ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

# Antioxidant status of fruit peel of *Citrus reticulata* Essential oil on 1, 2 Dimethyl hydrazine induced rat colon carcinogenesis.

**Burali Sanganna [1, 2]*and AR Kulkarni[3]**

[1] Research scholar, Karpagam University, Coimbatore, India
[2] RIPER College of Pharmacy, Anantpur, AP.
[3] Department of Pharmacology, SET's College of Pharmacy, Dharwad - 580 002, India

## ABSTRACT

In our study 1, 2 Dimethyl hydrazine is used as a chemical agent for producing specific type of colon cancer by methylation of the DNA, membrane lipid peroxidation, protein oxidation, cell destruction, altering the cell signaling etc. In cellular redox process, release the by-product reactive oxygen species and reactive nitrogen species free radicals. The enzymic antioxidant such as GSH, GPx, CAT etc scavenge free radicals and help to decrease the incidence of oxidative stress induced damage. Plants antioxidant can easily donate electrons to reactive free radicals and thus retard radical chain reactions. Hence the present study was selected an extract of *citrus reticulata* essential oil (CREO). Results were observed in Erythrocyte lysate levels of LPO increased and decreased the levels of GSH, GPx and CAT, where as in colon homogenate decreased the levels of LPO and CAT, where as increased the levels of GSH, GPx, levels of SGPT, SGOT, and ALP increased in DMH group. Also Fecal and colon homogenates bacterial enzymes β-Glucosidase, β-Glucuronidase and mucinase were increased levels in DMH group as compared to control. Administration of CREO to DMH treated rats significantly reverse the oxidative stress and may acts as a chemopreventive agent to DMH rats.

**Keywords:** DMH, *citrus reticulata* essential oil, Antioxidant, colon cancer.

***Corresponding author***

ISSN: 0975-8585

## INTRODUCTION

In India colorectal cancer incidence has been increasing in recent years because of change in lifestyle of diet and environment factors. The dietary is the one of the incidence factor for colon carcinogenesis due to contain high fat, high protein and low carbohydrates, low fibers are implicated as an inducing agent in epidemiological and animal studies [1]. Diet and nutrition play a major role in the etiology and prevention of chronic civilization diseases [2]. Earlier traditional herbal medicines and dietary foods were the main sources of antioxidants that protected people from the damaged caused by free radicals [3] and epidemiological studies suggest that generous consumption of fruits and vegetables is associated with reduced colon cancer risk [4].

In our study 1, 2 Dimethyl hydrazine is used as a chemical agent responsible for producing a specific type of colorectal carcinogenesis by methylation of the DNA, membrane lipid peroxidation, protein oxidation, cell distruction, altering the cell signaling etc. The free radicals species of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-product from the cellular redox process. Free radical induced oxidative stress is a fundamental mechanism of cancer, cardiovascular, neurological disorder and aging [3]. Which are protected by antioxidant defense system consisting of lipid peroxidation and the activities of enzymic antioxidant such as GSH, GPx, CAT etc by scavenge free radicals and help to decrease the incidence of oxidative stress induced damage [5]. They also play an important role in carefully balancing free radical producing and scavenging reactions in cell and thus maintaining optimum level of radicals and acts as a mutually supportive team of defense against reactive oxygen species (ROS). The colon and intestinal bacterial enzyme are incorporated in the metabolism of procacinogen and production of tumor promoter. In 1, 2 Dimethyl hydrazine induced colon cancer increased the fecal and colon mucosal enzyme activity of β-glucuronidase, β –glucosidase and mucinase. These are responsible for converting procarcinogens to carcinogens, which led to a higher amount of toxic compounds in the colon [6].

Ponkan, the fruit of *citrus reticulata* Blanco. ponkan, is produced in Asia as the thick skinned mandarin orange [7]. *Citrus* cultivation is probably one of the most important commercial and industrial agricultural activities of the world [8]. Traditional populations in several countries reference citrus species as useful in reducing symptoms of anxiety or insomnia [9].

*Citrus reticulata* Blanco (Rutaceae) is commonly known as narangi or santra (orange). It is a small spiny tree with dense top of slender branches, grown in India. The related species of *citrus reticulata* Blanco are also commonly used for Central Nervous system disorders[9], Antifungal activity[10], Antimicrobial activity[11], Antioxidant and antinociceptive effects of *Citrus limon* Essential Oil in Mice [12] and cancer related diseases [13].

The objective of this work is to use non-cytotoxic nutrients and having a potent chemo preventive agent that inhibit the transformation of normal cells to premalignant cells. The

potent beneficial role of *citrus reticulata* Blanco essential oil in DMH induced rats colon carcinogenesis has not been studied so far. Thus, our aim was to examine the effect of *citrus reticulata* Blanco essential oil on Erythrocyte lysate antioxidant estimation, Serum Liver Function test, Fecal and colon homogenates were used for antioxidant estimations and also attempt explain the mechanism of its anti-tumourigenic activity.

## MATERIAL AND METHODS

### Plant material:

The *Citrus reticulata* essential oil prepared by using fresh peels *citrus reticulata* Blanco was hydrolyzed in Clevenger type apparatus. The essential oils were collected and stored in the dark at 4 ºC and % yield was calculated [9].

### Chemicals:

DMH were purchased from Sigma Aldrich Company and all other chemical, reagents were used as AR grade.

### Animals:

Healthy Wister rats of four weeks were parched from Nijalingappa Medical College of Bagalkot, Karnataka. All rats were weighed approximately 80-120g housed in propylene cage under hygienic condition of temp (22±20 ºC), humidity (55±10%). 12 light/dark cycle in the departmental animal house. Necessary approval were obtained from IAEC (Reg. No.878/ac/05/CPCSEA) for the use of experimental animals in the present studies.

### Experimental Design:

All animal were divided in to four groups of six rats in each groups.

Group I served as Normal control was given normal saline & diet ad libitum. Group –II, III& IV received DMH (20mg/kg) subcutaneous once a week for first four weeks in addition group- III and IV received standard drug of capacitabine (200mg/kg) and *citrus reticulata* essential oil at the dose of (200mg/kg) respectively.

### Carcinogen administration:

The experimental animals were divided into four groups. The animals in groups II, III & IV received sub- cutaneous injections of DMH at a dose of 20 mg/kg body weight once a week for the first four consecutive weeks to induce colorectal cancer. Prior to subcutaneous injection, DMH was dissolved in 1mM EDTA the pH adjusted to 6.5 with 1mM NaOH to ensure the pH and stability of the chemical and was used immediately after preparation. Initial body

weights of all animals in this study protocol were ensured to be between 80 and 120g. The animal weights were recorded once a week throughout the experimental period and prior to sacrifice [14].

**Method of Preparation:**

At the end of the experimental period, rats were fasted overnight, body weight were taken and then anesthetized followed by cervical decapitation.

**1. Preparation of blood serum**

Blood was allowed to coagulate for 20 min and then centrifuged at 2000 x g for 15 min. The serum was separated and used for estimations.

**2. Preparation of erythrocyte lysate homogenate**

After sacrificing the rats, the erythrocyte lysate was prepared by lysing a known volume of erythrocytes by adding two volumes of distilled water to the packed erythrocytes and centrifuging the resulting solution at 3000g for 10 min at 4 ºC to separate the erythrocyte lysate and used antioxidant defense system [15].

**3. Preparation of colon homogenate**

The mucosa from the colon was collected by scarping with a slide and homogenized with phosphate buffer saline, centrifuged at 2000g for 10 min at 4c and the supernatant collected for the assays of mucosal bacterial enzyme [6].

**4. Preparation of faecal homogenate**

The last day of the experiment, feces were collected for 24h, weighed, frozen at 20 ºC and later homogenized with equal weight of water and lyophilized to a fine powder and suspected for assay of bacterial enzymes [6].

**Biochemical investigations:**

**1. Estimation of lipid peroxidation, GSH, GPx, and CAT oxidation in erythrocyte lysate homogenate and colon homogenate**

Lipid peroxidation is estimates by measuring thiobarbituric acid reactive substances, quantified in terms of MDA equivalents using a method of Fraga et al. [16]. The values are expressed in Mol of TBARS /ml of plasma. Reduced glutathione (GSH) was determined by the method of Ellman and the values are expressed as µM of TNB//ml of plasma [17]. Glutathione peroxidase (GPx) activity was assayed by the method of Rotruck et al and the values are

expressed as µM of GSH utilized/ml of plasma [18]. The activity of CAT was determined by the method of Sinha, the values of CAT activity are expressed as Mol of $H_2O_2$utilized/min/ml of plasma [19].

**2. Estimation of Serum Liver Function Parameters SGOT, SGPT and ALP.**

The serum was analyzed for SGOT, SGPT and ALP content by using the Biochemical methods. As follows serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) using Modified IFCC Method [20], alkaline phosphatase (ALP) by using Wilkinson et al of the Bessey, Lowry et al method [21].

**3. Estimations of fecal and colon bacterial enzyme activity:**

**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 [22].

**β-glucuronidase**

The activity was measured by the method of Freeman (1986). Known volume of 0.02 M phosphate-buffered saline (pH 7.0), 0.1 mM EDTA, 3.0mM p-nitrophenyl-b-D-glucopyranoside and the enzyme supernatant was made up to a final volume of 1 ml, and the mixture was incubated at 37ºC for 15 min. The reaction was arrested with 0.2 M glycine buffer (pH 10.4) and the amount of p-nitrophenol released was read at 540 nm with a spectrophotometer. All reactions were linear with respect to concentration and incubation time to 45 min. The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve [23].

**β-glucosidase**

The activity was measured by the method of Freeman (1986). The mixture of samples and substrate (p-nitrophenyl-b-D-glucoside) were incubated with 37ºC for 60 min. After incubation 0.2 M $Na_2CO_3$, was added to arrest the reaction. The released p-nitrophenol was measured at 400 nm. All reactions were linear with respect to concentration and incubation time to 60 min. The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve [23].

**Mucinase**

The activity was determined by the method of Shiau and Chang [24].The assay mixture contained 0.2 M porcine gastric mucin with a known amount of fecal suspension made up to 1

ml with distilled water. The mixture was then incubated at 37ºC for 25 min. The amount of reducing sugar was measured by the method of Nelson [25] at 520 nm. Values are expressed as mg of glucose liberated/min/mg protein.

## RESULT AND DISCUSSION

At the end of the sixteen weeks in our study result show that, the erythrocyte lysate level of the LPO increased significantly ($p<0.001$) and GSH, GPx and CAT level were decreased significantly ($p<0.001$) in DMH alone group compared with normal control group. Suggest that rapid Cell proliferation involved in colon cell. Hence, cancer cell have certain characteristics that promote proliferation and tend to faster cell proliferation. [26]. DMH itself can generate $H_2O_2$ in the presence of copper ions. In the presence of metal ions such as Fe2+ and Cu2+, $H_2O_2$ can react with $O_2$ to convert it into the more reactive OH radical. If sufficient amounts of CAT or GPx are not available to decompose $H_2O_2$, [15] the generated OH radicals are capable of attacking DNA basement. GSH an important non-protein thiol in conjunction with GPx and GST plays a significant role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species [27]. The present study, correlates with the decline in circulatory antioxidants such as GSH, GPx, and CAT. This may be due to their overutilization to scavenge the products of lipid peroxidation as well as sequestration by tumor cells. GPx uses $H_2O_2$ to catalyse the oxidation of GSH to GSSG, thereby nullifying the deleterious effects of $H_2O_2$ [28]. Diminished GPx activity indicates cellular accumulation of the lipid hydroperoxides, which can potentially turn on a chain reaction, wherein more polyunsaturated fatty acid become targets for further peroxidative tissue injury. In the present study, GPx level was reduced in erythrocyte lysate and neoplastic tissues, which could be due to the elevated levels of hydroperoxides, Supplementation with Citrus *reticulata* essential oil significantly ($p<0.001$) decrease the level of LPO in erythrocyte lysate of DMH treated rats, as well as significantly ($p<0.001$) increased the level of the antioxidants defense enzymatic system of GSH, GPx and CAT level.

The hepatic cell membrane damage releases the enzymes SGOT, SGPT, ALP into circulation, which can be measured in serum. High levels of SGOT indicate liver damage. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury. The present data indicate the efficacy of the *citrus reticulata* essential oil in protecting or attenuating DMH-induced liver toxicity, by the reduction of serum hepatic enzymes activity and significantly ($p<0.001$) enhanced antioxidant defense [29].

In the liver glucuronidation reaction take place with carcinogen which is detoxified and secreted, via bile into the intestine. When the conjugated carcinogen reaches the colon, it is hydrolyzed by bacterial enzymes β-glucuronidase and β-glucosidase and the colons get exposed to free carcinogen. In DMH induced group bacterial enzyme activities got enhanced in the presence of pro-carcinogen like DMH [30]. Treatment with *citrus reticulata* essential significantly ($p<0.001$) reduced the activities of these enzymes in fecal as well as in colon

homogenate and prevent the colon from the toxic effects by lowering the hydrolysis of glucouronide conjugates [30].

Chemicals probably need to reach the colonic mucosa before they can induce tumour formation. Mucinase is an enzyme present in the intestinal microflora, which hydrolyzes the protective mucins in the colon. Mucins are glycoproteins consisting of a larger number of carbohydrate side chains attached to a protein core. They also form gels coating the intestinal mucosa and function as a lubricant and probably as a chemical and mechanical barrier against bacteria, viruses, and toxins [24]. Significantly ($p<0.001$) increased mucinase activity leads to decreased protection of the underlying tissues. *Citrus reticulata* essential oil in DMH induced group was found to significantly ($p<0.001$) decrease this activity both in the colon and in the fecal contents thereby, exerting protective effect on colonic mucosa and decreasing the susceptibility of the colonic mucosa to attack by carcinogens [24].

## CONCLUSION

The present studies were concluded that the *citrus reticulata* essential oil may be a potential chemopreventive agent in DMH induced colon cancer by lowers the enzymatic and non-enzymatic antioxidant activity, as well as inhibiting bacterial enzyme levels.

**Table 01: Effect of *citrus reticulata* essential oil on the LPO, GSH, CAT, GPx antioxidant enzymes in Erythrocytes lysate DMH induced rats.**

| Groups | LPO Mol of TBARS /ml of plasma | GSH µM of TNB//ml of plasma | GPx µM of GSH utilized/ml of plasma | CAT Mol of $H_2O_2$ utilized min/ml of plasma |
|---|---|---|---|---|
| **Normal** | 0.098± 0.003 | 8.973± 0.142 | 13.94± 0.179 | 0.3977± 0.017 |
| **DMH** | 0.3485± 0.016 *** | 4.015± 0.132 *** | 8.027± 0.739 *** | 0.086± 0.006 *** |
| **Capacitabine** | 0.1602± 0.038 | 7.712± 0.275 | 11.62± 0.386 | 0.377± 0.025 |
| ***Citrus reticulata* Essential oil** | 0.2427± 0.025 *** | 5.792± 0.139 *** | 10.25± 0.520 *** | 0.3003± 0.039 *** |

The results are depicted as mean ± S.D. of six rats from each group and one way ANOVA analyzed by Bonferroni's Multiple Comparison test for coming to conclusion. ***P<0.001. All treatment groups compared to DMH group was considered as significant.

**Table02: Effect of *citrus reticulata* essential oil on SGPT, SGOT and ALP Serum Liver Function in DMH induced rats.**

| Groups | SGPT (U/L) | SGOT (U/L) | ALP (U/L) |
|---|---|---|---|
| **Normal Control** | 56.83± 4.262 | 60.5± 3.507 | 138.5± 3.619 |
| **DMH (20mg/kg)** | 125.3± 4.457*** | 124.7± 4.082*** | 284.8± 6.242*** |
| **Capacitabine(200mg/kg)** | 87.33± 4.676 | 79.67± 4.844 | 165.3± 9.092 |

ISSN: 0975-8585

| | | | |
|---|---|---|---|
| ***Citrus reticulata*** **Essential oil (200mg/kg)** | 97.17± 6.735 *** | 92.00± 1.414 *** | 205.8± 4.875 *** |

Each Value is mean ± SD of 6 animals *** P < 0.001: Normal control vs DMH, DMH vs *Citrus reticulata* Essential oil treatment groups.

**Table 03: Effect of *citrus reticulata* essential oil on tissue Lipid peroxidation, GSH, GPx and CAT in DMH induced rats.**

| Groups | LPO Mol of TBARS/g of tissue | GSH µM of TNB/g of tissue | GPX µM of GSH utilized/g of tissue | CAT Mol of $H_2O_2$utilized/min/g of tissue |
|---|---|---|---|---|
| **Normal** | 0.317± 0.0237 | 1.811± 0.3296 | 17.15± 0.6368 | 0.0991± 0.0076 |
| **DMH** | 0.103± 0.0460 *** | 5.761± 0.4074 *** | 34.37± 1.200 *** | 0.0446± 0.0092 *** |
| **Capacitabine** | 0.259± 0.0404 | 3.144± 0.4606 | 22.41± 0.9208 | 0.0718± 0.0115 |
| ***Citrus reticulata*** **Essential oil** | 0.258± 0.0690 *** | 3.859± 0.6613 *** | 27.23± 2.139 *** | 0.0716± 0.0075 *** |

Each Value is mean ± SD of 6 animals *** P < 0.001: Normal control vs DMH, DMH vs *Citrus reticulata* Essential oil treatment groups.

**Table 04: Effect of *citrus reticulata* essential on fecal and colonical bacterial enzyme activity in DMH induced rats.**

.

| Groups | Fecal -PH | β-glucuronidase µg of phenolphthalein liberated/g of protein in 45 min incubation time. | β–glucosidase µg of p-nitro phenol liberated/g of protein in 60 min incubation time. | Mucinase µg of reducing sugar liberated/g of protein in 15 min incubation time. |
|---|---|---|---|---|
| **Normal** | 5.567 ± 0.1633 | 6.033± 0.2302 | 11.56± 0.7172 | 3.365± 0.141 |
| **DMH** | 8.283 ± 0.1472 *** | 10.42± 0.4238 *** | 15.5± 0.7144 *** | 7.73± 0.231 *** |
| **Capacitabine** | 6.633 ± 0.2422 | 7.398± 0.1771 | 12.81± 0.1726 | 4.523± 0.1777 |
| ***Citrus reticulata*** **Essential oil** | 7.367 ± 0.3011 *** | 9.325± 0.6186 *** | 13.65± 0.3605 *** | 5.558± 0.3044 *** |

Each Value is mean ± SD of 6 animals *** P < 0.001: Normal control vs DMH, DMH vs *Citrus reticulata* Essential oil treatment groups.

ISSN: 0975-8585

**Table 05: Effect of *citrus reticulata* essential oil on the colon β- glucosidase, β - glucuronidase and Mucinase Enzymes activity in DMH induced rats.**

| Groups | β-glucuronidase µg of phenolphthalein liberated/g of protein in 45 min incubation time. | β–glucosidase µg of p-nitrophenol liberated/g of protein in 60 min incubation time. | Mucinase µg of reducing sugar liberated/g of protein in 15 min incubation time. |
|---|---|---|---|
| **Normal** | 1.482± 0.071 | 1.35± 0.087 | 1.445± 0.097 |
| **DMH** | 7.525± 0.175 *** | 3.905± 0.294 *** | 4.385± 0.173 *** |
| **Capacitabine** | 3.835± 0.346 | 1.99± 0.185 | 1.968± 0.070 |
| ***Citrus reticulata* Essential oil** | 5.175± 0.163 *** | 3.183± 0.196 *** | 3.455± 0.220 *** |

Each Value is mean ± SD of 6 animals *** P < 0.001: Normal control vs DMH, DMH vs *Citrus reticulata* Essential oil treatment groups.

## REFERENCES


[1] Gang Xu, Guijie Ren, Xia Xu. Food and Chemical Toxicolog. 2010; 48:390–395.
[2] Emília Hijova, Anna Chmelarova, Alojz Bomba. ACTA VET. BRNO. 2009;78:661-666.
[3] Himalya health for you.Vol LI-No 03. Jan-Mar 2012. Capsule.
[4] Venkatachalam K.K, Sreedharan V, Namasivayam N. Environ Toxicol Pharmacol 2010; 29:50–57.
[5] V Manju, N Nalini. J Biochem Tech 2010;2(2):161-167.
[6] Selvaraj A, Jayabal P.S, Narmasivayam N. J Pharm Pharmacol 2008;60:1385-1392.
[7] Qizhen Du, Hui Chen. The methoxyflavones in Citrus reticulata Blanco cv. Food Chem 2010; 119:567–572.
[8] F. Gulay Kirbaşlar, Aydin Tavman, Başaran Dülger and Gulen Turker. Pak J Bot 2009; 41(6):3207-3212.
[9] Andre C.G, Celso A.R. Almeida Costa, Mirtes Costa. Tree and forestry Science and Biotechnology 2008;1:121-124.
[10] M Chutia, P Deka Bhuyan, MG Pathak, TC Sarma, P Boruah. Food Sci Technol 2009; 42:777–780.
[11] GA Ayoola, OO Johnson, T Adelowotan, IE Aibinu. African J Biotechnol 2008; 7 (13):2227-2231.
[12] LidianneMayra L.C, Campelo, Antonia A.C. de Almeida, Rizangela L.Mendes de Freitas. J Biomed Biotechnol 2011, Article ID 678673, 8 pages.
[13] S. Aranganathan, N. Nalini. Food and Chemical Toxicol 2009; 47: 2594–2600.
[14] V. Manju and N. Nalini. Cell bio chemistry and function.10.1027/cbf:1305.
[15] Gunasekaran Sivagami, Venkatachalam Karthikkumar, Thangavel Balasubramanian Namashivayam Nalini. Chemico-Biological Interactions. 2012; 196:11–22.
[16] Fraga CG, Leibouitz BE, Toppel AL. Free Radical Bio Med 1988; 4:155–161.
[17] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82:70-77.

[18] Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Science. 1973; 179:588-90.
[19] Sinha AK. Anal Biochem 1972; 47(2):389-394.
[20] SGOT (ASAT) KIT (Modified IFCC Method) [package insert]. Goa: Crest Biosystems; 2011.
[21] Alkaline Phosphotase (Tris Carbonate Buffer, Kinetic) [package insert]. Baddi: Transasia Bio-Medicals ltd; 2011.
[22] Scott LS, Richard LN, Lloyd MN. J Roy Soc Med. 1985; 78: 230-34.
[23] Freeman HJ. Cancer Res 1986; 46: 5529-32.
[24] Shiau, Shi-Yen, Chang, George W. J Nutr 1983; 113: 138-44.
[25] Nelson,N. J Biol Chem 1944; 153:375–380.
[26] Vaiyapuri Manju, Vairappan Balasubramaniyan and Namasivayam Nalini. CELL. MOL. BIOL. LETT. 2005; 3: 10: 536.
[27] Vaiyapuri Manju, Namasivayam Nalini. Clinica Chimica Acta.2005; 358:60–67.
[28] Bains JS, Shaw CA. Brain Res Rev 1997; 25:335–58.
[29] Mohammed SA. CEMED.2010; 4: (1):129–140.
[30] Manju V, Nalini N. Cell Biochem Funct. 2007; 25(2): 189-94. 114