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ROLE OF Chlorella pyrenoidosa IN 1,2 DIMETHYL HYDRAZINE (DMH) INDUCED COLORECTAL CARCINOMA IN MALE WISTAR RATS

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A B S T R A C T

Colon cancer is the abnormal or uncontrolled growth of new cells in the colon, characterized by cells that tend to invade surrounding tissues and metastasize the new body sites. Colorectal cancers arise from adenomatous polyps in the colon. These mushroom-shaped growths are usually benign, but some develop into cancer over time Dimethylhydrazine (DMH) is metabolized to a methyl free radical and generates hydroxyl radical or hydrogen peroxide in the presence of metal ions that may contribute to the initiation of lipid peroxidation (Dudeja *et al* 1990). The products of lipid peroxidation are measured to find out the amount of oxidative damage in the cancer cells.

Chlorella's multi-layered cell wall contains the polysaccharides and beta carotene which can be attributed to much of the observed anti-cancer action.

A total of 36 male wistar rats were divided into six groups. Group 4,5 and 6 were given Dimethylhydrazine 20mg/kg once a week for four weeks along with Chlorella pyerenoidosa 500mg,750mg and 1000mg/kg respectively for 16 weeks. At the end of 16 weeks, the animals were sacrified and the blood samples were sent for determination of diagnostic marker enzymes. Results showed that the TBARS are reduced in DMH treated group (Group II) compared to control (group I), standard (group III and test groups (Groups IV, V & VI). results show a significant decrese in the levels of catalase and glutathione peroxidase in DMH treated group II rats whereas it is almost doubled in drug treated and standard group which is comparable to the control group. Our results suggest that the anticarcinogenic effect of chlorella may be mediated by the induction of reduced glutathione because this endogenous tripeptide molecule can detoxify various carcinogeus, serve as an intracellular antioxidant and also regulated DNA and protein synthesis . This substantiates the claim that chlorella can serve as a prophylactic agent in the management of colon cancer

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INTRODUCTION

Colorectal cancer, also called colon cancer or large bowel cancer includes cancerous growths in the colon, rectum and appendix. With 6,55,000 deaths worldwide per year, it is the fifth most common form of cancer in the United States and the third leading cause of cancer-related deaths in the Western world. The incidence rates of colon cancer are currently increasing in all industrialized countries as well as in the urban areas of developing countries (WHO., 1997). The epidemiology of large bowel malignancies has generated a lot of interest in recent years, mainly because the disease provides an excellent model for studying the interaction between specific genes and several environmental factors in the etiology of cancer (Ponz de leon *et al*., 1996). Typically, the rates of colon cancer incidence tend to be higher in developed countries that are economically privileged (Schottenfeld, 2005)Recent studies show high incidence of CRC in individuals living in Mumbai (India), based on the epidemiological studies it has been estimated that as much as 70-80% of CRC could be attributed to the contribution of environmental and life style factors (Parkin *et al*., 1992) Chlorella's multi-layered cell wall contains the polysaccharides which

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research has shown to be responsible for much of the plant's immune-stimulating and detoxification properties. The presence of beta carotene can be attributed much of the observed anti-cancer action, CGF is also implicated in cancer prevention and treatment. Dimethylhydrazine (DMH) is metabolized to a methyl free radical and generates hydroxyl radical or hydrogen peroxide in the presence of metal ions that may contribute to the initiation of lipid peroxidation (Dudeja *et al* 1990).

Aims and Objectives

Aim

The aim of the present study was to find out the effect of *Chlorella pyrenoidosa* in the management of colorectal cancer in male Wistar rats.

Objectives

Since research in animals & humans is very limited, this study is undertaken to

1. To access the serum biochemical parameters in control, test and reference animals.
2. To access the tissue biochemical parameters in control, test and reference animals

MATERIALS AND METHODS

Chemicals and Reagents

Drug/Chemicals	Source
Chlorella pyrenoidosa	E. Merck (Mumbai, India)
Irinotecan hydrochloride	E. Merck (Mumbai, India)
1,2-Dimethylhydrazine	Sigma-Aldrich (Delhi, India)

- All other chemicals and reagents were of the analytical grades and obtained locally.
- Biochemical and enzymatic kits were from Agappee diagnostics, Kerala.

Animal model used for investigation (Albino Wistar Rats)

Class : Mammalia
Family : Muridae
Order : Rodentia
Genus : Rattus
Scientific name : *Rattus norvegicus*

Fig. 5 Animal Model

The institutional animal ethical committee (Register No.160/ 1999/CPCSEA), Annamalai University, Annamalai Nagar, India approved the experimental design (Proposal No.633, dated 25.05.2009). *Albino wistar* male rats of 140-160g were used for the study. Animals were housed in well ventilated room (temperature 23 ± 2°C, humidity 65-70% and 12h light/dark cycle) at Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. Animals were fed with standard pellet diet and water *ad libitum*. All studies were conducted in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) norms and the National Institute of Health guidelines "Guide for the Care and use of Laboratory Animals". The Male wistar rats, housed in polypropylenecages under hygienic conditions adapted to the laboratory conditions for a week were used for the study.

The animals were divided into six groups. Each group had six rats and a total of 36 rats were used for this study. The experimental period was of 16 weeks duration. The animals were into the following groups.

Group – I	**Normal control**	**Distilled water and normal diet**
Group – II	Cancer control	DMH 20 mg/kg *s.c* once a week for four weeks
Group – III	Standard drug	DMH 20mg/kg *s.c* and Irinotecan 25mg/kg i.v. once a week for four weeks
Group – IV	Test drug low dose	DMH 20 mg/kg *s.c* once a week for four weeks and *Chlorella pyrenoidosa* 500 mg/kg/po daily (16 weeks)
Group –V	Test drug medium dose	DMH 20 mg/kg *s.c* once a week for four weeks and *Chlorella pyrenoidosa* 750 mg/kg /po daily (16 weeks)
Group –VI	Test drug high dose	DMH 20 mg/kg once a week *s.c* for four weeks and *Chlorella pyrenoidosa* 1000 mg/kg /po daily (16 weeks)

At the end of 16 weeks the animals were sacrificed by cervical decapitation under ketamine anesthesia.
Blood samples were collected in centrifuge tubes using sodium citrate as anticoagulant and the plasma separated was used for the determination of diagnostic marker enzymes.

The liver, colon tissue were excised immediately and washed with chilled isotonic saline. The tissue homogenates were prepared in ice cold 0.1 M Tris-HCl buffer, pH 7.2 separatly. The homogenate was centrifuged and the supernatant was used for the assay of clinical marker enzymes in the tissue & determination of biochemical parameters .

Biochemical Estimations

Measurement of Tissue Thiobarbituric Acid Reactive Substances (TBARS)

The extent of lipid peroxidation in tissues was determined by the method of Ohkawa *et al*. (1979).

Estimation of Plasma TBARS

Plasma TBARS was determined by the method of Ohkawa *et al*., (1979).

Assay of Catalase (CAT)

The activity of CAT was determined by the method of Sinha (1972).

Assay of glutathione peroxidase (GPx)

GPx was assayed by the method of Rotruck *et al*. (1973)

Estimation of glutathione reductase

GR activity was assayed by the method of Carlberg and Mannervik, (1985).

Faecal bacterial enzymes

Faecal homogenate (0.9 ml) was placed in tubes and incubated at 30C° for 2 minutes. Then 0.1 ml of 0.5% (w/w) porcine gastric mucin was added. Control incubations consisted of faecal homogenates to which 0.1 ml of distilled water was added and substrate control consists of 0.1 ml porcine gastric mucin and 0.9 ml distilled water. All tubes were incubated at 30°C for 25 minutes and then placed into boiling water for 3 min to stop enzymatic action and reducing sugar release was measured by the Nelson- Somogyi method (1994).

Colonic bacterial enzymes

To each 1 ml diluted faecal homogenate, 0.05 ml phenolphthalein glucuronide (0.01 M, pH 7.0, Sigma Chemical Co.) was added. Controls consisted of 0.05 ml distilled water and 1.0 ml faecal homogenate.

Faecal mucinase assay

Faecal homogenate (0.9 ml) was placed into tubes and incubated at 30°C for 2 min. Then 0.1 ml of 0.5% (w/w) porcine gastric mucin (Sigma Chemical Co., St. Louis, MO) was added. Control incubations consisted of faecal homogenates to which 0.1 ml of distilled water was added. Substrate controls consisted of 0.1 ml porcine gastric mucin and 0.9 ml distilled water. All tubes were incubated at 30°C for 25 min and then placed into boiling water for 3 min to stop enzymatic action. Reducing sugar release was measured by the Nelson- Somogyi method.

Determination of 0-glucuronidase activity

The assay was performed on bacterial suspensions prepared from either broth culture or feces. The activities found in the feces and in the cultures were called *in vivo* and *in vitro* activities, respectively; glucuronidase activity was determined spectrophotometrically by looking for the release of phenolphthalein from phenolphthalein mono-p-glucuronide (Sigma Chemical Co., St. Louis, Mo.), (Goldin and Gorbach, 1984). Protein amount was determined by the method of Lowry *et al* (1951For quantifying both *in vivo* and *in vitro*, glucuronidase activities, the same procedure was used, except that the reaction was incubated for 30 min instead of 4 h. The bacterial pellets were prepared by centrifugation of 20 ml of culture or the 10-*1* faecal dilution and washed three times with phosphate buffer. The clean packed cells were suspended in 2 ml of phosphate buffer and transferred to the micro chamber of a Sorvall Omnimixer (Sorvall, Newton, Conn.).

RESULTS AND DISCUSSION

Colonic oxidant - antioxidant status

Various studies (Dudeja and Brasitus 1990) have shown that DMH decreases the intestinal and colonic lipid peroxidation in rats. Our study also shows that the TBARS are reduced in DMH treated group (Group II) compared to control (group I), standard (group IIIand test groups (Groups IV, V & VI). Catalase IS considered to be the primary antioxidant enzyme and it will add be involved in the direct elimination of the reactive oxygen species (ROH

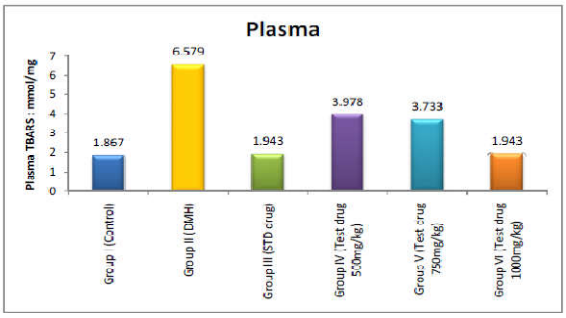

Fig 1 Effect of TBARS levels in plasma of the control and experimental rats

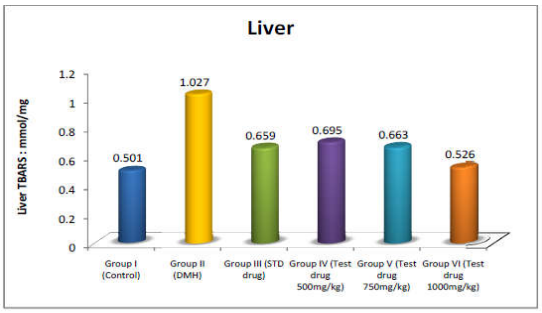

Fig 2 Effect of TBARS levels in liver tissue of the control and experimental rats

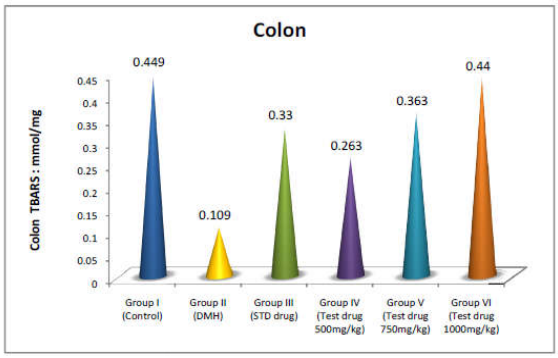

Fig 3 Effect of TBARS levels in colon tissue of the control and experimental rats

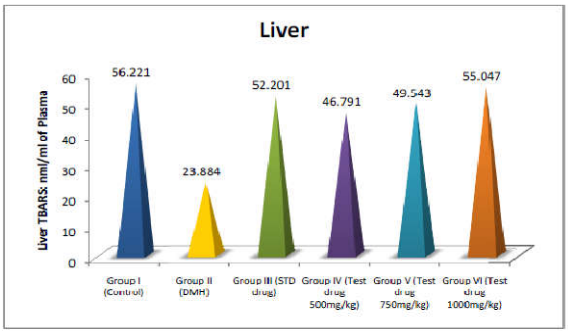

Fig 4 Effect of Catalase activites in liver tissue of the control and experimental rats

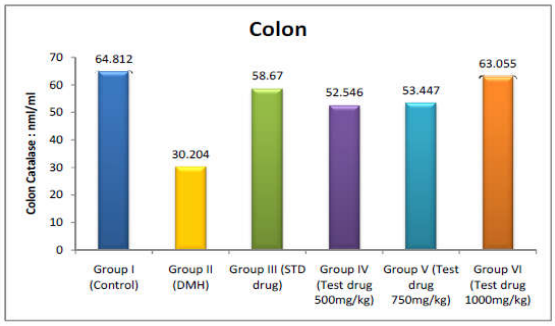

Fig 5 Effect of Catalase activites in colon tissue of the control and experimental rats

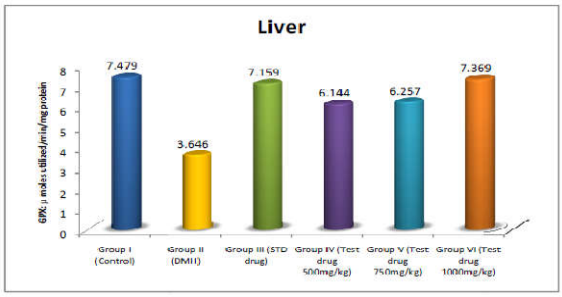

Fig 6 Effect of Glutathione peroxidase activites in liver tissue of the control and experimental rats

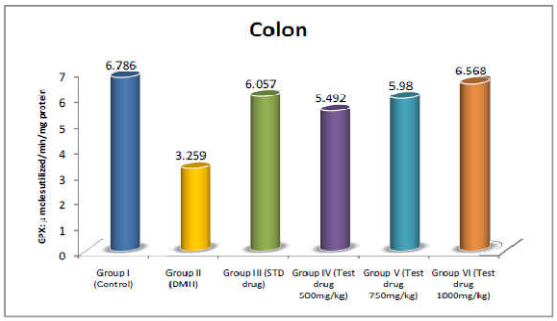

Fig 7 Effect of Glutathione peroxidase activites in tissue of the control and experimental rats

Reduced glutathione (GSH) helps in the primary defense mechanism and is the non-enzymic small molecular antioxidant. There is a strong association between the metabolic activity of the intestinal microflora and cancer of the large bowel (Reddy *et al* 1978). The activation of the procarcinogens could be mediated enzymatically by intestinal bacteria and the activities of colonic bacterial enzymes like β - glucuronidase are stimulated by DMH. Chlorella reduced the level of β - glucuronidase. Mucinase, a hydrolytic enzyme secreted by the intestinal microflora degrades the protective mucus layer of the colon (Mastromarino 1976).

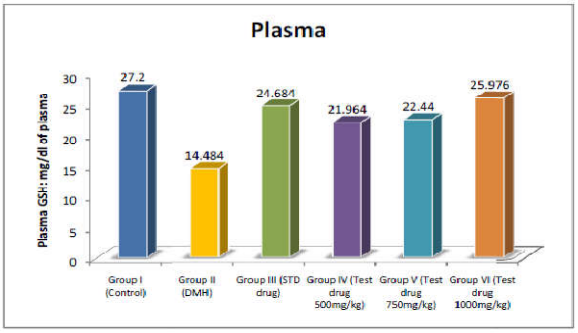

Fig 8 Effect of Glutathione reductase activites in plsma tissue of the control and experimental rats

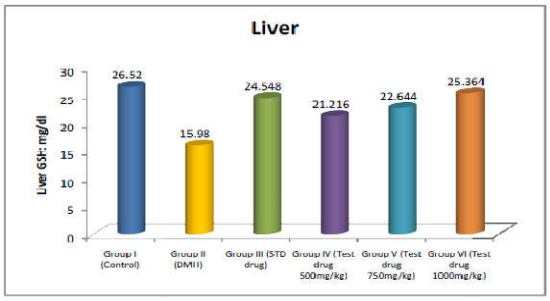

Fig 9 Effect of Glutathione reductase activites in liver tissue of the control and experimental rats

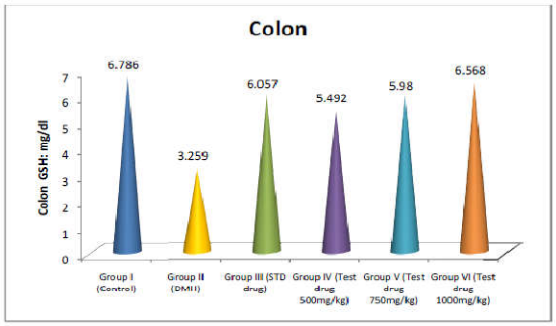

Fig 10 Effect of Glutathione reductase activites in colon tissue of the control and experimental rats

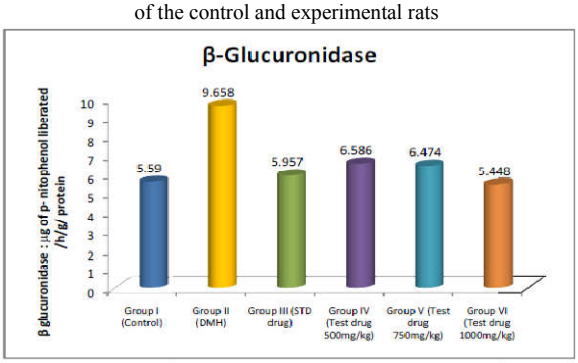

Fig 11 Effect of activites of colonic bacterial enzymes of the control and experimental rats

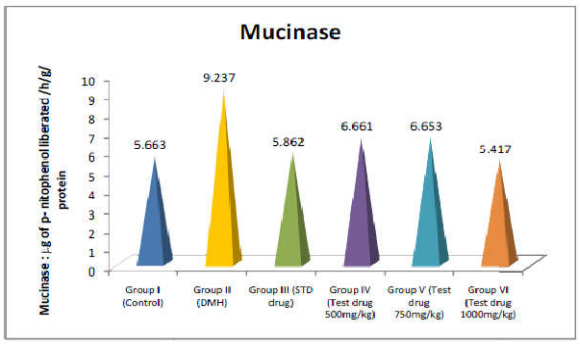

Fig 12 Effect of activites of colonic bacterial enzymes of the control and experimental rats

Dimethylhydrazine (DMH) is metabolized to a methyl free radical and generates hydroxyl radical or hydrogen peroxide in the presence of metal ions that may contribute to the initiation of lipid peroxidation (Dudeja *et al* 1990). The products of lipid peroxidation are measured to find out the amount of oxidative damage in the cancer cells. The equilibrium for cell survival is maintained by antioxidants. Any change in one of them can cause cell damage leading to malignancy. Improper antioxidant

defense mechanism will lead to considerable oxidative stress resulting in carcinogenesis.

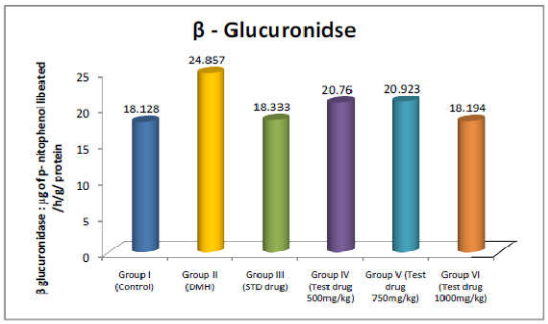

Fig 13 Effect of activites of faecal bacterial enzymes of the control and experimental rats

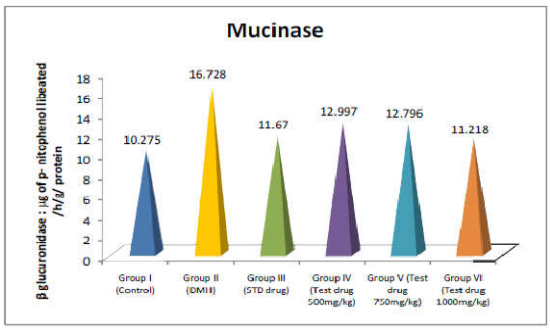

Fig 14 Effect of activites of faecal bacterial enzymes of the control and experimental rats

In this study, an accelerated generation of reactive oxygen species and toxic degradation products of lipid peroxidation have been noted in liver and plasma of DMH treated group which reflects endogenous lipid peroxidation. The level of the principal defense systems against oxidation like catalase, glutathione peroxidase and reduced glutathione are elevated in all the drug treated groups which is comparable to that of the standard drug (Irinotecan) group.

Chlorella deattenuates the hepatic damage induced by DMH possibly by

1. Enhancing reduced glutathione levels by inducing the GSH synthesing enzymes like γ glutanylcysteine synthetase and GSH synthetase.
2. Increasing adaptive response to compensate for the depleted GSH levels by inhibition of conversion of oxidised glutathione to reduced glutathione by glutathione reductase.
3. Increasing the rate of free radical utilization via the glutathione peroxidase system.
4. Inhibition of lipid peroxidation by elevating hepatic β carotene and vit - A levels.

An inverse relationship exists between the concentration of lipid peroxides and the rate of cell proliferation (Das 2002) and differentiation (Navarro *et al* 1999). This may be due to the low level of polyunsaturated fatty acids in tumor cells resulting in lower rate of lipid peroxidation. The enzymes catalase and glutathione peroxidase play a key role in the cellular defense against free radical damage. But tumor cells lack these enzymes and so the normal protection exerted by their enzymes by scavenging toxic oxygen species such as superoxide radicals, hydrogen peroxide and lipid hydro peroxides (Masoti *et al* 1988) are not possible.

Our results show a significant decrese in the levels of catalase and glutathione peroxidase in DMH treated group II rats whereas it is almost doubled in drug treated and standard group which is comparable to the control group. Catalase, glutathione peroxidase and reduced glutathione replenishment on chlorella supplementation throughout the experimental group reflects a favourable balance between potentially harmful oxidants and protective antioxidants. Furthermore elevated catalase levels can have an inhibitory role on cell transformation. Catalase has been found to significantly reduce the chromosomal aberrations and also to delay or prevent the onset of spontaneous neoplastic transformations in mouse fibroblasts and epidermal keratinocytes. Our results suggest that the anticarcinogenic effect of chlorella may be mediated by the induction of reduced glutathione because this endogenous tripeptide molecule can detoxify various carcinogeus, serve as an intracellular antioxidant and also regulated DNA and protein synthesis (Meister and Anderson 1983) The cytotoxic action of chlorella against cancer cells may be through the mobilization of endogenous copper and the consequent prooxidant action which might be one of the mechanisms involved in ROS mediated tumor cell apoptosis and cancer chemoprevention. Thus the significant increase in colon lipid peroxidation products observed on chlorella supplementation may be correlated with its prooxidant property.

CONCLUSION

Being an antioxidant chlorella altered the lipid peroxidation status and the oxidation induced cell damages. The anticancer effect may also be attributed its high β- carotene content.

Chlorella pyrenoidosa, the green algae used in our study is claimed to have many therapeutic benefits like immune stimulation, anticancer action, antianemic action and is claimed to be effective in the management of obesity, pancreatitis, gastritis, duodenal ulcer, sleep disorder and in other conditions. The nutritive value is very high in chlorella. It contains 50-60%. protein, chlorophyll, Vit A, β-carotene, vit-B_1, B_2, B_6, $B1_2$, niacin, pantothenic acid, folic acid, calcium phosphorus, magnesium, iron, zinc and copper. The immunostimulant action of chlorella (Macrophage stimulation T-Cell stimulation and interferon stimulation) has been well documented.

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