: Fresh leaves of *M. diplotricha* (about 1000g) were collected from Palakkad district, Kerala, South India in the month of July-August. The specimen was identified and authenticated at Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2018/Tech./ 2040). The collected leaf sample of *M. diplotricha* was shade dried, powdered and the powdered sample was extracted with ethanol in 1: 5 (W/V) ratio for 72 hr by mild maceration. The ethanolic extract was filtered and allowed to dry. The dried extract was collected and stored in refrigerator for further analysis.

Experimental animals: Healthy adult Wistar albino rats of (140-150 g) of same age, obtained from animal house facility, Karpagam Academy of Higher Education were used for the present study. The protocols used

in the study were approved by Institutional Animal Ethical Committee and Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Ethical approval reference number is: KAHE/IAEC/2019/15-06/004. The animals were maintained in clean, polyurethane cages (25 ± 3°C) under healthy and sterile conditions as per ethics of animal research. They were fed with commercial food pellets and water *ad libitum* and maintained under 12 h light and dark cycles.

Acute oral toxicity study: A total of 6 animals were used for acute toxicological analysis. The study was performed according to the procedure as per Organization for Economic Cooperation and Development (OECD, 2002) guidelines 423.[11] Experimental animals were divided into two groups of three animals in each group. Grouping is as follow:

Control: With normal food and distilled water

Group I: Treated with a single dose, 2000mg/kg.bw of ethanolic extract of *M.diplotricha* by oral gavage on the first day of experimental analyses.

Animals were monitored for any toxic sign during the first 30 min and then further monitored for every 24 hr, 48 hr and 72 hr for any signs of abnormalities in behavioral pattern or any evident mortality. After 14 days rats were kept in overnight fasting and then subjected to euthanasia. Blood sample was collected for analysis of serum biochemical parameters. Histolopathalogical analysis of Liver, kidney and colon tissues were carried out.

Induction of CRC by MNU: CRC was induced in Wistar albino rats chemically by using MNU as tumor inducer. MNU was administrated intrarectally to experimental animals. Dosage and duration of induction standardized based on previous literature as 2mg per rat in 0.5ml of distilled water three times a week for five weeks.[4] After five weeks of MNU administration CRC induction was confirmed by analyzing bacterial marker enzymes, β-glucosidase β-glucuronidase and mucinase in fecal homogenate of treated rats. Rats expressing elevated level of the respective marker enzymes were considered to be CRC induced as colorectal carcinoma always results in higher expression of the corresponding enzymes.

Experimental design for anticancer study: After conformation of induction animals were grouped for anticancer study. The induced animals were then randomly selected and grouped into the following groups with six animals in each group; Group I: MNU induced, Group II MNU induced + Standard drug (5-flurouracil 15mg/kg.bw), Group III: MNU induced

+ plant extract 200mg/kg and Group IV: MNU induced + plant extract 400mg/kg. Dose of standard drug was standardized based on previous literature.[4] Plant extract dosage was selected based on toxicology study. Apart from induced groups normal wistar rats of same age and weight were selected and grouped as follows with six rats in each group; Group V: normal rats treated with plant extract 200mg/kg and Group VI: normal rats treated with plant extract 400mg/kg. Control rats remained untreated with food and distilled water. The method of administration of plant extract and standanrd drug was done orally and the duration of treatment was for 60 days (2 months). The experimental grouping of animals and the methodology of treatment was depicted in Table 1. Upon completion of experimental duration the rats were kept in overnight fasting and euthanized. Blood samples were collected for biochemical analysis. Colon tissue were removed, a portion fixed in formalin for histological and immunohistochemical analysis and a portion was used for the analyses of tissue antioxidant and microbial enzymatic parameters.

Biochemical analyses: Serum markers like CCSA-4, CEA, TGF- β and TNF-α were estimated using enzyme linked immunosorbent assay (ELISA) kit as per manufactures instructions. Serum biochemical parameters like total protein, glucose, AST, ALT, ALP, urea, uric acid and creatinine were evaluated using Agappe diagnostics kit as per the manufacturer's instructions.

S.No	Description	No. of Animals
Table 1: Experimental animal design for anticancer study.		
Control	Food and water.	6
Group I	MNU Induced	6
Group II	MNU Induced + standard drug (5-flurouracil 15mg/kg.bw, orally in alternate days) for 60 days	6
Group III	MNU Induced + *M. diplotricha* ethanolic leaf extract (200mg/kg, oral gavage) for 60 days	6
Group IV	MNU Induced + *M. diplotricha* ethanolic leaf extract (400mg/kg, oral gavage) for 60 days	6
Group V	Normal rats + *M. diplotricha* ethanolic leaf extract (200mg/kg, oral gavage) for 60 days	6
Group VI	Normal rats + *M. diplotricha* ethanolic leaf extract (400mg/kg, oral gavage) for 60 days	6

Determination of antioxidants and bacterial enzymatic markers in colon tissues: Colon tissues were excised, cleansed in ice cold normal saline and subjected to homogenization using Tris-Hcl buffer, pH 7.4, centrifuged at 3000 rpm for 10 min. The supernatant was used for lipid peroxidation (LPO) and antioxidant assays; superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), vitamin C and E using standard protocols.[12-17] For assay of bacterial enzymes colon homogenate were prepared in PBS saline pH 7.4. Enzymes such as mucinase, β-glucosidase and β-glucuronidase were estimated by standard experimental protocols described by Freeman, 1986 and Somogyi, 1952.[18,19]

Histopathological findings on Colon tissues: For histopathological evaluation immediately after euthanazia, Colon tissues were removed and washed with ice-cold normal saline and then fixed in 10% formalin for 24 hr. Thin paraffin sections of 5µm thickness were made using a rotary microtome. Further to this, the sections were stained with Haematoxylin and Eosin.[20] The histopathological slides were examined and photomicrographs were captured and analysed with a Zeiss AxioSkop 40 photomicroscope at 40X magnification.

Immunohistochemical staining in colon tissues: Immunohistochemical staining for Bcl-2 anti-apoptotic indicator was done according to the manufacturer's protocol (Dako ARK™ USA), based on avidin, biotin and peroxidase methodologies. Deparaffinize and rehydrate formalin-fixed paraffin-embedded tissue section. Appropriate antigen retrieval buffer or enzyme (primary antibody dependent) were used to treat sections followed by appropriate washing in buffer. Then add enough hydrogen peroxide blocking solution to cover the sections and incubated for 10 min. Again washing in buffer was carried out followed by application of protein block or normal serum from same species as secondary antibody and incubated for 5 min at room temperature to block nonspecific background staining. Wash once in buffer followed by application of primary antibody in antibody diluent and incubate. Wash 4 times in buffer

and incubate slide with biotinylated secondary antibody and wash 4 times in buffer. Apply streptavidin-HRP and incubate for 10 min at room temperature. Rinse 4 times in buffer and place the slide in DAB substrate ab64238 substrate and incubate until desired color is achieved (1-10 min). Rinse 4 times in buffer. Add enough drops of hematoxylin to cover the section. Incubate for 1 min. Cover slipping was performed as the final step before slides were examined under the light microscope. Photographs were taken at 40X magnification. The pattern of expression of Bcl-2 in different groups are indicated by arrow mark

Statistics: Numerical Figures were represented as mean ± SD. Variations between the groups were performed using one-way ANOVA followed by Duncan's multiple range test to assess individual difference between groups. A probability value of $p < 0.05$ was considered as statistically significant.

RESULTS

Acute oral toxicity results of the ethanolic leaf extract of M.diplotricha: Results revealed that *M.diplotricha* is found to be nontoxic upto 2000mg/kg.bw. There were no significant alterations in biochemical parameters like protein, glucose, urea, uric acid. Creatinine, chlosterol, HDL, ALT and AST (Table 2). Statistical alterations of numerical Figures in biochemical parameters in treated animals are also coming within the normal range of respective parameter and hence the numerical deviations cannot be counted as sign of toxicity. Histopathological results revealed that there is no manifestation of hepato and renal toxicity (Figure 1). Hence LD_{50} for *M. diplotricha* ethanolic leaf extract was found to be above 2000mg/kg.bw.

Changes in body weight in control and induced rats: Animals belonging to almost same age and weight were chosen for the experimental analysis. Initial weight of experimental animals comes in almost same range with slight deviation. After the duration of experiment is over the induced group (group I) exhibit significant reduction in final body weight (123 ± 4.2g) compared

Groups	Glucose mg/dl	Protein mg/dl	Urea mg/dl	Uric acid mg/dl	Creatinine mg/dl	Cholesterol mg/dl	HDL mg/dl	AST U/L	ALT U/L
Control	102.46 ± 0.15[1]	5.47 ±0.21[1]	4.87 ± 0.08[1]	9.21 ± 0.17[1]	0.84±0.03[1]	102.19 ± 0.11[1]	53.74 ± 0.18[1]	49.48 ± 0.33[1]	26.24 ± 0.014[1]
Group I	104.21± 0.01[2]	4.87 ± 0.08[2]	41.54 ± 0.25[2]	8.21 ± 0.09[2]	0.77± 0.12[2]	109 ± 0.15[2]	49.48 ± 0.33[2]	49.48 ± 0.33[2]	30.52 ± 0.23[2]

Table 2: Serum biochemical parameters in acute toxicological analysis.

Values are expressed as Mean ± SD (*n*=3). Statistical analyses were carried out using one way ANOVA followed by DMRT. Values not sharing common superscript letters differ significantly at *p*< 0.05.

to control (156 ± 2.9) as an indicative of neoplasm. Plant extract treated group (group III and IV) exhibit restoration of body weight (133.3 ± 1.2g and 137 ± 2.9g) compared to induced animals, indicative of therapeutic effect of the extract. Group II (standard drug) also exhibit an increase in body weight compared to induced rats. Normal rats treated with plant extract alone (group V, 161.67 ± 2.4g and group VI, 161.23 ± 1.9g) have final body weight without any considerable deviation from control and exhibit no sign of toxicity (Figure 2).

Analysis of serum markers: Results indicates a significant elevation of serum markers in CRC induced animals (group I) compared to control, evident as CEA (1.36 ± 0.03 ng/ml), CCSA-4 (121.34 ± 0.82 pg/ml), TNF-α (1.04 ± 0.03 pg/ml) and TGF-β (41.12 ± 0.61pg/ml) in disease control groups (Table 3). Induced animals with plant extract and standard drug treatment exhibit a down regulation in tumor markers compared to induced animals. Normal rats treated with plant extract alone have tumor markers within the normal range.

Serum biochemical parameters: From the results of serum biochemical parameters like protein, glucose, urea, uric acid, creatinine, ALT, AST and ALP it was evident that treatment with plant extract exhibit significant anti-tumor efficacy compared to induced animals. Renal parameters, glucose and protein were found to be significantly deviated from control rats in induced groups (Table 4). Plant extract treated groups as well as treatment with standard drug exhibit significant positive modulation in the above mentioned parameters. Normal rats treated with plant extract (group V and VI) elucidate no sign of toxicity in the respective biochemical parameters.

Effect of treatment on oxidative stress parameters: From the data (Table 5) it is evident that induced animals (group I) exhibit a significant down regulation in antioxidant parameters and an elevation in malondialdehyde (MDA) liberation (12.66 ± 0.1MDA liberated/min/mg of protein tissues) compared to control. However induced rats administrated with plant extract and standard drug showed significant elevation in respective antioxidant parameters and reduction in MDA liberation. Plant extract supplementation was found to be effective in normalizing the oxidative stress parameters.
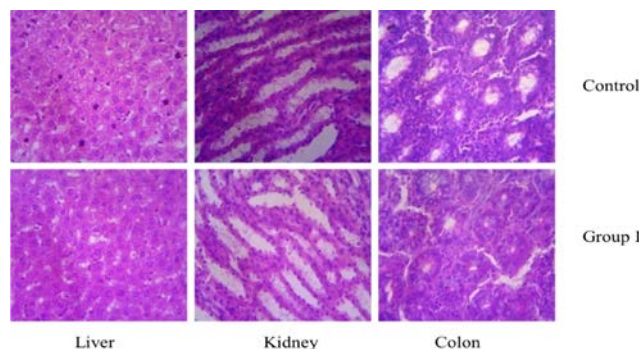

Figure 1: Morphology of liver, kidney and colon tissues in acute toxicity study.
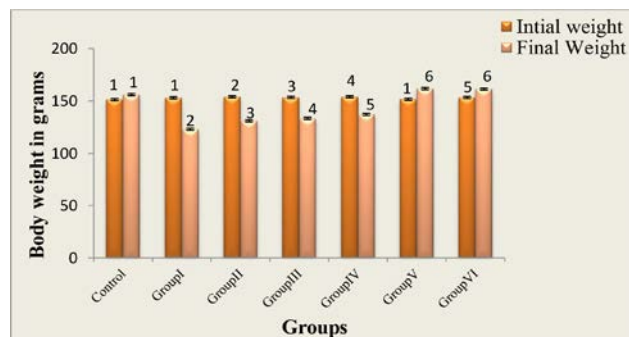

Figure 2: Effect of plant extract on body weight parameters in experimental rats.

Values are expressed as Mean ± SD (*n*=6). Statistical analyses were carried out using one way ANOVA followed by DMRT. When compared to control values not sharing common superscript letters differ significantly at *p*< 0.05.
Group I- MNU induced, Group II-MNU induced+5-flurouracil, Group III- MNU induced+ *M. diplotricha* 200mg/kg.bw, Group IV- MNU induced+ *M. diplotricha* 400mg/kg.bw, Group V-Normal+ *M. diplotricha* 200mg/kg.bw and Group VI-Normal+ *M. diplotricha* 400mg/kg.bw

Table 3: Serum level of CEA, CCSA-4, TNF-α and TGF-β in experimental animals.						
Parameters	**Control**	**Group I**	**Group II**	**Group III**	**Group IV**	**GroupVI**
CEA (ng/ml)	0.82 ± 0.02[1]	1.36 ± 0.03[2]	0.98 ±0.05[3]	1.02 ± 0.01[4]	0.94 ± 0.01[3]	0.84 ± 0.05[1]	0.88 ± 0.02[1]
CCSA-4 (pg/ml)	52.98 ± 0.32[1]	121.34 ± 0.82[2]	88.97 ± 0.43[3]	93.65 ± 0.32[5]	98.72 ±0.31[5]	56.88 ± 0.45[6]	58.06 ± 0.37[7]
TNF-α (pg/ml	0.63 ± 0.02[1]	1.04 ± 0.03[2]	0.88 ± 0.05[3]	0.96 ± 0.01[4]	0.94 ± 0.01[4]	0.59 ± 0.05[1]	0.61 ± 0.02[1]
TGF-β (pg/ml)	26.58 ± 0.27[1]	41.12 ± 0.61[2]	36.77 ± 0.38[4]	33.08 ± 0.45[3]	34.22 ± 0.27[3]	24.57 ± 0.29[1]	22.9 ± 0.43[5]

Values are expressed as Mean ± SD (*n*=6). Statistical analyses were carried out using one way ANOVA followed by DMRT. When compared to control values not sharing common superscript letters differ significantly at *p*< 0.05.
Group I- MNU induced, Group II-MNU induced+5-flurouracil, Group III- MNU induced+ *M. diplotricha* 200mg/kg.bw, Group IV- MNU induced+ *M. diplotricha* 400mg/kg.bw, Group V-Normal+ *M. diplotricha* 200mg/kg.bw and Group VI-Normal+ *M. diplotricha* 400mg/kg.bw

Table 4: Serum biochemical parameters in experimental animals.

Biochemical Parameters	Control	Group I	Group II	Group III	Group IV	Group V	Group VI
Protein g/dl	6.6 ± 0.0.1[1]	10.3 ±0.04[2]	8.2 ± 0.08[3]	7.8 ± 0.0.03[4]	7.3 ± 0.03[5]	6.7 ± 0.02[1]	6.5 ± 0.03[1]
Glucose mg/dl	123.5 ± 0.1[1]	83.1 ± 0.65[2]	126.5 ± 0.39[3]	102.3 ± 0.28[4]	103.8 ± 0.23[4]	112.9 ± 0.21[5]	114.6 ± 0.19[5]
Urea mg/dl	34.5 ± 0.15[1]	42.6 ± 0.5[2]	36.5 ± 0.35[3]	31.5 ± 0.3[4]	32.4 ± 0.21[5]	36.5 ± 0.2[6]	34.8 ± 0.18[1]
Uric acid mg/dl	5.89 ± 0.13[1]	5.16 ± 0.3[2]	6.73 ± 0.07[3]	6.1 ± 0.05[4]	6.3 ± 0.07[5]	6.5 ± 0.16[6]	5.9 ± 0.03[1]
Creatinine mg/dl	0.68 ± 0.01[1]	0.73 ± 0.02[2]	0.67 ± 0.02[3]	0.59 ± 0.01[4]	0.63 ± 0.22[5]	0.7 ± 0.1[1]	0.69 ± 0.04[1]
ALT U/L	57.1 ± 0.15[1]	73.3 ± 0.3[2]	59.8 ± 0.6[3]	50.9 ± 0.49[4]	52.3 ± 0.3[5]	58.2 ± 0.6[6]	57.1 ± 0.5[1]
ASTU/L	148.2 0.4[1]	116.3 ± 0.25[2]	159.9 ± 0.68[3]	126.1 ± 0.35[4]	132.4 ± 0.3[5]	144.6 ± 0.4[6]	138.7 ± 0.03[7]
Protein g/dl	6.6 ± 0.0.1[1]	10.3 ±0.04[2]	8.2 ± 0.08[3]	7.8 ± 0.0.03[4]	7.3 ± 0.03[5]	6.7 ± 0.02[1]	6.5 ± 0.03[1]

Values are expressed as Mean ± SD (*n*=6). Statistical analyses were carried out using one way ANOVA followed by DMRT. When compared to control values not sharing common superscript letters differ significantly at *p*< 0.05.
Group I- MNU induced, Group II-MNU induced+5-flurouracil, Group III- MNU induced+ *M. diplotricha* 200mg/kg.bw, Group IV- MNU induced+ *M. diplotricha* 400mg/kg.bw, Group V-Normal+ *M. diplotricha* 200mg/kg.bw and Group VI-Normal+ *M. diplotricha* 400mg/kg.bw.

Table 5: Enzymatic and Non-enzymatic parameters in experimental animals.

Parameters	Control	Group I	Group II	Group III	Group IV	Group V	Group VI
Supreroxide dismutase	1.84 ± 0.13[1]	0.82 ± 0.02[2]	1.26 ± 0.03[3]	1.28 ± 0.05[3]	1.29 ± 0.09 [4]	1.4 ± 0.01[4]	1.35 ± 0.02[4]
Catalase	1.02 ± 0.05[1]	0.85 ± 0.0.03[2]	1.58 ± 0.07[3]	1.13 ± 0.04[4]	1.27 ± 0.05[3]	1.16 ± 0.06[1]	1.59 ± 0.22[3]
Glutathione peroxidase	1.4 ± 0.03[1]	0.65 ± 0.03[2]	1.13 ± 0.04[3]	1.24 ± 0.03[4]	1.25 ± 0.03[4]	1.61 ± 0.07[5]	1.55 ± 0.02[6]
Glutathione S transferase	66.34 ± 0.09[1]	32.65 ± 0.08[2]	48.44 ± 0.09[3]	62.79 ± 0.05[4]	54.96 ± 0.05[5]	67.74 ± 0.11[6]	69.41 ± 0.07[7]
Glutathione reductase	4.44 ± 0.08[1]	2.33 ± 0.04[2]	3.37 ± 0.06[3]	3.48 ± 0.07[4]	3.42 ± 0.03[4]	3.77 ± 0.04[5]	3.88 ± 0.05[6]
Total reduced glutathione	6.78 ± 0.07[1]	2.56 ± 0.10[2]	4.61 ± 0.06[3]	5.48 ± 0.11[4]	4.7 ± 0.06[3]	5.56 ± 0.05[5]	5.66 ± 0.08[6]
Vitamin C	1.88 ± 0.05[1]	0.65 ± 0.25[2]	1.58 ± 0.22[3]	1.73 ± 0.06[4]	1.68 ± 0.45[4]	1.68 ± 0.45[1]	1.93 ± 0.04[5]
Vitamin E	1.07 ± 0.04[1]	0.55 ± 0.03[2]	1.04 ± 0.02[1]	1.28 ± 0.04[3]	1.23 ± 0.03[3]	1.35 ± 0.02[4]	1.12 ± 0.02[1]
Lipid peroxidation	8.51 ± 0.24[1]	12.66 ± 0.1[2]	10.33 ± 0.08[3]	11.15 ± 0.04[4]	8.96 ± 0.02[5]	8.51 ± 0.21[1]	8.48 ±0.4[1]

Values are expressed as Mean ± SD (*n*=6). Statistical analyses were carried out using one way ANOVA followed by DMRT. When compared to control values not sharing common superscript letters differ significantly at *p*< 0.05.
Group I- MNU induced, Group II-MNU induced+5-flurouracil, Group III- MNU induced+ *M. diplotricha* 200mg/kg.bw, Group IV- MNU induced+ *M. diplotricha* 400mg/kg.bw, Group V-Normal+ *M. diplotricha* 200mg/kg.bw and Group VI-Normal+ *M. diplotricha* 400mg/kg.bw
Units: Supreroxide dismutase - Inhibition of 50% nitrite formation/min/ mg protein tissues; Catalase - µmoles of H202 utilized/min/ mg protein tissues; Glutathione peroxidase - µg of glutathione oxidized /min/mg protein tissues; Glutathione S transferase - µmoles of CDNB-GSH conjugate formed/min/mg protein; Glutathione reductase - µmoles of NADPH oxidized /min/mg protein tissues; Total reduced glutathione - g/mg protein tissues; Vitamin C - µg/mg protein tissues; Vitamin E - µg/mg protein tissues and MDA liberated/min/mg of protein tissues

Bacterial enzymatic markers (β-glucosidase, β-glucuronidase and mucinase) in colon tissues of experimental groups: The level of enzymes in colonic tissue homogenate in induced rats evident as, mucinase (91.29 ± 0.13 µg/ml), β-glucosidase (65.21 ± 0.43 µg/ml) and β-glucuronidase (33.51 ± 0.24 µg/ml) was significantly elevated from control as represented in Figure 3. Elevation in colonic microbial enzymes is an indicative of carcinoma progression. The plant extract treated group exhibit a significant reduction in bacterial enzymes compared to induced rats indicating anti-neoplastic activity of the respective species.

Histopathology of colon tissues in experimental animals: Histopathology of colon tissues in control rats (A) display normal mucosal and submucosal layers with no sign of adenoma or polyps. Normal rats with plant extract administration alone (F-200mg/kg bw and G- 400mg/kg bw) also exhibit normal colonic architecture. Induced group (B) exhibit neoplasm with inflammation of intestinal crypts along with inflammatory infiltrates. In induced groups treated with standard drug (C) and plant extract (D-200mg/kg bw and E- 400mg/kg bw) there were evident reduction in
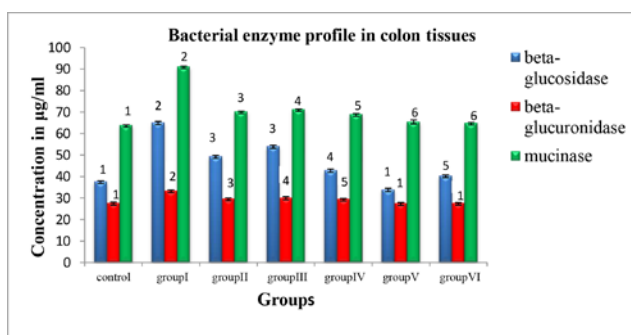
Figure 3: Level of bacterial enzymes β-glucosidase β-glucuronidase and mucinase in colon tissues.

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Group I- MNU induced, Group II-MNU induced+5-flurouracil, Group III- MNU induced+ *M. diplotricha* 200mg/kg.bw, Group IV- MNU induced+ *M. diplotricha* 400mg/kg.bw, Group V-Normal+ *M. diplotricha* 200mg/kg.bw and Group VI-Normal+ *M. diplotricha* 400mg/kg.bw
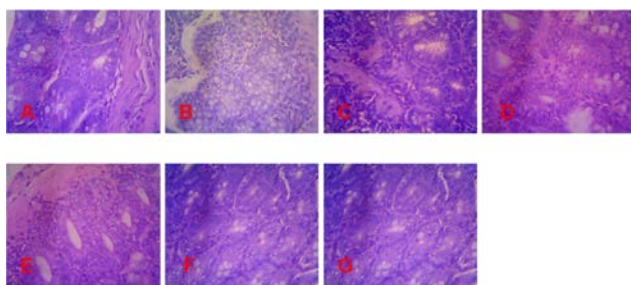

Figure 4: Histopathology of colon tissues; A- control, B-Induced, C-Induced + 5-flurouracil, D- Induced +Plant extract 200mg/kg bw, E- Induced +Plant extract 400mg/kg bw, F- Plant extract 200mg/kg bw and G- Plant extract 400mg/kg bw.

inflammatory infiltrates and neoplastic cells in colonic mucosa (Figure 4).

Immunohistochemical staining for Bcl-2 in colon tissues of control and experimental groups:
Immunohistochemistry of colon tissues using antibody against Bcl-2 revealed that MNU induced rats (Group B) exhibit severe positivity for Bcl-2, evident as darker staining intensity representing elevated expression of anti-apoptotic protein Bcl-2. Induced rats treated with standard drug, 5-FU (Group C) and plant extract 200mg/kg bw (Group D) exhibit moderate positivity for Bcl-2 staining. Animals treated with plant extract 400mg/kg bw (Group E) exhibit mild postivity for Bcl-2. Control rats (Group A), normal rats treated with plant extract alone (Group F and G) exhibit mild positive reaction (Figure 5).

DISCUSSION

Present investigation is aimed to focus on analysing the anti-cancer activity of *M. diplotricha* ethanolic leaf extract
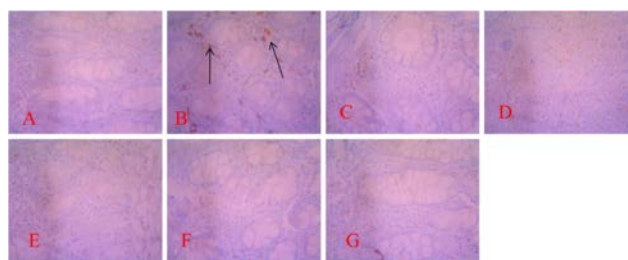

Figure 5: Immunohistochemical staining of colon tissues; A- control, B-Induced, C-Induced + 5-flurouracil, D- Induced +Plant extract 200mg/kg bw, E- Induced +Plant extract 400mg/kg bw, F- Plant extract 200mg/kg bw and G- Plant extract 400mg/kg bw. Arrow mark represent colonic tissues exhibiting severe positivity for Bcl-2 staining.

on MNU induced colorectal carcinoma using rat model system by evaluating biochemical, oxidative, histological and immunohistochemical data. As evident from result the extract exhibits significant anticancer effect.

As an initial step to address the efficacy of *M. diplotricha* on MNU induced colon carcinogenesis the animal models were subjected to acute oral toxicological analysis. Results evident by analysing biochemical and histological parameters revealed no observable changes in tissue morphology and serum parameters and hence it can be concluded that oral administration of *M. diplotricha* is safe upto 2000mg/kg.bw.

In anticancer study initial analyses were made on body weight changes in experimental animal models. A reduction in body weight in MNU induced groups was observed (Figure 2). Treatment with *M. diplotricha* significantly improved the body weight. The colonic lesions observed in MNU induced rats could have led to reduced nutrient uptake and there by reduction in body weight. Infiltration of immune cells in the colonic tissue suggested the inflammatory microenvironment which again could have contributed to the weight loss in MNU induced rats. The restoration of body weight in plant extract treated group is an indicative of medicinal attributes of potential anticancerous phytochemicals harbouring the species.

Elevated level of tumour specific markers CEA and CCSA-4 and inflammatory markers TNF-α and TGF-β in disease induced group is an implication of tumour progression and chronic inflammation. Enhanced level of CEA is an indicative of CRC poor prognosis and metastasis. CEA is a membrane bound glycoprotein found to be expressed highly in tumour tissues and in low levels in normal intestinal epithelial cells and is used for clinical diagnosis.[21,22] The release of membrane associated glycoprotein may be due to cleavage by proteases, cell lysis, shedding of plasma membrane

vesicles and due to action of phospholipases.[4,23] CCSA-4 is a highly specific and sensitive serum marker for colon cancer.[4] Elevated level of TGF- β promotes the transformation of normal intestinal epithelial cell by activation of TGF- β signalling pathway resulting in cell proliferation, angiogenesis, apoptotic inhibition, immunosuppression and constitute as one of the major altered signalling pathway in colorectal carcinogenesis.[24,25] Elevated expression of NF-kB signalling pathway may contribute to increased level of inflammatory cytokine, TNF-α leading to chronic inflammation which in course serves as a major factor for carcinoma initiation and progression.[26] In the present study *M. diplotricha* treated groups exhibit a reduction in tumor and inflammatory markers indicating the phytochemicals of corresponding species have the potentiality in modulating inflammatory pathway and carcinoma progression. Urea, uric acid and creatinine was found to be elevated in serum of tumour induced animals as a result of increase in free radicals such as reactive oxygen species which contributes to mesangial cell contraction and an associated decrease in glomerular filtration.[27] In plant extract treated groups a protective effect was noticed by a reduction in serum concentration of urea, uric acid and creatinine, attributed due to antioxidant effect exerted by phenolic compounds.

Oxidative stress parameters (tissue antioxidants) are up regulated in cancer induced rats receiving *M. diplotricha* treatment which supports the antioxidant activity of phytochemicals harbouring the plant species. Management of oxidative stress is a major concern in CRC onset and progression as colorectal carcinoma is directly correlated with oxidative stress in intestinal mucosa due to dietary risk factors. Endogenous antioxidant enzymes exhibit a promising role in scavenging free radicals. CAT prevents the oxidative damage by O_2 and H_2O_2. Superoxide radicals are eliminated by SOD. GSH provides reducing ability to peroxidases and thioltransferase and protect cell membrane from oxidative damage.[28]

Microbial enzymes can be considered as metabolic markers in CRC progression. In carcinogenic condition there will be an imbalance in normal intestinal microbial flora. This shift in intestinal microbial homeostasis leads to corresponding increase in microbial enzymes which can be considered as a sign of progression of neoplasm as these enzymes play a significant role in tumor progression. Carcinogenic and toxic substances are usually detoxified in liver and excreted via bile into intestinal lumen as glucuronide conjugates.[29] Elevated level of bacterial enzyme β-glucuronidase hydrolyses the conjugates and colon gets exposed to free carcinogens leading to tumor onset. Enzyme mucinase is involved in the hydrolyses of mucosal envelope of colon thereby exposing the underlying colonic epithelial to toxic and xenobiotic compounds. Increase in mucin level also stimulates inflammatory response.[30] Increased enzymatic markers in cancer induced rats is a sign of tumor progression. Plant extract treated group show a positive correlation with control animals in enzyme level thereby reducing the risk of carcinogenesis. Anatomical elucidation of colon histological features also supports the anti-cancer efficacy of *M. diplotricha* extract. Compared to induced animals plant extract treated group display a decrease in carcinoma progression as evident by reduced inflammatory infiltrations and adenomatous lesions.

Immunohistochemical staining revealed a significant upregulation in expression of anti-apoptotic protein Bcl-2 in cancer induced groups. Apoptosis have got a significant role in the tumor progression and metastasis. Bcl-2 is a direct marker of apoptosis and is usually used in grading tumor.[31] Bcl-2 is an apoptotic inhibitor that binds to mitochondrial outer membrane and blocks the release of cytochrome C, a pre-requisite for apoptosis. Bcl-2 is a proto-oncogene which prevents the oligomerisation of BAX/BAK protein which stimulates the expression of several apoptogenic proteins in mitochondria. Usually apoptosis is mediated by release of mitochondrial cytochrome C and activation of procaspase 9 to active caspase and subsequent activation of caspase 8 and 3 involved in apoptosis.[4] In the present study downregulation of Bcl-2 in plant extract treated carcinoma induced group indicates the apoptotic and antiproliferative potential of *M. diplotricha*. Apoptotic initiation can be considered as one major mechanism involved in the anticancer attribute of *M. diplotricha*. The phtyochemicals present in the species are able to induce apoptosis and prevent carcinoma progression. There are already reports of phytochemicals involved in executing anticancer potential by inducing apoptosis targeting activator caspases, activating protein-1 and by generating ROS (reactive oxygen species) thereby facilitating the generation of apoptogenic proteins to cytoplasm through mitochondrial membrane.[32] The plant extract is administrated orally in two respective doses 200 mg/kg.bw and 400 mg/kg.bw. Both the doses exhibit anticancer potential even though groups treated with dose 400 mg/kg.bw proved to be more effective in anti-neoplastic potential as evident from the results of corresponding parameters analyzed. Anticancer efficacy of plant extract in comparison with standard drug 5-flurouracil is also found to be significantly convincing and modulating.

CONCLUSION

Results of the present study suggest promising therapeutic efficacy of *M. diplotricha* against CRC induced by MNU in Wistar rats as observed from the analysis of biochemical, oxidative, enzymatic, histological and immunohistochemical data. This paves opportunity for intervention of *M. diplotricha* in advanced therapeutic research by analyzing the active ingredients and their action on molecular genetics in tumor microenvironment to find a better phytochemical alternative for CRC.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALT: Alanine aminotransferase**; ALP:** Alkaline phosphatase; **APC:** Adenomatous polyposis coli; **AST:** Aspartate transaminase; **BAX:** Bcl-2-associated X protein; **Bcl-2:** B-celllymphoma-2; **CAT:** Catalase; **CCSA-4:** Colon cancer specific antigen; **CEA:** Carcinoembryogenic antigen; **COX-2:** Cyclooxygenase-2; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **CRC:** Colorectal cancer; **DMRT**: Duncan's Multiple Range Test; **ELISA:** Enzyme-linked immunosorbent assay; **GSH:** Reduced glutathione; **HDL:** High-density lipoprotein; **K-ras:** Kirsten rat sarcoma virus oncogene homolog; **LPO:** Lipid peroxidation; **MNU:** N-methyl-N-nitrosourea; **NF-kB:** Nuclear factor kappa-light-chain-enhancer of activated B cells; **OECD:** Organisation for Economic Co-operation and Development; **ROS:** Reactive oxygen species; **SOD:** Superoxide dismutase; **TGF-β:** Transforming growth factor beta; **TNF-α:** Tumor necrosis factor alpha; **Wnt:** Wingless-type.
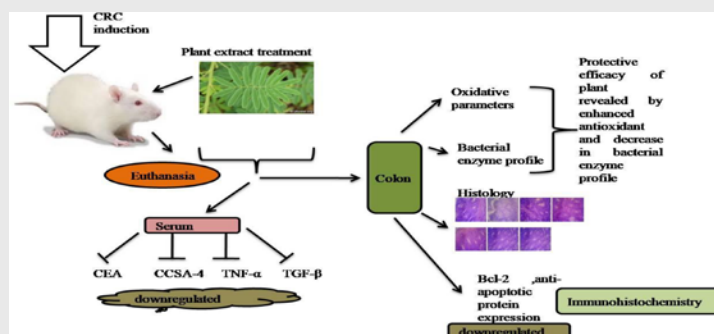
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SUMMARY

The present research evaluated and revealed the anti-colorectal cancer (CRC) potential of *Mimosa diplotricha* ethanolic leaf extract in chemically induced carcinogenesis in Wistar albino rats. The plant species belong to fabaceae and reported to be rich in polyphenolic compounds having significant antioxidant potential as oxidative stress is considered as a major risk factor in carcinoma onset. CRC is induced by intrarectal instillation of N-methyl-N-nitrosourea, 2mg per rat in 0.5ml of distilled water three times a week for five weeks. After carcinoma induction rats were subjected to treatment for a period of 60 days and euthanized for evaluation of biochemical, histological and immunohistochemical parameters. Results revealed that the plant extract treatment significantly downregulated the expression of CRC specific markers CEA and CCSA-4. The extract was also found to bring down the expression of inflammatory marker TNF-α and tumour related cytokine TGF-β. The oxidative stress parameters and microbial enzyme profile was also found to be positively modulated in plant extract treated group. Histological analysis of colon tissues also supports the protective efficacy of species. Immunohistochemical staining for anti-apoptotic protein Bcl-2 indicates an elevated expression of Bcl-2 in induced groups and a down regulation in plant extract treated group. Decrease in Bcl-2 expression can be an indicative of apoptotic potential of the species. From the data analyzed it can be concluded that *M. diplotricha* have promising therapeutic efficacy against CRC induced by MNU in Wistar rats as observed from the analysis of biochemical, oxidative, enzymatic, histological and immunohistochemical data. This paves opportunity for intervention of the species for advanced therapeutic research to find a better phytochemical alternative for CRC.

PICTORIAL ABSTRACT