ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2013; 4(4):758 - 770

ORIGINAL RESEARCH ARTICLE

Linalool Exerts Dose Dependent Chemopreventive Effect Against 1, 2-Dimethylhydrazine Induced Rat Colon Carcinogenesis

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Received 09 May 2013; Revised 10 Aug 2013; Accepted 20 Aug 2013

ABSTRACT

Our aim is to identify the chemopreventive potential of linalool against 1, 2-dimethylhydrazine (DMH) induced rat colon carcinogenesis. Colon tumours were induced in rats by subcutaneously injecting DMH (20 mg/kg body weight [b.w.]) once a week for first 4 consecutive weeks and supplemented with linalool at three different doses of 25, 50 and 100 mg/kg b.w./day for the total experimental period of 16 weeks. DMH alone administered rats showed increased tumour incidence, ACF formation and phase I xenobiotic metabolizing enzymes activities accompanied by decreased tissue lipid peroxidation and antioxidants which were reversed on linalool supplementation suggesting its optimal anticarcinogenic effect at the dose of 50 mg/kg b.w.

Key words: Colon cancer, chemoprevention, linalool, aberrant crypt foci, xenobiotics, oxidative stress.

1. INTRODUCTION

Cancer continues to be one of the major causes of death worldwide; it is a genetic disease that usually arises from the accumulation of several gene mutations in healthy cells, resulting in the failure of cell cycle control and uncontrolled growth of abnormal cells. Colorectal cancer (CRC) occurs when abnormal tissues grow on the inner walls of the colon or rectum. These abnormal tissues are commonly present in the form of polyps. Polyps grow as a projection of tissue away from the colon wall, remaining connected to the colon wall by way of a thin stalk. Their shape is similar to that of a mushroom. The rate of colon cancer incidence was low in India but is presently increasing; out of 3.5 million cancer cases, 35,000 suffer from colon cancer [1]. Colon cancer can be induced in experimental animals with 1,2-dimethylhydrazine (DMH). DMH is a procarcinogen which induces colon tumour formation after undergoing various metabolic changes in the colon and liver [2]. DMH produces tumours of the large bowel in susceptible strains of both rats and mice. DMH is an indirectly acting colon carcinogen that must be activated to DNA-reactive metabolites [3]. DMH provides a powerful model for studying the pathogenesis of this disease. The cells at the subcutaneous site do not possess the enzymes capable of reacting with DMH. Damage to DNA from reactive oxygen species (ROS) is a consequence of oxidative stress and several oxidative DNA adducts, including 8-oxodG, has been implicated in the tumourigenic process [4]. ROS may be involved in the pathogenesis of various human diseases because they induce damage to biological macromolecules such as DNA, carbohydrates and proteins. Glutathione-S-transferase (GST) is a ubiquitous enzyme, which provides cellular protection against a wide variety of xenobiotics. This enzyme is an attractive candidate biomarker for both cancer susceptibility and chemopreventive activity. GST primarily functions in conjugating "functionalized P450 metabolites" with GSH, thereby favouring carcinogen elimination. In the presence of xenobiotics, GST and GPx generally help in the detoxification, by conjugating GSH with toxic electrophiles conferring a selective growth advantage to cancer cells [5]. Induction of these enzymes has been become essential in determining the potency of many anti-carcinogenic substances [6]. Xenobiotic metabolizing enzymes are probably involved in the development of colonic carcinoma [7]. The

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colonic epithelium is in continuous contact with potentially carcinogenic compounds, which enter the body usually as part of the diet. Xenobiotics may be activated or detoxified by phase I and phase II biotransformation enzymes including cytochrome P450, epoxide hydrolases, and glutathione-S-transferases. Metabolism may occur in the gastrointestinal tract, including the colon or the liver, or both [8].

Linalool is a monoterpene, found as a major component of the essential oils of several aromatic plant species. Linalool is the main ingredient in coriander. It is naturally occurring in many flowers, plants and spices with many commercial applications. It has other names such as linalool, linalyl alcohol, linaloyl oxide, p-linalool, allo-ocimenol and 2, 6-dimethyl-2, 7-octadien-6-ol (**Fig 1**). It is an appreciably water-soluble, organic liquid at room temperature. It is used in vitamin E synthesis, added to processed food and beverages, and also used in perfumes, cosmetics and soaps as well as in household detergents and waxes. Linalool was used to control food pest traditionally. The aim of the present study was to investigate the dose dependent chemopreventive effects of linalool on the development of aberrant crypt foci, xenobiotic metabolizing enzymes, lipid peroxidation, and antioxidant status in DMH induced rat colon carcinogenesis.

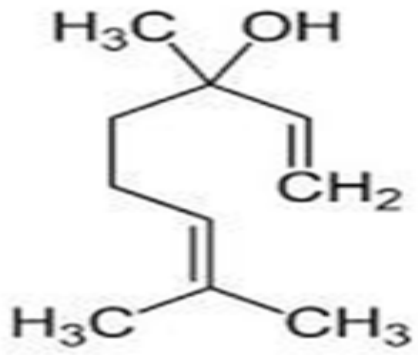

Fig 1: Structure of linalool

2. MATERIALS AND METHODS

Chemicals

Linalool and DMH were purchased from Sigma Chemicals Co., St. Louis, MO, USA. All other chemicals and reagents used were of analytical grade and obtained from Hi-Media Laboratories Ltd., Mumbai, India.

Preparation of linalool

Linalool was suspended in corn oil and was administered orally by intragastric intubation daily at three different doses of 25, 50, and 100 mg/kg b.w.

Tumour induction

DMH was dissolved in 1 mM EDTA just prior to use and the pH adjusted to 6.5 with 1 mM NaOH to ensure the stability of the carcinogen. The rats were given subcutaneous injections of DMH for first 4 consecutive weeks at the dose of 20 mg/kg b.w.

Animals and experimental design

Male Wistar rats (n=12 per group) were used for this study. They were all maintained in air-conditioned animal facility (Central Animal House, RMMCH, Annamalai University) with a 12-h light/dark cycle, and provided with modified pellet diet along with 20% fat (peanut oil) (**Table 1**). The rats were cared in compliance with the principles and guidelines of Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Reg. No 160/1999/CPCSEA).

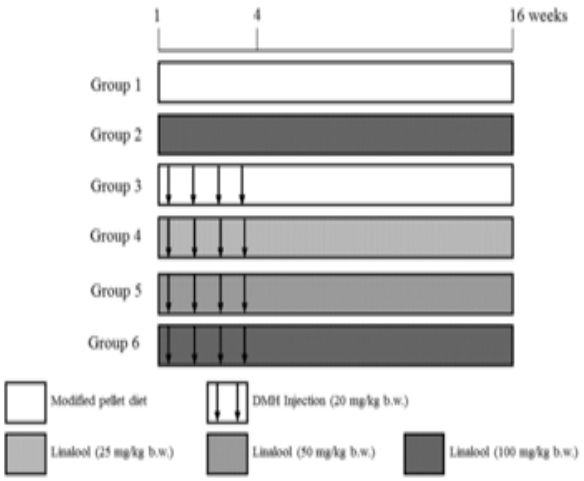

Fig 2: Schematic representation of the experimental design.

Table 1 Composition of the experimental diet

Nutrient	Commercial pellet diet (84.2%)	Peanut oil (15.8 %)	Total (100%)
Protein	17.7	-	17.7
Fat	4.2	15.8	20.0
Carbohydrates	50.5	-	50.5
Fiber	3.4	-	3.4
Minerals	6.7	-	6.7
Vitamins	1.7	-	1.7

The rats were treated as follows;

Group 1 (Control)	Rats received modified pellet diet.
Group 2 (Control + Linalool)	Rats received modified pellet diet + linalool (100 mg/kg b.w. p.o) every day for 16 weeks.

Group 3 (DMH)	Rats received DMH injection s.c. (20 mg/kg b.w.) once a week for the first 4 weeks then kept without any treatment for the remaining 12 weeks
Group 4 (DMH + Linalool)	Rats received DMH as in group 3, in addition to linalool (25 mg/kg b.w. p.o.) for the entire period of experiment.
Group 5 (DMH + Linalool)	Rats received DMH as in group 3, in addition to linalool (50 mg/kg b.w. p.o.) for the entire period of experiment.
Group 6 (DMH + Linalool)	Rats received DMH as in group 3, in addition to linalool (100 mg/kg b.w. p.o.) for the entire period of experiment.

The total experimental period was 16 weeks, which is diagrammatically represented in (**Fig 2**). Body weights of rats were recorded initially, at weekly intervals and also at the end of the experiment. Animals were fasted overnight prior to the day of termination of the experiment.

Preparation of plasma/lysate and tissue homogenate

Animals were anaesthetized with an intra-peritoneal injection of ketamine hydrochloride (30 mg/kg b.w.) and sacrificed by cervical decapitation and the tissue samples (liver, proximal colon, and distal colon) were immediately rinsed with 0.9% NaCl and homogenized using the appropriate buffer in a tissue homogenizer. Blood was collected in heparinized tubes and plasma was separated by centrifugation at 2000g for 10 min. After the separation of plasma, the buffy coat was removed and packed red blood cells were washed thrice with cold physiological saline. Erythrocyte lysate was prepared by lysing a known volume of red blood cells with hypotonic phosphate buffer, pH 7.4. Centrifuging at 3000g for 10 min at 4 °C separated the hemolysate.

Preparation of cytosolic and microsomal fractions

Cytosolic and microsomal fractions prepared from individual tissues (liver and colonic mucosa) were homogenized in 0.25 M sucrose and centrifuged at 9000g for 20 min. The supernatant was collected, 0.2 volumes of 0.1 M $CaCl_2$ in 0.25 M sucrose was added to each and the sample were kept on ice for 30 min, centrifuged at 27,000g for 20 min which yielded clear cytosolic fractions that were promptly assayed for phase II xenobiotic metabolizing enzymes. Microsomal pellets were washed twice by suspending in 7 ml of 10 mM Tris-HCl (pH 7.4) in 0.25 M sucrose, centrifuged at 9000g for 20 min which yielded microsomal fractions that was promptly assayed for phase I xenobiotic metabolizing enzymes.

Determination of aberrant crypt foci (ACF)

At the end of the 16 week study, rat colons were removed and flushed with potassium phosphate buffered saline (0.1 M, pH 7.2). Colons were split open longitudinally and placed on strips of filter paper with their luminal surface open and exposed. Another strip of filter paper was placed on top of the luminal surface. The colons were then secured and fixed in a tray containing 10% buffered formalin overnight. Each of the fixed colons was cut into proximal and distal portions of equal lengths and each portion was further cut into 2 cm long segments. Each segment was placed in a petri dish and stained with 0.2% methylene blue solution for 2 min. The segments were then transferred to another petri dish containing buffer to wash off excess stain. The segments were examined using a light microscope at low magnification to score the total number of ACF as well as the number of crypts per focus. ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells. ACF in the colon were counted as described by [9].

Xenobiotic metabolizing enzymes

Cytochrome P450 was assayed by the method of [10]. Cytochrome P450 was determined using carbon monoxide (CO) difference spectra. The absorbance of CO adducts formed by the reaction of reduced cytochrome P450 with CO were measured at 450 nm. Cytochrome P450 was determined using the absorption coefficient of 104 mM^{-1} respectively.

Cytochrome P4502E1 (CYP4502E1) activity was assayed by the method of [11]. The assay mixture contained 100 µl microsomal protein, 40 mM p-nitrophenol and 0.1 M phosphate buffer. The reaction was initiated by the addition of 10 mM of NADPH and incubated at 37 °C for 60 min. The reaction was stopped with 20% TCA and centrifuged at 1000rpm for 5 min, 10 mM NaOH

was added to the supernatant and the absorbance was measured at 450 nm.

Glutathione-S-transferase (GST, EC. 2.5.1.18) activity was assayed spectrophotometrically by the method of [12]. The reaction mixture contained 1.7 ml phosphate buffer (100 mM; pH 6.5), 0.1 ml GSH (30 mM), and 0.1 ml 1-chloro-2, 4-dinitrobenzene (CDNB, 30 mM). After pre-incubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 ml erythrocyte lysate and the absorbance was followed for 5 min at 340 nm. The specific activity of GST was expressed as mmoles of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6×10^{-4} $mM^{-1}cm^{-1}$.

Estimation of peroxidation markers

Lipid peroxidation (LPO) is one of the basic mechanisms of cellular damage caused by reactive oxygen species. A well-established mechanism of cellular injury is used as an indicator of oxidative stress. Total LPO levels and key enzymes that indicate the antioxidant status of animals were analysed using established biochemical procedures as follows: The levels of tissue thiobarbituric acid reactive substances (TBARS) were measured using the method of [13]. The pink chromogen produced was measured at 532 nm. The level of conjugated dienes (CD) was assessed by the method of [14].

Estimation of antioxidants markers

Superoxide dismutase (SOD, EC. 1.15.1.1) activity was assayed by the method of [15]. based on 50% inhibition of the formation of NADH-phenazinemethosulphate(PMS)nitrobluetetrazolium (NBT) formazan at 520 nm.

Catalase (CAT, EC. 1.11.16) was assayed by the method of [16]. The reaction mixture contained 0.1 ml of tissue homogenate, 1 ml of phosphate buffer (0.01 M, pH 7.0) and 0.2 M H_2O_2 The reaction was arrested by the addition of dichromate acetic acid reagent and the chromic acetate formed was measured at 590 nm.

Glutathione peroxidase (GPx, EC. 1.11.1.9) was assayed by the method of [17]. The reaction mixture comprised of 0.2 ml EDTA (0.8 mM), 0.1 ml sodium azide (10 mM), 0.2 ml erythrocyte lysate, 0.2 ml reduced glutathione (GSH; 30 mM), and 0.1 ml H_2O_2 (0.2 M). The contents were incubated at 37 °C for 10 min and the reaction was arrested by adding 0.5 ml of 10% trichloroacetic acid and centrifuged. To the supernatant, 0.2 ml of phosphate buffer (0.4 M; pH 7.0) and 1.0 ml Ellman's reagent were added and read at 520 nm.

Glutathione reductase (GR, EC 1.6.4.2) was assayed spectrophotometrically by the method of [18]. Glutathione reductase (GR) is a glutathione regenerating enzyme, which permits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD^+ was used as the index of enzyme activity. The assay mixture contained1.0 ml of potassium phosphate buffer (50 mM; pH 6.6), 0.5 ml of GSSG (0.8 mM), 0.5 ml of NADPH (0.48 mM), and 20 ml of erythrocyte lysate. The absorbance was recorded every 10 min for 1 h, relative to a control in which GSSG was omitted.

Reduced glutathione (GSH) was assayed according to the method of [19]. GSH estimation was based on the development of yellow colour when 5,5-dithiobis (2-nitro benzoic acid) was added to compounds containing sulfhydryl groups.

Statistical analysis

The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $p<0.05$. All statistical analyses were made using SPSS 11.0 software package (SPSS, Tokyo, Japan).

3. RESULTS

Mortality

During this study we did not observe any evidence of mortality, all the animals survived during the entire period of the experiment.

Colon polyps/tumour analysis

In the carcinogen treated group (group 3), polyps/tumour incidence was 100%. Administration of linalool at the doses of 25, 50 and100 mg/kg b.w. to DMH treated rats resulted in reduced tumour incidence (**Table 2**).

Effect of linalool and DMH on ACF

Aberrant crypt foci (ACF) are stereoscopically distinct from normal crypts as evident by their darker staining, larger size, elliptical shape, thicker epithelial lining, and larger pericryptal zone. Total numbers of crypts in the different experimental groups are shown in (**Table 3**) and fig.3. No ACF were detected either in the control rats or in the linalool alone supplemented control rats. DMH treated rats (group 3) showed a significantly higher number of total aberrant

crypts as compared to control rats. Supplementation with linalool at different doses of 25, 50, and 100 mg/kg b.w. (group 4, 5 & 6) to DMH treated rats significantly reduced the total number of aberrant crypts. The reduction in ACF incidence was 44.1%, 68.7% and 58.8% in rats supplemented with linalool at the doses of 25, 50 and 100 mg/kg b.w. respectively. The reduction in ACF as well as the tumour incidence was more pronounced in the rats supplemented with 50 mg/kg b.w. linalool (group 5).

Effect of linalool and DMH on phase I enzymes of control and experimental rats

The activities of xenobiotic metabolizing enzymes (cytochrome P450 and cytochrome P4502E1) in the liver and colonic mucosa of control and experimental rats are shown in (**Fig 4 & 5**). Cytochrome P450, and cytochrome P4502E1, activities were significantly elevated in DMH treated rats (group 3) as compared to control rats (group 1). On supplementation with linalool at different doses of 25, 50, and 100 mg/kg b.w. to DMH treated rats the activities of DMH metabolizing phase I enzymes were significantly decreased as compared to the DMH alone treated rats. More pronounced effect was observed in rats supplemented with linalool at the dose of 50 mg/kg b.w. (group 5).

Effect of linalool and DMH on the phase II enzyme of control and experimental rats

The effect of linalool and DMH on the phase II enzyme, glutathione-S-transferase (GST) in the liver and colonic mucosa of control and experimental rats is represented in fig.6. DMH treatment caused a significant reduction in the GST activities of group 3 rats as compared to the control rats (group 1 and 2). Supplementation with linalool produced significant increase in the activity of GST in groups 4, 5 and 6 as compared to DMH alone treated rats. Supplementation with linalool at the doses of 25, 50, 100 mg/kg b. w. to DMH treated rats restored the GST activities. More pronounced effect was observed when linalool was supplemented at the dose of 50 mg/kg b.w.

Effect of linalool on lipid peroxidation byproducts

(**Tables 4 & 5**) also show the effects of linalool and DMH on the levels of lipid peroxidation byproducts TBARS and CD of control and experimental rats. The lipid peroxidation byproducts such as TBARS and CD were significantly decreased ($p<0.05$) in the colonic tissues whereas in the circulation and liver the levels were significantly increased (group 3) as compared to the control rats (group1 and 2). The lipid peroxidation levels were restored to near those of the control values on supplementation with linalool at different doses such as 25, 50 and 100 mg/kg b.w. to DMH treated rats. More pronounced effect was observed in the rats supplemented with linalool at the dose of 50 mg/kg b.w.

Effect of linalool and DMH on SOD and CAT of control and experimental rats

The activities of SOD and CAT of control and experimental rats are shown in (**Table 6**). The activities of these enzymes were significantly lowered in the tissues (liver, proximal colon, distal colon and lysate) of DMH treated rats as compared to the control groups at the end of 16 weeks. Linalool supplementation at different doses of 25, 50 100 mg/kg b.w. (group 4, 5 & 6) significantly increased the activities of SOD and CAT as compared to DMH alone treated rats. More pronounced effect was observed in the rats supplemented with linalool at the dose of 50 mg/kg b.w. (group 5).

Effect of linalool and DMH on GSH and glutathione dependent enzymes

The activities of glutathione dependent enzymes (GPx and GR) and the levels of GSH of control and experimental rats are shown in table 7 and 8. Activities of glutathione dependent enzymes and the levels of GSH were significantly decreased in the circulation, liver and colon tissues of DMH treated rats (group 3) as compared to the control rats (group 1). Supplementation with linalool at different doses (25, 50 and 100 mg/kg b.w.) to carcinogen exposed rats (groups 4, 5 & 6) significantly elevated the activities/levels of glutathione dependent enzymes and GSH as compared to the DMH treated rats (group 3). More pronounced effect was observed in the rats supplemented with linalool at the dose of 50 mg/kg b.w. (group 5).

4. DISCUSSION

Linalool, the main component of coriander seed oil extract is a monoterpene which is being used in fragrance industry and in traditional medicine [20]. The present study clearly indicates that linalool present in essential oils of many medicinal and aromatic plants at moderate doses can appreciably attenuates the alterations in the activities of xenobiotic metabolizing enzymes lipid peroxidation and antioxidants in various tissues of male Wistar rats. In general, the medium dose (50

mg/kg b.w.) was more effective than the low (25 mg/kg b.w.) and the high dose (100 mg/kg b.w.).

Aberrant crypt foci (ACF) have been reported to be a preneoplastic marker by a number of investigators and are used extensively to identify the modulators of colon carcinogenesis [21]. In the present study, after four injections of DMH rat colons were examined for ACF at the end of 16 weeks. The data indicates that rats treated with DMH showed higher number of ACF, ACF multiplicity and the degree of the luminal alterations. Supplementation with linalool at three different doses (25, 50, 100 mg/kg b.w.) for 16 weeks showed reduced ACF incidence, ACF multiplicity, with a concomitant reduction in average tumour size. In fact the maximum inhibitory effect was observed in the colon of rats treated with 50 mg/kg b.w. of linalool (group 5). These suggest that the significant anticancer property of linalool is partly due to its antiproliferative property via suppression of the number of crypts in DMH treated rats. Moreover supplementation with different doses of linalool for 16 weeks showed reduced tumour incidence, tumour multiplicity, with a concomitant reduction in average tumour size, suggesting the forte at be beneficial role of linalool in inhibiting tumourigenesis.

Human colonic mucosa possesses enzymes necessary for the biotransformation of xenobiotics (phase I and phase II enzymes) and therefore can activate foreign compounds present in the diet into ultimate carcinogens in close proximity to their target. These metabolites might then activate oncogenes (ras gene mutations) or inhibit anti-oncogenes in colonic epithelial cells[22].

The cytochrome P450dependent monoxygenases (phase I enzymes) are a multigene superfamily of microsomal hemoproteins, that play a key role in the biotransformation of a wide variety of xenobiotics and are involved in their detoxification [23]. Cytochrome P450 isoenzymes, are essential for the initial conversion of lipophilic xenobiotics/carcinogens into more polar, hydrophilic and water-soluble metabolites, at the same time for the induction of procarcinogens to ultimate carcinogens [24]. The activity of cytochrome P4502E1 is known to be mainly regulated by transcriptional and post transcriptional protein stabilization; It is known that the metabolic activation of DMH to methyl azoxymethanol a proximate metabolite of DMH by cytochrome P4502E1, occurs mainly in the liver [25]. The activities of cytochrome P450 and cytochrome P4502E1 were significantly increased in the liver and colonic tissues of DMH treated rats as compared to the control at the end of 16 weeks.

In this present study we have found that linalool supplementation at the doses of 25, 50 and 100 mg/kg b.w. significantly decreased the activities of cytochrome P450 and cytochrome P4502E1. The inhibitory activity of linalool against DMH induced tumourigenesis is probably due to the inhibition of the bioactivation of these carcinogens. Thus, one of the ways by which linalool acts as an effective chemopreventive agent may be by inhibiting the cytochrome P4502E1 dependent monooxygenase system as observed in our study.

Glutathione-S-transferase (GST) is an ubiquitous enzyme, which provides cellular protection against a wide variety of xenobiotics. This family of enzymes is attractive candidate biomarkers for both cancer susceptibility and chemopreventive activity. GST primarily functions in conjugating “functionalized P450 metabolites” with GSH, thereby favouring carcinogen elimination. In the presence of xenobiotics, GST and GPx generally help in detoxification by conjugating GSH with toxic electrophiles conferring a selective growth advantage to cancer cells[5]. Induction of these enzymes has become essential in determining the potency of many anti-carcinogenic substances [6].

In our study the activity GST was significantly decreased in the liver and colonic mucosa of DMH alone treated rats, while linalool supplementation enhanced the GST activity thereby enhancing the detoxification of carcinogens, resulting in the inhibition of colon carcinogenesis.

Lipid peroxidation (LPO) is one of the basic mechanisms of cellular damage caused by reactive oxygen species. Free radical mediated lipid peroxidation leads to accumulation of lipid peroxidation products such as malondialdehyde, hydrogen peroxide and also hydroxyl radicals which inturn propagate lipid peroxidation process and causes serious damage to the membranes and intracellular enzymes resulting in the loss of cell function and cell death [27]. Oxidative stress is implicated during all the stages of cancer development [28]. specifically, there is evidence indicating the generation of reactive oxygen species in various carcinogenic processes [29].

DMH is a procarcinogen which requires metabolic activation to its active electrophilic carcinogenic form through a series of oxidative steps in the liver [30]. LPO as evidenced by the increased levels of thiobarbuturic acid reactive substances (TBARS) and conjugated dienes (CD) in the liver of DMH treated rats could be attributed to DMH induced oxidative stress, and production of reactive oxygen metabolites. On the other hand the levels of TBARS and CD were significantly decreased in the colonic tissues of DMH treated rats. Previous findings in our lab have also shown similar results in short term [31]. as well as in the long term studies [32,40].

Lowered levels of lipid peroxidation in the colon of carcinogen exposed rats may be due to an inverse relationship between the levels of cellular lipid peroxidation and rate of cell proliferation and/or the extent of differentiation. [33]. suggested that highly proliferating dedifferentiated tumour cells have notably low levels of lipid peroxidation products as compared with untreated control rats. Previous studies have shown reduced rates of lipid peroxidation in the tumour tissues of various types of cancers [26, 34]. Thus, malignant tissues are less susceptible and more resistant to free radical attack and hence lipid peroxidation is less intense [35]. It is possible that the changes in lipid peroxidation status may reflect cell growth rate rather than malignancy perse. Important changes will nevertheless occur at some earlier stage during the progression of normal cells to malignant cells. Linalool supplementation at three different doses (25, 50 and 100 mg/kg b.w. groups 4, 5 and 6 respectively) significantly restored the levels of TBARS and CD and a more pronounced effect was observed in the 50 mg/kg b.w., of linalool supplemented rats (group 5). The hydroxyl groups present in linalool could have played a significant role in scavenging the free radicals **[36]**. The activity of linalool to significantly reverse the lipid peroxidation status to near normal levels could be due to its strong antioxidant and antiproliferative properties.

Antioxidants and detoxification enzymes can block carcinogenesis by acting as inhibitors of environmental carcinogens and/or mutagens [37]. The antioxidant defence system maintains a relatively low rate of reactive and harmful ROS and thus the powerful antioxidant defence mechanism of our body plays a crucial role against the toxic effects of reactive oxygen species. The enzymic antioxidants (SOD, CAT, GPx and GR), and non enzymic antioxidants (GSH) constitute a mutually supportive team of defence playing an important role in preventing and neutralizing free radicals by alleviating tissue damage.

SOD and CAT are two important enzymic antioxidants that act against toxic oxygen free radicals such as superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$) ions in the biological system; they are involved in the direct elimination of reactive oxygen metabolites, which is probably one of the most effective defences of living body against various diseases. SOD and CAT work together to eliminate reactive oxygen species and small deviation in physiological concentrations may have a dramatic effect on the resistance on cellular lipids, proteins and DNA to oxidative damage [38]. Exposure to carcinogens or tumour promoters usually decreases the activities of SOD and CAT [39]. The decreased activities of SOD and CAT observed in carcinogen exposed rats may be due to the dangerously increased levels of reactive oxygen species. Our present results are in line with our previous reports [40]. Linalool supplementation to DMH treated rats at three different doses (groups 4, 5 and 6) significantly increased the activities of SOD and CAT, a more pronounced effect being observed in the rats supplemented with 50 mg/kg b.w. linalool (group 5). Linalool is known to offer various beneficial biological effects, and by virtue of its ability to scavenge free radicals and toxic carcinogenic electrophiles may spare the burden of the antioxidant enzymes which may be the cause for the enhanced activities of SOD and CAT in the circulation, colon and liver tissues of DMH + linalool supplemented rats. These results prove the excellent antioxidant property of linalool against DMH induced colon carcinogenesis.

Reduced glutathione (GSH) is a major non enzymic antioxidant and a non-protein thiol which plays a significant role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species [41]. GSH also plays a vital role in the detoxification of many environmental carcinogens, and maintains the integrity of the gastrointestinal tract and liver [42]. It is generally accepted that reduced GSH and GSH-related enzymes play an important role in the protection of mammalian cells against the harmful effects of chemical carcinogenesis and other alkylating agents.

Glutathione peroxidase (GPx) is a selenoprotein enzyme that catalyses the reduction of a spectrum of peroxides ranging from H_2O_2 to organic hydroperoxides. GPx plays a crucial protective role by removing hydrogen peroxide and lipid (organic) hydroperoxides. Glutathione reductase (GR) is a glutathione regenerating enzyme, which permits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD^+.

In this study the levels/activities of GSH, GPx and GR were significantly lowered in the liver, circulation and colonic tissues of DMH treated rats as compared to the control rats at the end of 16 weeks, which is consistent with our previous results [43]. The depleted levels of GSH on DMH administration suggests that the tripeptide may be involved in the detoxification and possibly repair mechanism in the liver and colonic tissues. Moreover,[44]. Reported decreased activities of GPx in the circulation, colon and liver of DMH treated rats, and this reflects increased detoxification capacity of GPx. This may be due to reduced transcription of GPx in tumour cells or due to suppression of GPx activity by hydroperoxide products of lipoprotein. Previous reports from our lab [32]. suggested that an increase in the activities of the enzymes involved in detoxification process might be considered as beneficial, since this could enhance the excretion of carcinogens. In the presence of xenobiotics, GST and GPx generally helps in detoxification by conjugating GSH with toxic electrophiles conferring a selective growth advantage to cancer cells [5]. Linalool supplementation to DMH treated rats significantly elevated the levels / activities of GSH, GPx and GR activity in the circulation, liver and colonic tissues. Induction of these enzymes has been evaluated as a means for determining the potency of many anticarcinogenic substances [45]. This may be due to the reduced oxidative stress and possibly enhanced repair mechanisms in the colonic mucosa. In this context linalool is known to suppress reactive oxygen species and enhance the levels of GSH [46].

Table 2 Effect of linalool on colonic tumour incidence and the number of tumours per tumour bearing rat

Groups	No. of rats	No. of tumours/ polyps bearing rats	Tumours/Polyps incidence (%)[a]	Total number of tumours/polyp	No. of tumours (polyps) /tumour bearing rat[b]
Control	12	0	Nil	Nil	Nil
Control+Linalool (100 mg/kg b.w.)	12	0	Nil	Nil	Nil
DMH	12	12	100	21	1.7
DMH+ Linalool (25 mg/kg b.w.)	12	9	71.42	15	1.6
DMH+ Linalool (50 mg/kg b.w.)	12	5	28.57	6	1.2
DMH+ Linalool (100 mg/kg b.w.)	12	6	42.82	9	1.5

[a](Total number of polyps in treatment group/total number of polyps in DMH group) x 100. [b](Total number of polyps/number of polyps bearing rats in each group).

Table 3: Effect of linalool and DMH on ACF

Groups	Total No. of ACF	No. of foci containing			% Inhibition of ACF
		1 crypt	2 crypts	> 4 crypts	
DMH	105.3±10.7[a]	35.8±3.4[a]	23.7±2.1[a]	44.8±4.5[a]	---
DMH+ Linalool (25 mg/kg b.w.)	58.8±6.7[b]	20.6±2.1[b]	15.3±1.6[b]	18.9±2.0[b]	44.1
DMH+ Linalool (50 mg/kg b.w.)	32.9±3.2[c]	17.4±1.6[c]	13.5±1.3[c]	---	68.7
DMH+ Linalool (100 mg/kg b.w.)	43.3±4.3[d]	18.1±1.8[bc]	15.91.5[b]	6.1±0.6[c]	58.8

Data are presented as the means ± S.D. of 6 rats in each group. Values not sharing a common superscript letter (a,b,c,d) differ significantly at p<0.05,DMRT.

Table 4: Effect of Linalool on tissue TBARS and CD of control and experimental colon cancer rats

	Control	Control+Linalool (100 mg/kg b.w.)	DMH	DMH+Linalool (25 mg/kg b.w.)	DMH+Linalool (50 mg/kg b.w.)	DMH+Linalool (100 mg/kg b.w.)
TBARS (mmoles/mg tissue)						
Liver	0.44±0.04[a]	0.45±0.04[a]	0.68±0.07[b]	0.46±0.04[a]	0.46±0.04[a]	0.53±0.05[c]
Proximal colon	0.35±0.03[a]	0.34±0.03[a]	0.19±0.02[b]	0.24±0.02[c]	0.30±0.03[ad]	0.29±0.03[d]
Distal colon	0.25±0.02[a]	0.26±0.03[a]	0.12±0.01[b]	0.18±0.02[c]	0.24±0.02[a]	0.21±0.02[d]
CD (mmoles/mg tissue)						
Liver	50.69±4.88[a]	52.54±5.06[a]	75.46±7.26[b]	65.65±6.32[c]	53.79±5.18[a]	67.75±6.52[c]
Proximal colon	59.97±5.77[a]	61.04±5.88[a]	35.72±3.44[b]	44.27±4.26[c]	58.83±5.66[a]	50.65±4.88[c]
Distal colon	35.98±3.46[a]	36.27±3.49[a]	12.03±1.16[b]	25.31±2.44[c]	35.13±3.38[a]	28.13±2.71[c]

All the values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c) differ significantly with each other (p< 0.05, DMRT).

Table 5: Effect of linalool on circulatory lipid peroxidation markers of control and experimental rats

	Control	Control+Linalool (100 mg/kg b.w.)	DMH	DMH+Linalool (25 mg/kg b.w.)	DMH+Linalool (50 mg/kg b.w.)	DMH+Linalool (100 mg/kg b.w.)
TABARS (pmoles/mL)						
	1.94±0.19[a]	1.97±0.19[a]	4.86±0.47[b]	2.88±0.28[c]	2.10±0.20[a]	2.75±0.27[c]
CD (µmoles/mL)						
	0.97±0.09[a]	0.95±0.09[a]	2.17±0.21[b]	1.52±0.15[c]	1.00±0.10[a]	1.62±0.16[c]

All the values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c) differ significantly with each other (p< 0.05, DMRT).

Table 6: Effect of linalool on tissue SOD and CAT enzymes of control and experimental colon cancer rats

	Control	Control+Linalool (100 mg/kg b.w.)	DMH	DMH+Linalool (25 mg/kg b.w.)	DMH+Linalool (50 mg/kg b.w.)	DMH+Linalool (100 mg/kg b.w.)
SOD (Enzyme required for 50% inhibition of NBT reduction/min/mg protein/Hb for tissues and lysate)						
Liver	3.38±0.33[a]	3.49±0.34[a]	2.15±0.21[b]	2.67±0.27[c]	3.25±0.31[a]	2.83±0.29[c]
Proximal colon	7.56±0.73[a]	7.65±0.74[a]	5.57±0.54[b]	6.49±0.62[c]	7.39±0.71[a]	6.37±0.61[c]
Distal colon	7.43±0.71[a]	7.04±0.68[a]	4.81±0.46[b]	5.89±0.57[c]	7.09±0.68[a]	5.70±0.55[c]
Lysate	5.67±0.55[a]	5.78±0.56[a]	3.05±0.29[b]	4.83±0.43[c]	5.55±0.53[a]	4.95±0.48[c]
CAT (µmoles of H_2O_2utillized/min/mg protein/Hb for tissues and lysate)						
Liver	53.27±5.13[a]	53.88±5.19[a]	27.45±2.64[b]	42.97±4.14[c]	52.92±5.09[a]	46.69±4.49[c]
Proximal colon	59.96±5.77[a]	61.04±5.88[a]	35.72±3.44[b]	44.27±4.26[c]	58.83±5.66[a]	50.65±4.88[d]
Distal colon	35.98±3.46[a]	36.27±3.49[a]	12.02±1.16[b]	25.31±2.44[c]	35.13±3.38[a]	28.13±2.71[c]
Lysate	29.91±2.88[a]	30.10±2.90[a]	13.50±1.30[b]	25.16±2.42[c]	29.23±2.81[a]	26.15±2.52[c]

All the values are expressed as means ± SD of 6 rats in each group. Values that have different superscript letter (a,b,c,d) differ significantly with each other (p< 0.05, DMRT).

Table - 7: Effect of linalool of tissue GPx, GR and GSH of control and experimental colon cancer rats

	Control	Control+Linalool (100 mg/kg b.w.)	DMH	DMH+Linalool (25 mg/kg b.w.)	DMH+Linalool (50 mg/kg b.w.)	DMH+Linalool (100 mg/kg b.w.)
GPX (µmoles of GSH utilized/min/mg protein for tissues)						
Liver	7.86±0.76[a]	7.27±0.70[a]	3.90±0.38[b]	5.21±0.50[c]	7.36±0.71[a]	5.82±0.56[c]
Proximal colon	2.74±0.26[a]	2.85±0.27[a]	0.87±0.08[b]	1.63±0.14[c]	2.37±0.23[d]	1.76±0.16[c]
Distal colon	2.73±0.26[ab]	2.82±0.27[b]	1.34±0.13[c]	1.82±0.17[d]	2.54±0.24[a]	1.94±0.19[d]
GR (µmoles of NADPH oxidized/min/mg protein for tissues)						
Liver	25.45±2.45[ab]	27.53±2.65[a]	10.19±0.98[b]	17.83±1.72[c]	23.71±2.28[d]	18.44±1.78[c]
Proximal colon	14.35±1.38[ad]	15.10±1.45[a]	7.55±0.73[b]	10.13±0.97[c]	13.18±1.27[d]	10.96±1.05[c]
Distal colon	14.46±1.39[ad]	15.80±1.52[a]	6.10±0.59[b]	9.22±0.89[c]	14.16±1.36[d]	10.37±1.00[c]
GSH (mmoles/mg tissue)						
Liver	24.07±2.32[a]	23.60±2.27[a]	16.80±1.62[b]	19.58±1.89[c]	23.05±2.22[a]	20.13±1.94[c]
Proximal colon	25.23±2.43[a]	25.43±2.45[a]	16.05±1.54[b]	19.18±1.85[c]	24.96±2.40[a]	20.94±2.02[c]
Distal colon	17.68±1.70[a]	17.27±1.66[a]	12.24±1.18[b]	14.55±1.40[c]	17.07±1.64[a]	14.96±1.44[c]

All the values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other (p< 0.05, DMRT).

Table 8: Effect of linalool on circulatory antioxidant GSH and glutathione dependent enzymes of control and experimental rats

	Control	Control+Linalool (100 mg/kg b.w.)	DMH	DMH+Linalool (25 mg/kg b.w.)	DMH+Linalool (50 mg/kg b.w.)	DMH+Linalool (100 mg/kg b.w.)
GPx (µmoles of GSH utilized/min/mg Hb for erythrocyte lysate)						
	3.67±0.35[a]	3.70±0.36[a]	1.91±0.18[b]	2.39±0.23[c]	3.60±0.35[a]	2.71±0.26[c]
GR (µmoles of NADPH oxidized/min/mg Hb for erythrocyte lysate)						
	3.53±0.34[a]	3.70±0.36[a]	1.82±0.18[b]	2.48±0.24[c]	3.34±0.32[d]	2.71±0.26[c]
GSH (mg/dL for erythrocyte lysate)						
	25.50±2.45[a]	25.64±2.47[a]	16.46±1.58[b]	19.18±1.85[c]	24.89±2.40[a]	21.28±2.05[c]

All the values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other (p< 0.05, DMRT).

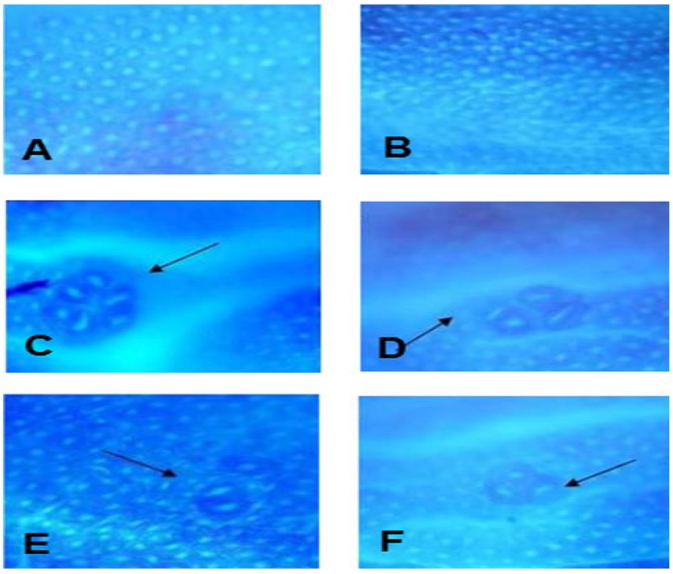

Fig. 3. Topographical view of aberrant crypt foci (ACF).
A and B: control and control+ linalool treated rats showing normal crypts. C: DMH alone treated rat showing six aberrant crypts. D, E and F: DMH + Linalool treated rat at the doses of 25, 50 and 100 mg/kg b. w. showing four, two and three aberrant crypts respectively.

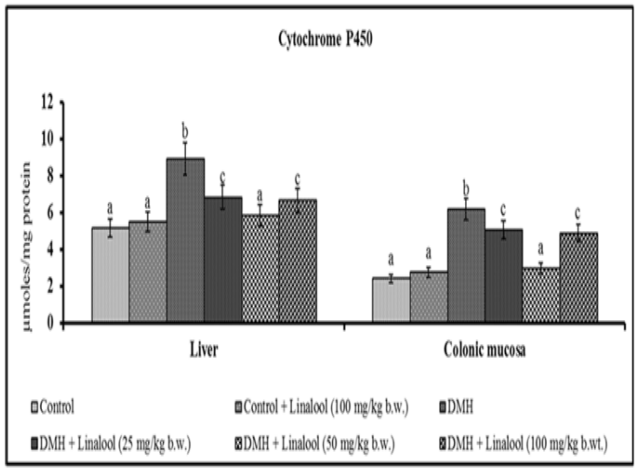

Fig 4: Effect of linalool on tissue cytochrome P450 of control and experimental rats
All the values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c) differ significantly with each other (p< 0.05, DMRT).

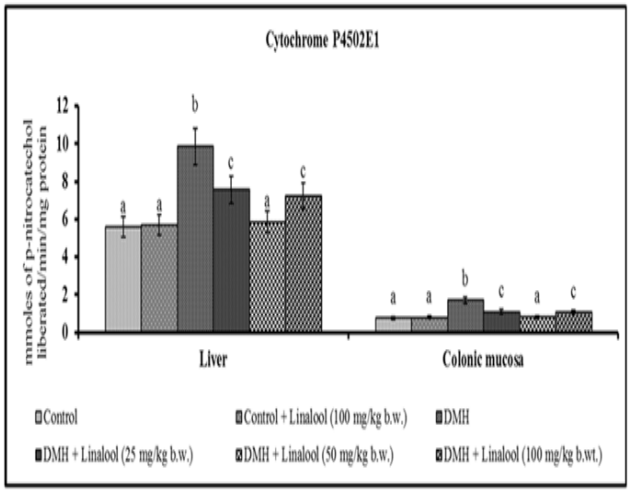

Fig 5: Effect of linalool on tissue cytochrome P4502E1 of control and experimental rats
The values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c) differ significantly with each other (p< 0.05, DMRT).

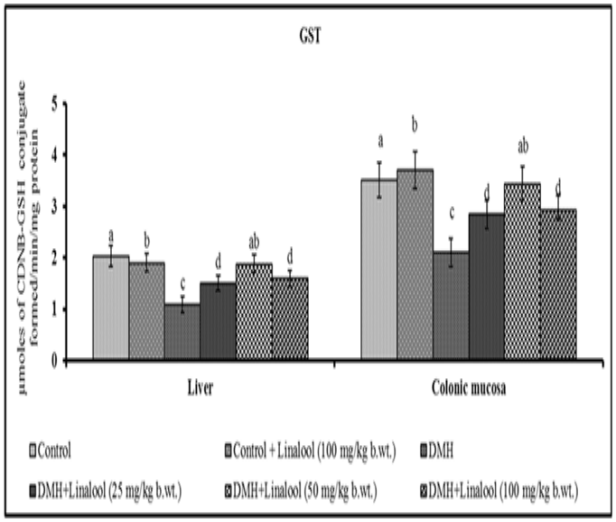

Fig 6: Effect of linalool on tissue glutathione-S-transferase(GST) activities of control and experimental rats
All the values are expressed as means ± SD of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other (p< 0.05, DMRT).

CONCLUSION

In conclusion, the results of this study suggest that linalool has a beneficial effect on the progression of chemically induced colon cancer in rats. Further our data suggests that linalool even at a medium dose of 50 mg/kg b.w. is able to exert an inhibitory effect on the formation of the preneoplastic lessons, Overall linalool markedly modulates the oxidative stress by activating the antioxidant defence system. Thus linalool may be an attractive candidate as an antioxidant supplement for anticancer therapy. However, a long-term bioassay is needed to confirm the results, which is now being planned in our laboratory.

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