RESEARCH COMMUNICATION

Chemoprevention by *Triticum Aestivum* of Mouse Skin Carcinogenesis Induced by DMBA and Croton Oil - Association with Oxidative Status

Priyanka Arya, Madhu Kumar*

Abstract

Chemopreventive action of wheat grass (Triticum astivum) leaf extract in Swiss albino mice was evaluated. Oral administration of wheat grass leaf extract at a dose level of 20 ml/kg body weight per day at pre, peri, and post-initional phases and in combination group, caused significant variation in tumour incidence and tumour yield as compared to the control group. Moreover, the average latent period was significantly increased from 9.87±0.12 to 13.4±0.23 weeks in the combination group, together with significant elevation of reduced glutathione (GSH), superoxide dismutase (SOD) catalase (CAT) and reduction in lipid peroxidation (LPO) was observed as compared to the control group.

Keywords: Chemoprevention - wheat grass leaves extract - papillomas - GSH - SOD - CAT - LPO

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Introduction

Cancer is still a major cause of mortality and morbidity in developing as well as in developed countries (Greenwald et al., 2002; Soria et al., 2003) . From more than 12 million newly diagnosed cancer cases in 2007, the number of newly diagnosed global cancer cases is expected to reach over 17 million by 2020 (New York Times, 2008). The purpose of cancer prevention is to cause delay in onset of cancer, its progression from precancerous lesion or recurrence after treatment. Therefore ultimate goal of cancer prevention is preferably to live without cancer or with cancer without suffering from symptoms until the natural termination of life (Tsuda et al., 2004). The majority of cancer are reported to be caused by environmental carcinogenic agents (Wenk et al., 2001; Stefanie et al., 2010) occupational environmental and dietary habits (Shukla and pal, 2004).There are increasing rise in incidence of skin cancer patients throughout the world (Greenlee et al., 2001; Gupta et al., 2001).It has been reported that variety of physical and chemical insults making it most accessible organ to environmental contaminants (Green et al., 1999). Epidemiological and experimental evidence suggests that diets rich in fruits and vegetables helps in the prevention of chronic disease, such as cancer (Park and Pezzuto, 2002; Aderson et al., 2003; Balder et al., 2006; Michelse et al., 2006; Ferruzia and Blakslee, 2007; Kumar et al., 2010).

Wheat grass (Triticum aestivum) refers to the young grass of the common wheat plant which belongs to gramineae family.and has shown potential anti-inflammatory, antioxidant and antiaging properties (Smith2000). Benarya et al., (2002), Devogel et al (2005) Ferruzia and Blakslee (2007) had reported that regular ingestion of leaves extract improve the digestive system ,promote general well being ,detoxify the blood stream. Wheat grass juice is an effective alternative of blood transfusion. Its use in terminally ill cancer patients should be encouraged (Dey et al., 2006). Wheat grass has been used as nutritional alternative to chemotherapy for primary peritoneal cancer (Guisseppi, 2005). Recently Indian researchers examined the use of wheat grass juice by children with thalassemia patients who consumed 100 ml of wheat grass juice daily, reduced their blood transfusion requirement by up to 400% with no adverse effect (Marwaha et al., 2004).

In the present study an attempt has been made to study the chemopreventive activity of wheat grass (Triticum aestivum) on the skin papillomagenesis induced by DMBA and croton oil.

Materials and Methods

Animals

Random-bred, male Swiss albino mice, (7-8 weeks) were used for experiments. These animals were maintained in the animal house at temperatures of 24 ± 3°C and a light of 14:10 hours of light and dark. These animals were housed in polypropylene cages and fed standard mice fed from Aashirwad industries, Chandigarh India. Tap water was provided to the animals ad libitum and tetracycline was given monthly to the animals against infections.

*Cell and Molecular Biology Lab, Centre for Advance Studies, Department of Zoology, University of Rajasthan, Jaipur, India *For correspondence : mamsjpr@yahoo.com*

Chemicals

DPPH, 7, 12-Dimethyl benz(a) anthracene (DMBA) and croton oil were obtained from Sigma Chemicals Co., USA. Croton oil, 5,5-Dithio bis-2-nitro benzoic acid (DTNB), thio barbituric acid (TBA), Sodium Dodecyl Sulphate (SDS), methanol, acetic acid, sodium chloride, phosphate buffers, Pyrogallol, n- Butanol, TCA, Tri chloro acetic acid, TMP, tetra methoxy propane and MPA, meta phosphoric acid were obtained from Qualigens, HiMedia Laboratories Ltd, India and Sigma Chemicals Co. (USA).

Preparation of Wheat Grass (T. aestivum) Leaves Extract

Wheat grass leaves were collected locally after its proper identification from herbarium, department of Botany, University of Rajasthan, Jaipur, India (RUBL No-20597). The leaves were cut and freshly squeezed and juice was collected. The juice was administered orally to the animals.

Evaluation of antioxidant capacity

Radical scavenging activity of leaves extracts against stable DPPH (1,1-diphenyl-2-picryl hydrazyl radical) was determined spectrophotometrically. The DPPH assay was carried out as described by Cuendet et al., (1997). Stock solutions of crude extracts were prepared as 1 mg/ml in methanol. 50 µl of different concentration samples were added to 5 ml of 0.004 % methanol solution of DPPH. After 30 min of incubation in the dark at room temperature, the absorbance were read against a blank at 517 nm. The assay were carried out in triplicate and percentage of inhibition were calculated using the following formula:

% Inhibition = [(AB - AA)/ AB] x 100

Where AB = Absorbance of blank; AA = Absorbance of test.

Induction of Tumours

The hair on the dorsal region (back) of the animals were shaven 3 days before the commencement of the experiment and only those animals on the resting phase of the hair cycle were chosen.

For the induction of the tumours, a two stage protocol consisting of initiation with a single topical application of carcinogen DMBA followed two weeks later by a promoter, croton oil, three times a week, were employed as per our previous modified method of Berenblum,1941 (Prashar and Kumar, 1994). Animals devided into following groups to investigate anti- tumour potential of wheat grass:

Group 1 (Control group): 10 animals were applied topically with a single dose of DMBA (100μl/ 50μl of acetone) over the shaven area of the skin of the mice. Two weeks later, croton oil (1% in 100 μl of acetone) was applied as a promoter 3 times in a week till the end of the experiment (i.e. 16 weeks)

Group 2 (Pre group): Animal received wheat grass leaves extract orally (20ml/kg body weight) for 7 days. Before the application of DMBA (100μl/ 50μl of acetone) applied topically over the shaven area of the skin of the mice and two weeks later, promoted by repeated application of croton oil (1% of 100ul acetone)three times per week till the end of experiment.

Group 3 (Peri group): Animal were treated with DMBA (100μl/ 50μl of acetone) and then received wheat grass leaf extract orally (20ml/kg body weight) for 15 days, followed by croton oil three times a week as in group 1.

Group 4 (Post group): DMBA (100μl/ 50μl of acetone) was given as in group 1. Animal received wheat grass leaves extract orally (20ml/kg body weight), starting from the time of croton oil treatment and continued till the end of the experiment.

Group 5 (Combination group): The animal received wheat grass leaves extract orally (20ml/kg body weight) throughout the experimental period i.e., 7 days before and after DMBA (100μl/ 50μl of acetone) application as well as at the promotion stage till the end of the experiment Croton oil was given as in group 1.

Tumour incidence, tumour yield, and tumour burden were calculated after the termination of the experiment. Average latent period was calculated as time lag between the application of the promoting agents and the appearance of tumour in 50% of the animals.

Biochemical studies

Preparation of Homogenates. Animals were killed by cervical dislocation after the termination of the experiment and the entire liver was then perfused in situ immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free extraneous tissue and rinsed in chilled 0.15M Tris KCl buffer (PH 7.4) to yield a 10% (w/v) homogenate. An aliquot 0.5 ml of this homogenate were used for assaying reduced glutathione, lipid peroxidation, superoxide dismutase and catalase.

Determination of Reduced Glutathione (GSH). Hepatic level of reduced glutathione was determined by the method of Moron et al., (1979). Reduced glutathione was used as a standard to calculate μ mole GSH/100 gm tissue.

Estimation of Lipid Peroxidation (LPO). The lipid peroxidation level was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkawa et al., (1979) and is expressed in terms of malondialdehyde (MDA) formed per mg protein.

Determination of Superoxide Dismutase (SOD) Activity. Superoxide dismutase was assayed using the method of Marklund and Marklund (1974), involving inhibition of pyrogallol auto oxidation at pH 8.0. A single unit was defined as the quantity of superoxide dismutase required to produce 50% inhibition of auto oxidation.

Determination of Catalase (CAT) Activity. Catalase was estimated at 240 nm by monitoring the disappearance H_2O_2 as described by Aebi (1984). Catalase enzyme specific activity has been expressed as μ mole of H_2O_2 decomposed/min/mg protein.

Statistical analysis

The data are expressed as mean ± SE. Statistical significance between the groups was determined by one way ANOVA.

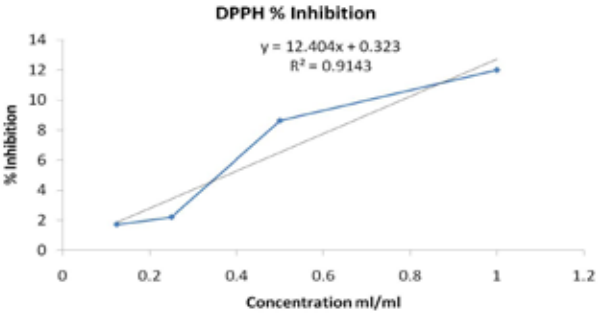

Figure 1. Inhibition of DPPH by Triticum Astivium Extract at Different Concentrations

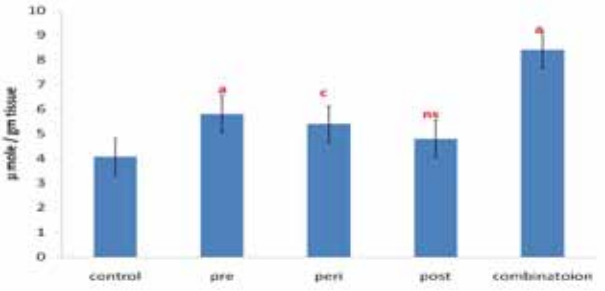

Figure 2. Modulatory Influence of *T. aestivum* on Mice Hepatic Antioxidant Status (GSH)

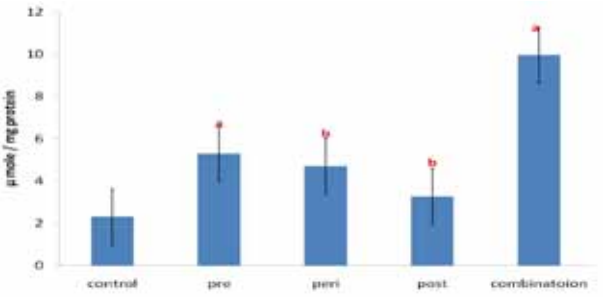

Figure 3. Modulatory Influence of *T. aestivum* on Mice Hepatic Antioxidant Enzyme (SOD)

Results

Antioxidant study

To evaluate the chemopreventive effect of *T.aestivum* extract the free radical scavenging capcity of the extract was observed. *T.aestivum* extract showed to be good radical scavengers with the inhibition of 12% at a 1ml/ml concentration of the extract in methanol (see Figure 1).

Biochemical study

Wheat grass leaves extract given orally (20ml/kg body weight) significantly increased reduced gluthione (GSH), superoxide dismutase (SOD) and catalase (CAT) as compared to control group (Figures 2, 3, 4), whereas lipid peroxidation (LPO) in all the experimental groups was significantly decreased (Figure 5).

Tumour study

Tumour incidence, yield, and burden in all groups i.e pre, peri, post and in combination (with *T.aestivum*) were significantly decreased as compared to control (alone with DMBA & croton oil) (Figures 6, 7, 8), whereas the average latent period was increased, being highest in the group 5 combined treatment group (Figure 9).

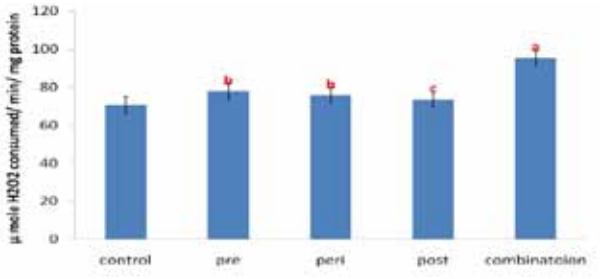

Figure 4. Modulatory Influence of *T. aestivum* on Mice Hepatic Antioxidant Catalase (CAT)

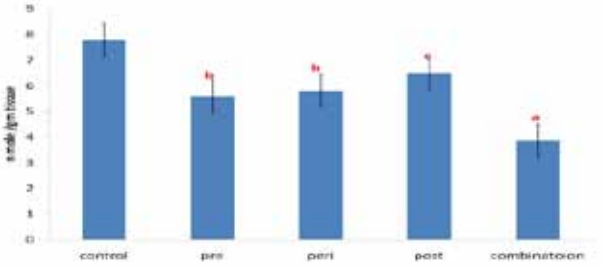

Figure 5. Modulatory Influence of *T. aestivum* on Mice Hepatic Lipid Per Oxidation (LPO)

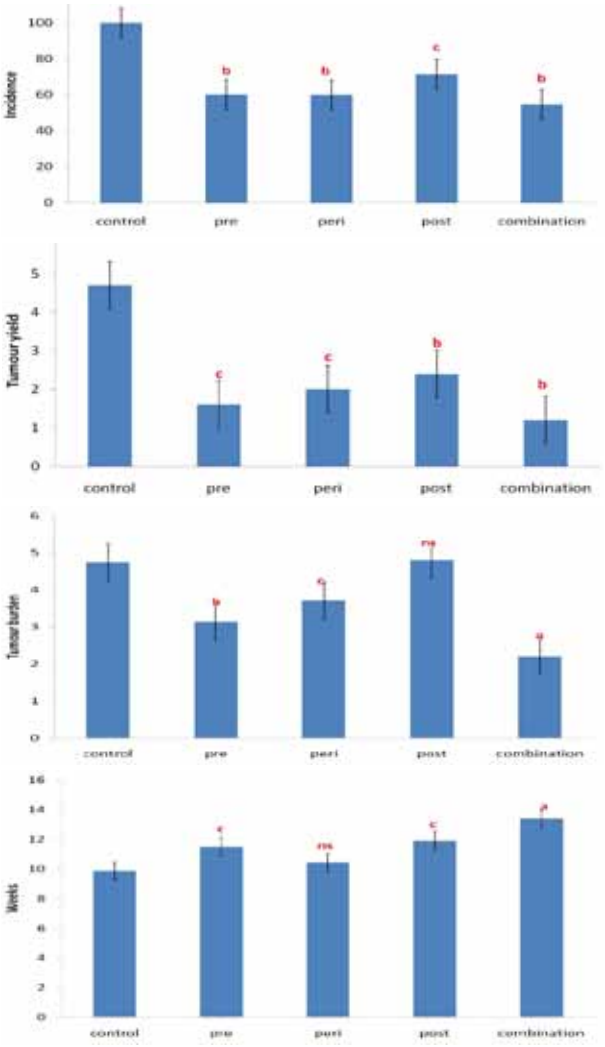

Figure 6. Tumour Data. a) Incidence; b) yield; c) burden; d) average latent period.

Discussion

The skin carcinogenesis model in experimental animals has been found to be a very good test system for investigating the influence of dietary (fruits, vegetable and herbs) and chemopreventors both mechanistically and operationally (Kausar et al., 2003). In our laboratory plant extracts of *Ocimum* (Prashar and Kumar, 1995), *Spirulina* (Mittal et al., 1998), *Brassica* (Qiblawi and Kumar, 1999), *Mentha* (Yasmeen and Kumar, 2001), *Ginseng* (Panwar et al., 2005), combination of *Mentha* and *Brassica* (Sharma and Kumar, 2006) and *Acacia nilotica* (Meena et al., 2006) have been proved to be chemopreventive and many showed DPPH activity (Smarth et al 2008).

The present investigation showed inhibition of tumour incidence, tumour yield, and tumour burden in the *T.aestivum* extract treated groups in comparison to control group.

There were significantly increased levels of GSH, SOD and CAT and decrease in LPO content in *T.aestivum* extract treated groups during pre peri and post initialional stage as compared to control group.

Reactive oxygen species (ROS) formed during DMBA metabolism or secondarily during tumor formation can diffuse from the site of generation to other targets within the cells or even propagate the injury to other intact cells. DMBA and its metabolites are documented to mediate their mutagenic and carcinogenic effect via ROS generation that acts complementary to the mutation induced by diol epoxides (Rubin, 2001). However the increased ROS load and loss of cellular redox balance can promote carcinogenesis. (Allen et al., 2000; Finkel and Holbrook, 2000). An excessive exposure to ROS could shift the prooxidant-antioxidant balance of skin toward a more oxidative state i.e., increased oxidative stress. The resulting oxidative stress causes damages to cellular components and changes the pattern of gene expression leading to skin pathologies (Ananthaswamy and Kanjial, 1996). Thus, in the present study enhanced hepatic LPO in DMBA treated animals and increased tumour burden may be due to the generation of ROS exacerbated by decreased efficiency of host antioxidant defense mechanisms. GSH maintains the integrity of the liver when the organ is challenged by a wide variety of xenobiotics, ROS and toxic compounds (Lu, 1999).The liver, which is rich in GSH, supplies this antioxidant to various extra hepatic tissues via a distinct GSH transport system (Locigno and Castronovo, 2001). GSH resulting from increased utilization to scavenge lipid peroxides may shift the redox status towards oxidative stress.

SOD and CAT play an important role in the detoxification of reactive oxygen species such as O2-, OH-, and H_2O_2 which are involved in genotoxicity and various stages of chemical carcinogenesis (Troll et al., 1984; Cerutti, 1985; Oberley and Oberley, 1986). SOD and CAT activities maintains the physiological level of oxygen and hydrogen peroxide by dismutation of oxygen radicals and decomposition of hydrogen peroxide (Gonzales et al., 1984; Sijun et al., 2000; Dasgupta et al., 2004) hence offering a protective role against the free radicals.

T.aestivum is a source of chlorophyll, selenium, amino acid, abscisic acid caroteniods, vitamins (A, E, B_{12} C and K) and antioxidant enzymes such as SOD, P4D1(Gloria, 2007; Bcronin2009). Its shows anti-inflammatory, antioxidant, antiaging, antimutaganic properties. SOD present in the *T.aestivum* extract plays an important role in the antioxidant enzyme defense system. The generation of reactive oxygen is inhibited and the dismutation of superoxide radical is accelerated by the catalyzing role of SOD. Catalase helps in removing the hydrogen peroxide produced by the action of SOD. Thus increased SOD activity, along with that of Catalase in all combination groups explain the protective effect of *T.aestivum* along with significant decrease in lipid per oxidation .

P4D1 and Abscisic acid are compounds that eat away the protective coating of cancer cells, making them vulnerable to the immune system. P4D1 also has an antiaging effect known to rebuild damaged DNA. Both compounds are found in wheatgrass extract (Gloria, 2007; Philippine, 2009).

Natural chlorophyll derivatives have demonstrated biological activities in vitro and in vivo consistent with the prevention of cancer including antioxidant activity, antimutaganic activity,modulation of xenobiotic enzyme, and induction of apoptotic events in cancer cell lines.(Egner et al., 2003). Chlorophyll may deliver more systemic physiological effects consistent with the prevention of cancer. Promising data on the ability of chlorophyll to modulate xenobiotic metabolizing enzymes. (Dingley et al., 2003; Fahey et al., 2005) and cartotenoids were shown to be effective in reducing tumour development in organ such as lung , liver,colon ,and skin in experimental animals.They posses antioxidant action as one of the mechanism for their cancer preventive effects (Nishino et al., 2002; Lynnette et al., 2004).

Vitamins C quenches reactive oxygen species and inhibits DNA synthesis and lipid peroxidation (Kalka et al., 2000; Biesalski and Obermueller-Jevic, 2001). Many epidemiological studies indicate that vitamin C intake is clearly related with low cancer incidence (Lee et al., 2003; Liu et al., 2003). Vitamins E is a promising chemopreventive and pharmacologically safe agent,which can be exploited or tested against skin cancer (Shakilur et al., 2008) Vitamins A, (its physiological metabolite and synthetic derivatives retinoids) have been shown to have protective effects against the development of skin cancer (Niles, 2000; Digionvanojj, 2001).

Anticancer properties found in wheat grass include selenium. Selenium builds the immune system. Strong immune system can lead to a decreased risk of diseases including cancer (Scott, 2009). Selenium is an essential element with physiological non enzymatic antioxidant anticarcinogenic and anti-inflammatory properties (Zelina et al., 2008). Antioxidants have been suggested to scavenge free radical and prevent their interaction with cellular DNA (Ferguson et al., 2004).

In conclusion, results of the present study suggest that *T.aestivum* leaves extract affects liver enzyme activities as well as lipid per oxidation and has modulatory effect on the two stage skin carcinogenesis and exhibit chemopreventive activity, which may be due to its pharmacological properties.

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