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Evaluation of aqueous and hydro alcoholic extracts of *Clerodendrum viscosum* V. leaves & *Macrotyloma uniflorum* L. seeds against DMH-induced colon cancer

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ABSTRACT

Evaluation of anti-neoplastic activity of aqueous and hydro alcoholic extracts of *Clerodendron viscosum* V. leaves & *Macrotyloma uniflorum* L. seeds against DMH-induced colon cancer is the objective of this study. Extracts were obtained using cold maceration process. Six groups of animals, each containing ten male albino wistar rats (80 – 100 g) were administered with respective treatment to evaluate the anti-neoplastic activity. On the last day of experimental period, fecal matter was collected and colons were removed. Fecal matter was homogenized, and subjected to the measurement of fecal pH and bacterial enzymes. Colons of five animals were subjected for the presence of MPLs, ACFs in each colon, and histopathology by using standard methodology. Colons of remaining five animals were subjected to homogenization to measure the bacterial enzymes such as β-Glucosidase, β-Glucuronidase, and mucinase. The extracts showed significant protective effect by showing significant decrease in the levels of fecal and colon β-glucosidase, β-glucuronidase, mucinase; reduction in total number of ACFs in each colon and crypt multiplicity. Although, all the plant extracts has shown protective effect against colon carcinogenesis, treatment with hydro alcoholic extracts has produced more pronounced effect as compared with aqueous extracts.

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INTRODUCTION

According to the International Agency for Research on Cancer (IARC) around 555,000 people died of cancer in 2010 in India (IARC 2012). Based on the 979,786 cancer cases reported in the year 2010 by National Cancer registry Programme (NCRP), recent studies stated that the cancer cases are likely to go up to 1,148,757 by the year 2020 in India (Takiar, Nadayil & Nandakumar 2010).

Natural products are being explored extensively as alternatives to conventional therapies partly due to their multiple beneficial effects, less cost and minimal adverse effects. Traditionally, roots and leaf extracts of *Clerodendrum viscosum* V. have been used for the treatment of cough, scrofulous infection, venereal and skin diseases, tumors, rheumatism, asthma and other inflammatory diseases (Shrivastava & Patel 2007; Bhattacharjee *et al.* 2011). *Macrotyloma uniflorum* L. seeds traditional uses include asthma, bronchitis, fever, heart conditions, inflamed joints, leucoderma, localized abdominal tumors, sinus wounds and urinary discharges (Kawsar *et al.* 2009; Muthu *et al.* 2006). Traditional healers use these plant parts for treating tumors without any scientific rationale. The major phytochemical constituents reported in these plants are steroids, terpenes, flavonoids and glycosides.

The DMH (1,2-Dimethylhydrazine) induced colon carcinogenesis was selected for the evaluation of the anti-neoplastic activity, since it induces cancer in colon with high specificity which resembles to that of human colon cancer morphologically and histologically (Perse & Cerar 2005).

In literature, there are no reports regarding the antitumor activity of these two plants against DMH induced colon cancer. So, the present study is aimed to assess their antitumor activity of specific parts of the plants against DMH induced colon cancer.

MATERIALS & METHODS

Collection and Authentication of the Plant

The *Clerodendrum viscosum* V. leaves & *Macrotyloma uniflorum* L. seeds were collected from the natural population growing in the area of Erattayal, Palakkad dist., Kerala, India. The leaves were identified and authenticated in Regional Medical Research Centre (RMRC), Indian Council of Medical Research (ICMR), Belgaum by Dr. Harsha Hegde. RMRC-1123 and RMRC-1167 accession numbers has been provided to these plants respectively and the specimens were deposited in the herbarium of RMRC.

Preparation of the Extracts

Leaves of C*lerodendrum viscosum vent* and seeds of *Macrotyloma uniflorum* were shade dried for 7 days at 28±2 ºC and subjected to drying in hot air oven at 50±3 ºC. The dried plant materials were grounded and sieved to get fine powder and subjected to cold maceration with water and hydro alcohol (1:1 Water: Ethanol) separately in a shaker system at room temperature to obtain aqueous and Hydro alcoholic extracts respectively. Each extracts were filtered, and the filtrate was subjected to evaporation under reduced pressure to obtain dry extract. All the four aqueous and hydro alcoholic extracts were used for the evaluation of anticancer activity against DMH induced colon cancer.

Chemicals and Drugs

DMH (1,2 Dimethyl Hydrazine), p – nitro phenyl-β– D-glucopyranoside, Phenolphthalein –β-D – glucuronide, Porcine gastric mucin, Sodium arsenate heptahydrate and Tris-Base were obtained from Sigma chemicals, USA; whereas Glycine, and Methylene blue were obtained from Himedia laboratories, Mumbai.

Experimental Animals

Male Albino Wistar rats weighing 80-100 g were procured from St. Xavier's College, Ahmedabad (139/99/CPCSEA). The animals were housed and maintained as per CPCSEA guidelines. The Institutional Animal Ethics Committee of L J Institute of Pharmacy (IAEC Reg. No. 221/CPCSEA), Ahmedabad has approved the experimental protocol to conduct the experiments. All the protocols and the experiments were conducted in strict compliance with the ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Induction of Colon Cancer

Animals were administered with 20 mg/Kg of DMH subcutaneously once a week for 4 weeks (Nirmala & Ramanathan 2011). DMH was dissolved in 1mM EDTA just prior to use and the pH was adjusted to 8 with 1 M sodium bicarbonate to ensure the stability of the chemical.

Experimental Design

Animals were divided into six groups each containing ten animals. The experimental design has been mentioned in the Table.1.

One day prior to sacrifice, rats were kept in individual metabolic cages and the next day fresh faecal matter was collected. The rats were sacrificed by cervical decapitation using ether anaesthesia. Liver and colon were excised and rats were examined for the presence of any abnormal growth in other tissues. Fecal matter was homogenized, and subjected to the measurement of fecal pH and bacterial enzymes. Colons of five animals were subjected for the presence of MPLs, ACFs in each colon, and histopathology by using standard methodology. Colons of remaining five animals were subjected to homogenization to measure the bacterial enzymes such as β-Glucosidase, β-Glucuronidase, and mucinase.

Preparation of faecal homogenate

The fecal pellets were weighed and then mixed with 0.01 M sodium phosphate buffer (pH 7.4, 0.02 M) in a ratio of 1:10 (w/v) and the resultant mixture was placed on ice for approximately 20 minutes to promote softening of the pellet. The pellets were then homogenized at 2000 x g for 2 minutes and used for the further assessments (Shiau & Chang 1983).

Washing of colon

The colons were removed, tied at one end with thread and flushed with ice cold normal saline for three times and with buffered formalin for once to distend it. Followed by colons were opened longitudinally and washed to remove fecal matter (if remaining). The colons were laid flat on whatman filter paper and divided into proximal and distal segments to evaluate the occurrence of multiple plaque lesions (MPL) (Wei *et al.*2003).

Preparation of colon homogenate

The colons (5 out of 10 colons in each group) were homogenized in an ice cold phosphate buffer saline (0.02M pH 7.4) in 1:10 ratio, centrifuged at 5000 x g for 15 minutes and used for assays.

Determination of Fecal pH

The pH meter was calibrated to neutral pH using Sodium Phosphate Buffer pH 7; then fecal pH was determined by dipping the electrode of pH meter in freshly prepared fecal homogenate (Samelson, Nelson & Nyhus 1985).

Determination of Bacterial enzymes β-glucuronidase, β-glucosidase, Mucinase

β-glucuronidase

β-glucuronidase assay was performed by the method of Freeman with some modifications (Freeman 1986). The reaction mixture included 0.8 ml of 0.02 M phosphate-buffered saline, pH 7.4; 0.1 ml of 0.1 mM EDTA, 0.1 ml of 1mM phenolphthalein β-D-glucuronide and 0.1ml of enzyme supernatant. The test tubes were incubated at 37^{0}C for 45 minutes in a shaking water bath. The reaction was stopped with 4.0 ml of 0.2 M glycine buffer, pH 10.4, in 0.2 M sodium chloride and absorbance was read at 540 nm against blank (distilled water + remaining reagents similar to test). The results were expressed as µg of phenolphthalein liberated/g of protein in 45 minutes incubation time. Absorbances of the prepared dilutions were recorded at 540 nm against blank (distilled water). A standard graph was plotted between absorbance vs. concentration of phenolphthalein and represented in Fig.1. From this graph, the concentrations of fecal and mucosal β-glucuronidase, was determined by interpolation.

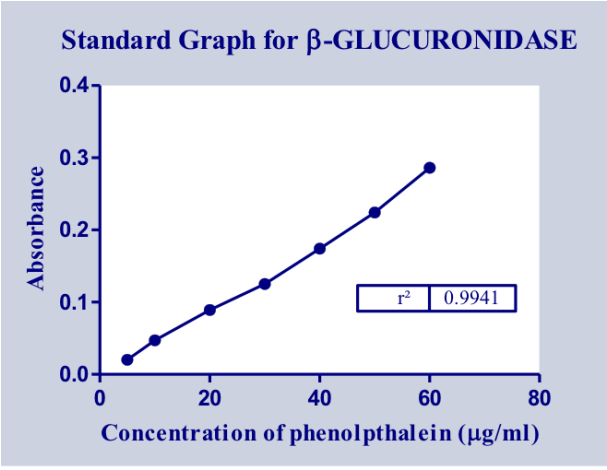


Figure 1: Standard graph for β-glucuronidase

β-glucosidase

β-glucosidase assay was performed by the method of Freeman with some modifications (Freeman 1986). The reaction mixture included 0.8 ml of 0.02 M phosphate-buffered saline, pH 7.4; 0.1 ml of 1mM p-nitro phenyl-β-D-glucopyranoside and 0.1ml of enzyme supernatant. The test tubes were incubated at 37^0 C for 60 minutes in a shaking water bath. The reaction was stopped with 5.0 ml of 0.01 M sodium hydroxide and absorbance was read at 450 nm against blank (distilled water + remaining reagents similar to test). The results were expressed as µg of p-nitro phenol liberated/g of protein in 60 minutes incubation time. Absorbance of the prepared dilutions was recorded at 450 nm against blank (PBS pH 7.4, 0.02M). A standard graph was plotted between absorbance vs. concentration of p-Nitro phenol and represented in Fig.2. From this graph, the concentration of fecal and mucosal β-glucosidase was determined by interpolation.

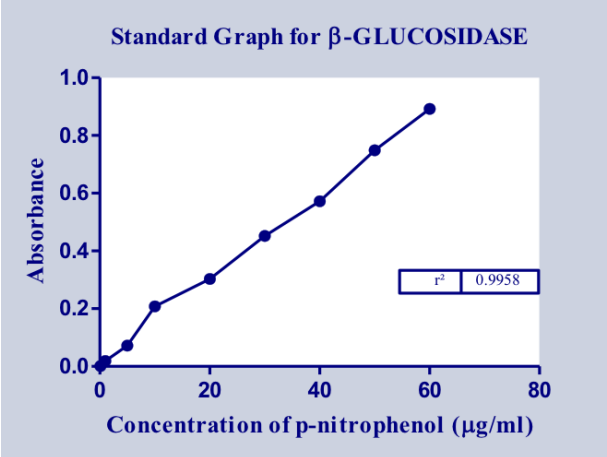


Figure 2: Standard graph for β-glucosidase

Mucinase

0.9 ml of fecal homogenate was placed in a test tube and incubated at 30° C for 2 minutes. Then 0.1 ml of 0.5% (wt/wt) porcine gastric mucin was added followed by incubated at 30° C for 25 minutes. The test tubes were then placed into boiling water for 3 minutes to stop enzymatic action. Reducing sugar released was measured by the Nelson method with some modifications as described (Somogyi 1952). To each test tube containing 1ml of reaction mixture, 9 ml of 5% TCA was added for the precipitation of protein (Remington 2006). The mixture was centrifuged at 5000 x g for 5 minutes and supernatant was collected. To 1ml of supernatant, 1 ml of a mixture of 25 parts of Reagent A and 1 part of Reagent B was added; the resultant solution was mixed and heated for 20 minutes in a boiling water bath and allowed to cooling. Then to each test tube, 1 ml of the arsenomolybdate reagent was added. The mixture was then diluted up to 10 ml and absorbance was read at 500 nm against blank (0.9 ml of distilled water + 0.1 ml of porcine gastric mucin). The results were expressed as µg of reduc-

ing sugar liberated/g of protein in 25 minutes incubation time. Absorbance of the prepared solutions were recorded at 500 nm against blank (Distilled water). A standard graph was plotted between absorbance vs. concentration of dextrose (reducing sugar) and represented in Fig.3. From this graph, the concentrations of fecal and mucosal mucinase, was determined by interpolation (Shiau & Chang 1983).

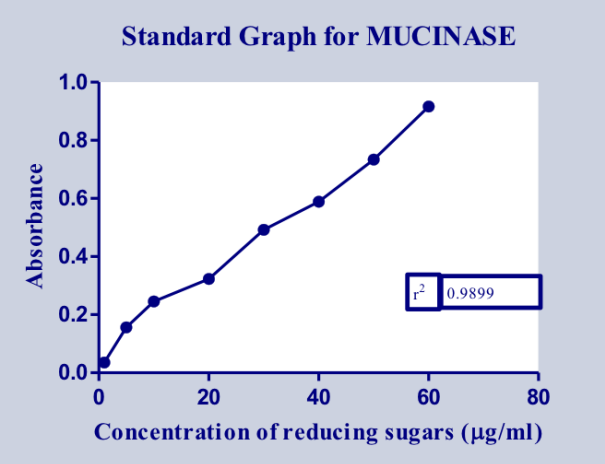


Figure 3: Standard graph for Mucinase

To express the results of these enzymatic activities as per gram of protein, fecal and colonic protein content was determined using Biuret method (Lubran 1978).

Multiple Plaque Lesions

Multiple Plaque Lesions were clearly recognized by the appearance of either raised or non-raised stretches of tissues in the form of identifiable tissue growth, often appearing singly or in multiple forms throughout the length of the colon (Kaur *et al.* 2009). MPL incidence (the percentage of animals having MPLs), MPL burden (the total number of MPLs counted/total number of rats), and MPL multiplicity (the total number of MPLs counted/number of MPL bearing rats) has been calculated.

Aberrant crypt foci

ACFs has been evaluated using the method of Bird with some modifications (Bird 1995). The ACF were recognized by increased size, thicker epithelial cell layer and increased pericryptic zone. The proximal and distal part of each colon was marked and fixed with 10% buffered formalin solution overnight. The colon was then stained with 0.2% methylene blue for 3–5 min and washed with PBS pH 7.4, 0.02 M to wash off excess stain. The number of aberrant crypt foci per colon, the number of aberrant crypts in each focus, and the location of each focus were determined by microscopy at a magnification of x40.

Histopathological Examination

Colons containing ACF were marked and embedded in paraffin, sectioned and stained with hematoxylin and eosin, in order to obtain the Histopathological information.

Statistical Analysis

Results were expressed as Mean ± S.D., where n=10. Differences among data were determined using one way ANOVA followed by "Bonferroni's Multiple Comparison Test" by using Graph Pad Prism software, version 5.01. $p<0.05$ was considered statistically significant. For each parameter, the proposed increase or decrease in response to DMH or treatment evolved from a comparative analysis of data obtained for normal control vs. DMH group and treatment vs. DMH group respectively. It was assumed that the comparison of changes occurring in one group with changes in another within an identical time frame can minimize the variation among individual animals within a group.

RESULTS AND DISCUSSION

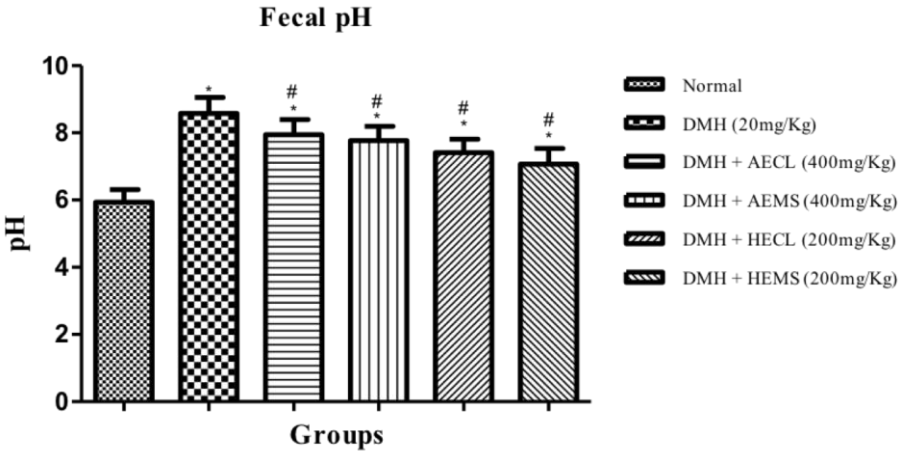
In the present study, colon cancer was induced using DMH to assess the anti neoplastic activity of aqueous and hydro alcoholic extracts of *Clerodendron viscosum V. leaves* and *Macrotyloma uniflorum L. seeds.* Bacterial enzymes were used as metabolic markers, where as MPL, ACFs and Histopathological analysis was used as markers for colon cancer status.

General Observations

Neither of the animals displayed any signs of stress or behavioral changes, nor were there any signs of toxicity during the experimental period. Gross examination of other organs showed no primary tumors. No tumor was observed at the site of the DMH injections. All animals survived up to the final sacrifice.

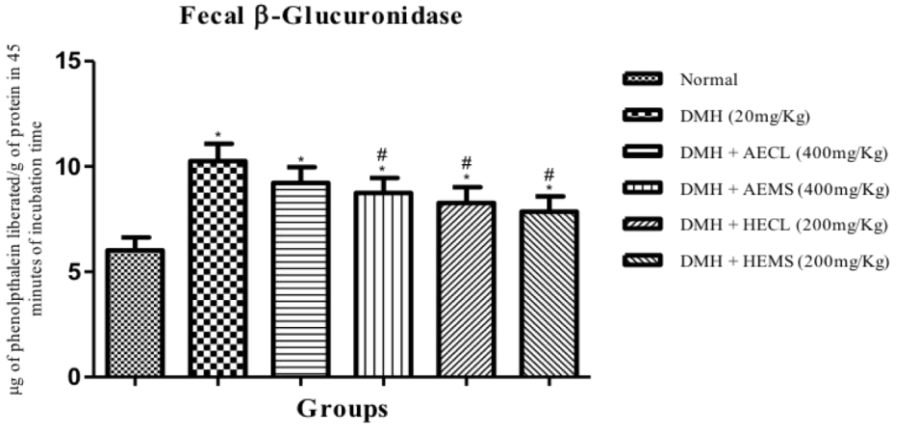
Effect of extracts on fecal pH

The mean fecal pH value of normal control group and disease control group was found to be 5.94 and 8.58 respectively. These values are further potentiating the earlier studies that the increased risk of colon cancer results into a higher fecal pH (Thornton 1981; Newmark & Lupton 1990; Malhotra 1982). The alkaline pH observed in the disease control group may be due to the increase in the amount and flow rate of bile and pancreatic juice entering the intestinal lumen (Malhotra 1982). Groups treated with DMH and plant extracts



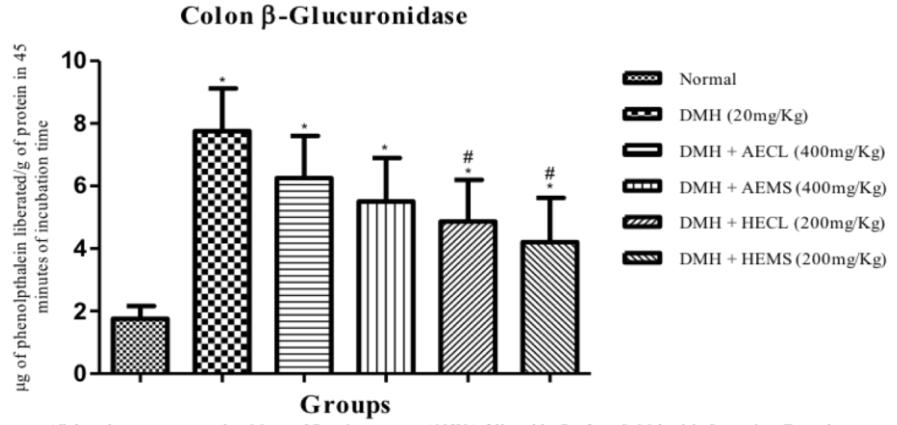
All the values were expressed as Mean ± S.D. using one way ANOVA followed by Bonferroni's Multiple Comparison Test, where n=10; *P<0.05 -when compared with Normal; #P<0.05 -when compared with DMH (20mg/kg)

Figure 4: Effect of extracts on fecal pH



All the values were expressed as Mean ± S.D. using one way ANOVA followed by Bonferroni's Mult iple Comparison Test, where n=5; *P<0.05-when compared with Normal; #P<0.05 -when compared with DMH (20mg/kg)

Figure 5: Effect of extracts on Fecal β-glucuronidase



All the values were expressed as Mean ± S.D. using one way ANOVA followed by Bonferroni's Mult iple Comparison Test, where n=5; *P<0.05-when compared with Normal; #P<0.05 -when compared with DMH (20mg/kg)

Figure 6: Effect of extracts on Colon β-glucuronidase

(AECL, AEMS, HECL, HEMS) did not acidify the fecal pH rather the pH was brought to neutral range. This neutral pH can be attributed to the anti-neoplastic activity of the plant extracts. HEMS showed lowest mean fecal pH among the other plant extracts, there by demonstrating that this extract is having higher potential of anti-neoplastic activity in comparison to remaining extracts. The values of fecal pH of each group were tabulated in Table. 2 and plotted in Fig. 4.

Effect of extracts on Bacterial Enzymes (β-glucuronidase and β-glucosidase) in fecal and colon homogenate

The level of bacterial enzymes (β-glucuronidase, β-glucosidase and Mucinase) in sample of fresh fecal homogenate was significantly high in disease control group when compared with normal control group. DMH is a colon carcinogen which is detoxified by glucuronidation in liver and secreted via

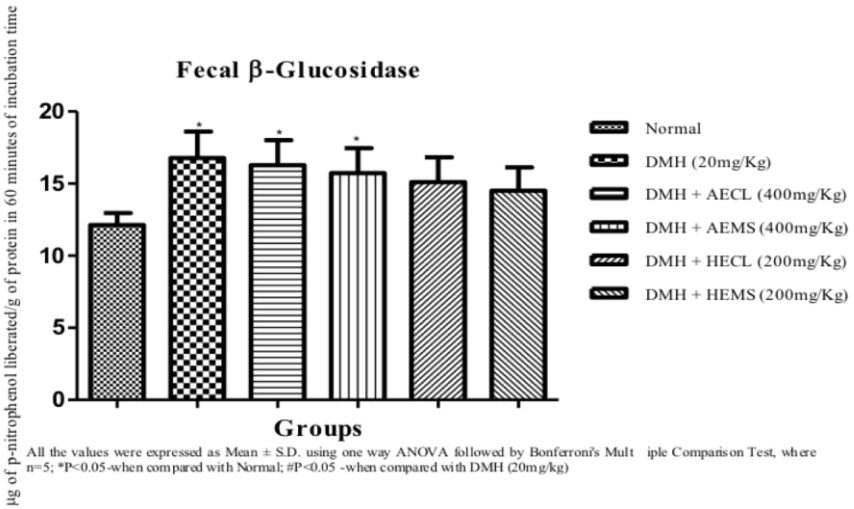


Figure 7: Effect of extracts on Fecal β-glucosidase

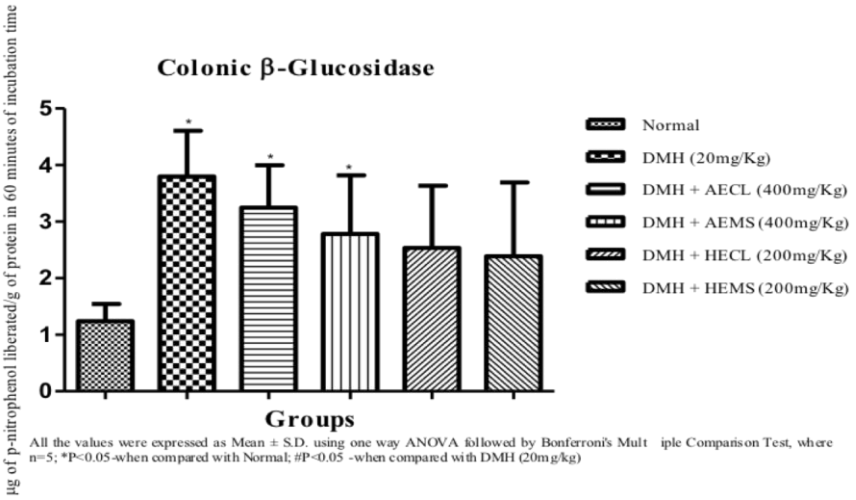


Figure 8: Effect of extracts on Colon β-glucosidase

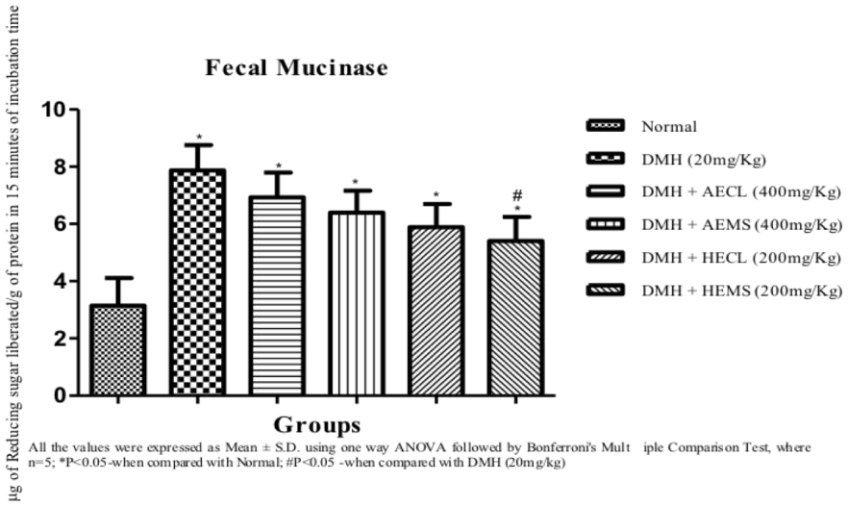


Figure 9: Effect of extracts on Fecal Mucinase

bile into the intestine. When the conjugated carcinogen reaches the colon, it is hydrolyzed by bacterial enzymes and the colon gets exposed to free carcinogen. In the present study, elevated levels of fecal and colonic β-glucuronidase and β-glucosidase were observed in DMH induced group which confirms that bacterial enzyme activities got enhanced in the presence of pro-carcinogen like DMH (Manju & Nalini 2007). Enhanced level of these enzymes, hydrolyze glucuronides of DMH as well as activates DMH metabolites to toxic car-

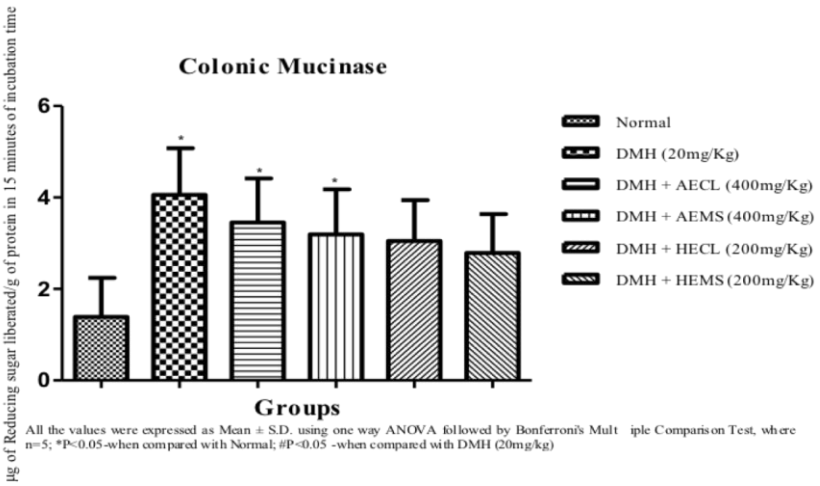


Figure 10: Effect of extracts on Colon Mucinase

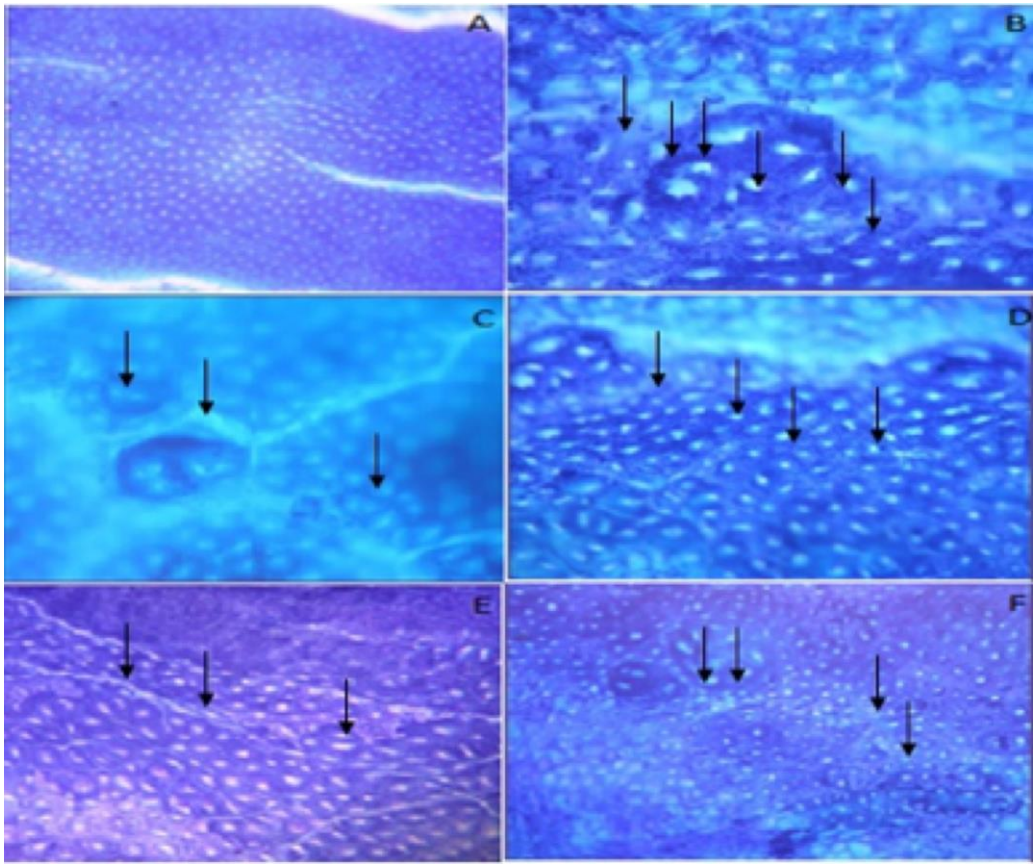


Figure 11: Effect of extracts on Aberrant Crypt Foci A: Normal, B: DMH (20 mg/Kg), C: DMH + AECL (400 mg/Kg), D: DMH + AEMS (400 mg/Kg), E: DMH + HECL (200 mg/Kg), F: DMH + HEMS (200 mg/Kg); Arrow represent ACF

cinogens in the colon, a prerequisite for colon carcinogenesis thereby eliciting the conversion of normal epithelial cells to neoplastic cells. Treatment with plant extracts showed significantly low level of these enzymes in comparison to disease control group. Among the plant extracts, HEMS has shown lowest β-glucuronidase and β-glucosidase levels, there by demonstrating that this extract is having highest potential of anti-neoplastic activity in comparison to remaining plant extracts. The levels of β-glucuronidase and β-glucosidase in fecal and colon homogenate were tabulated in Table. 3 and plotted in Fig. 5, 6, 7 and 8 respectively.

Effect of extracts on Bacterial Enzyme - Mucinase in fecal and colon homogenate

The fecal and colonic activity of mucinase was significantly high in disease control groups in comparison to normal control group. Mucins form gels coating the intestinal mucosa and function as a lubricant and probably as a chemical and mechanical barrier against bacteria, viruses, and toxins. The

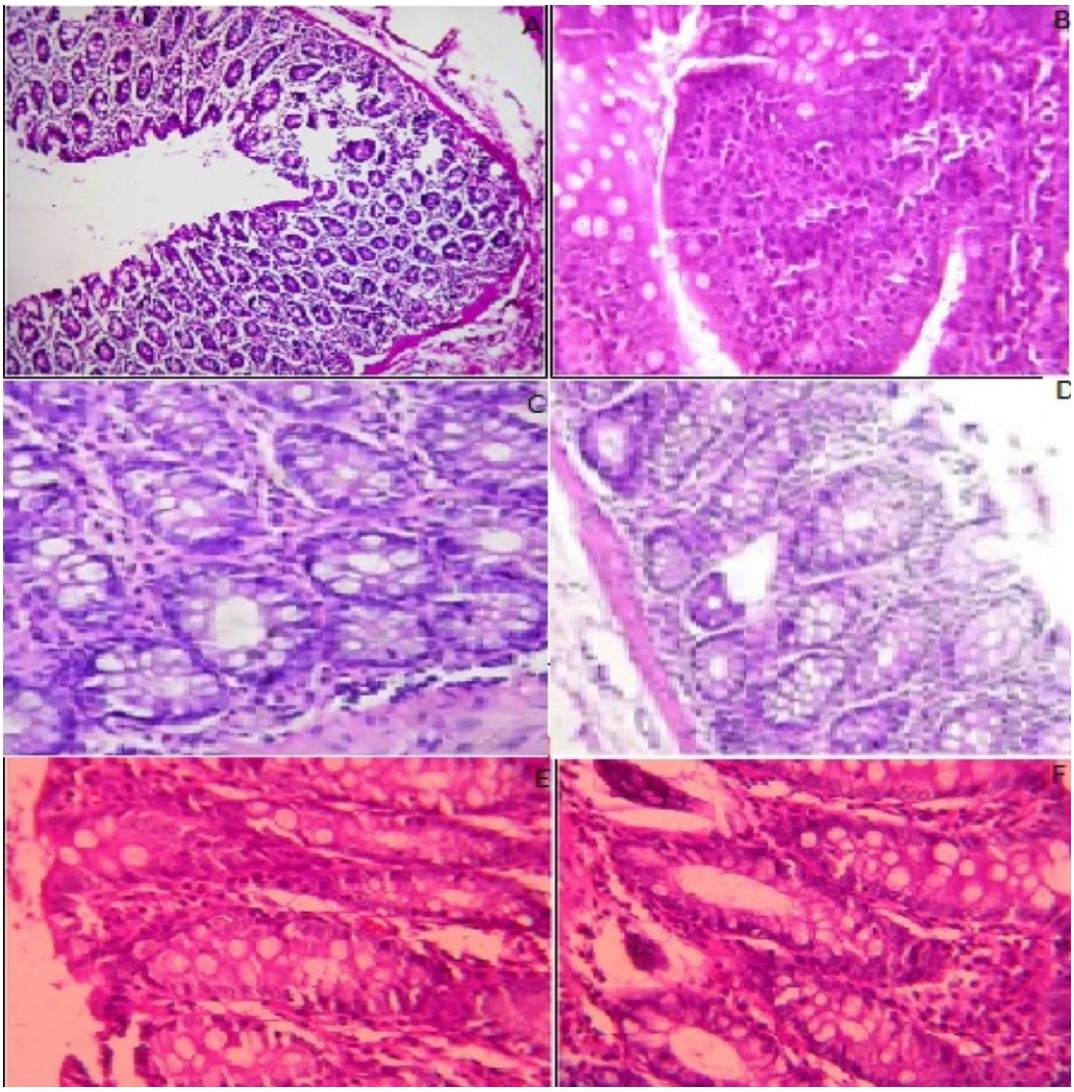


Figure 12: Effect of extracts on Histopathology of colon A: Normal, B: DMH (20 mg/Kg), C: DMH + AECL (400 mg/Kg), D: DMH + AEMS (400 mg/Kg), E: DMH + HECL (200 mg/Kg), F: DMH + HEMS (200 mg/Kg)

high mucinase activity in disease control group leads to decreased protection of the underlying tissues. Treatment with plant extracts has shown low levels of mucinase activity in comparison to disease control group suggesting the protective effects of plant extracts; thereby, exerting protective effect on colonic mucosa and decreasing the sus- ceptibility of the colonic mucosa to attack by car- cinogens. Hydro alcoholic extracts has shown lower level of mucinase activity in comparison to aqueous extracts. The values of mucinase in fecal and colon homogenate were tabulated in Table. 4 and plotted in Fig. 9 and 10 respectively.

Effect of extracts on MPL

There were no MPL in the normal control group, whereas in disease control group, MPL incidence in the colon was 100%. Gross examination of the disease control group rat's colons revealed that the MPL's were observed throughout the colon, but were more commonly found in the descending portion. Hemorrhages of various severities were associated with MPL's and were observed in most of the DMH induced rat's colon Treatment with plant extracts markedly suppressed MPL formation and the incidence was reduced to 60% in comparison to disease control group. The number as well as size of MPL was decreased in the group receiving plant extract along with DMH. Hydro alcoholic extracts showed a potential effect in contrast to aqueous extracts. The MPL counts of each group were mentioned in the Table. 5.

Effect of extracts on Aberrant Crypt Foci (ACF)

ACFs considered as an index of early neoplastic induction. Gross examination of the DMH induced group colon revealed the presence of severe dysplasia and numerous malignancy related features along with the development of pre neoplastic events such as the occurrence of aberrant crypt

Table 1: Experimental Design

Group No.	Description	Treatment	Duration
I	Normal Control (N)	EDTA (1mM, s.c.)	Once a week for 4 weeks
II	Disease Control DMH (20 mg/kg)	DMH (20 mg/kg, s.c.)	Once a week for 4 weeks
III	DMH + AECL (400 mg/Kg)	DMH (20 mg/kg, s.c.) + AECL (orally)	DMH: Once a week for 4 weeks, AECL: For 8 weeks daily
IV	DMH + AEMS (400 mg/Kg)	DMH (20 mg/kg, s.c.) + AEMS (orally)	DMH: Once a week for 4 weeks, AEMS : For 8 weeks daily
V	DMH + HECL (200 mg/Kg)	DMH (20 mg/kg, s.c.) + HECL (orally)	DMH: Once a week for 4 weeks, HECL : For 8 weeks daily
VI	DMH + HEMS (200 mg/Kg)	DMH (20 mg/kg, s.c.) + HEMS (orally)	DMH: Once a week for 4 weeks, HEMS : For 8 weeks daily

AECL: Aqueous extract of *Clerodendron viscosum* V. Leaves; AEMS: Aqueous extract of *Macrotyloma uniflorum* L. Seeds; HECL: Hydro alcoholic extract of *Clerodendron viscosum* V. Leaves; HEMS: Hydro alcoholic extract of *Macrotyloma uniflorum* L. Seeds.

Table 2: Effect of extracts on fecal pH

Group	Fecal pH
Normal	5.94 ± 0.3688
DMH (20 mg/Kg)	8.58 ± 0.478*
DMH + AECL (400 mg/Kg)	7.95 ± 0.4428*#
DMH + AEMS (400 mg/Kg)	7.77 ± 0.4244*#
DMH + HECL (200 mg/Kg)	7.41 ± 0.3985*#
DMH + HEMS (200 mg/Kg)	7.07 ± 0.4692*#

All the values were expressed as Mean ± S.D. using one way ANOVA followed by Bonferroni's Multiple Comparison Test, where n=10; *P<0.05-when compared with Normal; #P<0.05-when compared with DMH (20mg/kg).

Table 3: Effect of extracts on Bacterial Enzymes (β-glucuronidase and β-glucosidase) in fecal and colon homogenate

Group	β-Glucuronidase		β-Glucosidase	
	Fecal	Colon	Fecal	Colon
Normal	6.02 ± 0.6083	1.75 ± 0.4123	12.12 ± 0.8408	1.24 ± 0.305
DMH (20 mg/Kg)	10.25 ± 0.8246*	7.75 ± 1.369*	16.77 ± 1.836*	3.8 ± 0.8062*
DMH + AECL (400 mg/Kg)	9.214 ± 0.7537*	6.254 ± 1.351*	16.27 ± 1.744*	3.247 ± 0.7509*
DMH + AEMS (400 mg/Kg)	8.743 ± 0.7076*#	5.5 ± 1.395*	15.72 ± 1.733*	2.782 ± 1.034*
DMH + HECL (200 mg/Kg)	8.269 ± 0.7509*#	4.859 ± 1.334*#	15.12 ± 1.701	2.536 ± 1.098
DMH + HEMS (200 mg/Kg)	7.851 ± 0.7343*#	4.204 ± 1.417*#	14.51 ± 1.622	2.388 ± 1.305

All the values were expressed as Mean ± S.D. using one way ANOVA followed by Bonferroni's Multiple Comparison Test, where n=5; *P<0.05-when compared with Normal; #P<0.05-when compared with DMH (20mg/kg).

foci (ACF) all over the length of the colonic segments. The rats in the normal control group showed no evidence of ACF formation in the colon, whereas rats induced with DMH developed ACF in their colon with 100% incidence However, the group treated with plant extracts has showed different levels of inhibition of ACF. Compared with the disease control group, rats receiving plant extracts alter the mean total number of ACF per rat significantly. However, the groups receiving hydro alcoholic extracts reduced the mean total no. of ACF more effectively All the groups except normal control showed more number of aberrant crypt foci in the distal part of the colon as compared to proximal part. The results suggest that plant extracts inhibited the growth of colonic ACF and suppressed progression of pre neoplasia to malignant

Table 4: Effect of extracts on fecal and colon mucinase

Group	Mucinase Fecal	Mucinase Colonic
Normal	3.147 ± 0.9618	1.39 ± 0.8544
DMH (20 mg/Kg)	7.87 ± 0.886*	4.06 ± 1.02*
DMH + AECL (400 mg/Kg)	6.926 ± 0.8731*	3.454 ± 0.9639*
DMH + AEMS (400 mg/Kg)	6.393 ± 0.7702*	3.191 ± 0.9862*
DMH + HECL (200 mg/Kg)	5.889 ± 0.8074*	3.047 ± 0.8935
DMH + HEMS (200 mg/Kg)	5.401 ± 0.8449*#	2.786 ± 0.8537

All the values were expressed as Mean ± S.D. using one way ANOVA followed by Bonferroni's Multiple Comparison Test, where n=5; *P<0.05-when compared with Normal; #P<0.05-when compared with DMH (20mg/kg)

Table 5: Effect of extracts on Multiple Plaque Lesions

Group	Total MPL	Rats with MPL	MPL Incidence	MPL Burden	MPL Multiplicity
Normal	0	0	0	0	0
DMH (20 mg/Kg)	31*	5*	100*	6.2*	6.20*
DMH + AECL (400 mg/Kg)	23*	4*	80*	4.6*	5.75*
DMH + AEMS (400 mg/Kg)	21*	4*	80*	4.2*	5.25*
DMH + HECL (200 mg/Kg)	16*	3*#	60*#	3.2*	5.33*
DMH + HEMS (200 mg/Kg)	13*#	3*#	60*#	2.6*#	4.33*#

All the values were analyzed using one way ANOVA followed by Bonferroni's Multiple Comparison Test, where n=5; *P<0.05-when compared with Normal; #P<0.05-when compared with DMH (20mg/kg).

neoplasia. Microscopical images of ACF were shown in Fig. 11.

Effect of extracts on Histopathology of colon

The Histopathological analysis of the colon of normal control rats revealed no signs of malignancy in terms of dysplasia, adenoma or adenocarcinoma and normal epithelium were observed. Histologically, the colorectal carcinomas in disease control group were moderately to well differentiated, and their growth was frequently limited to the mucosa. Severe dysplasia was also observed in the colon's of disease control group. The crypt cells in the dysplasia showed the enlarged nuclei, round or ovoid and deeply stained. Also, the number of mitotic cells is far more. The regressive effect of the plant extracts were observed in the DMH-induced carcinogenesis and the epithelium was moderately hyper plastic. Histopathological images of all the groups has been showed in Fig. 12.

CONCLUSION

In conclusion, the study shows that treatment with hydro alcoholic extracts significantly inhibits ACF, colon tumor incidence and multiplicity. All the extracts probably prevent colon carcinogenesis by inhibiting bacterial β-glucuronidase, β-glucosidase and mucinase levels, thus decreasing the recycling of glucuronide-conjugated carcinogens in the lower intestine. Moreover, treatment with HECL and HEMS at 200mg/kg was comparatively more effective than treatment with AECL and AEMS at 400mg/Kg. Thus, this study demonstrates the potential usefulness of hydro alcoholic extracts as a chemo-preventive agent for colon cancer development. But it should be noted that the crude extract containing numerous active constituents was used to evaluate the effect; thus, to understand the mechanism of action against DMH induced colon cancer in a better way, further investigations are essential for the isolation of the active principles of hydro alcoholic extracts of *Clerodendron viscosum* V. leaves & *Macrotyloma uniflorum* L. seeds.

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