

## Aggravation of Oxidative Stress by Ethanolic Extract of *Ocimum gratissimum* in DMH Induced Colon Injury.

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### ABSTRACT

Context: Medicinal and pharmacological properties of *Ocimum* (Lamiaceae) have been exploited since ancient times. However, concerns are raised regarding its toxic properties and need attention.

Objectives: The present study evaluates effect of *Ocimum gratissimum* leaves' ethanolic extract on N,N-dimethylhydrazine dihydrochloride (DMH) induced colon toxicity.

Material and methods: Male Wistar rats were divided into 4 groups and sacrificed after 10 weeks. Group 1: Control. Group 2: DMH (40mg/kg body weight). Group 3: extract+DMH. Group 4: extract only. Histopathology and oxidative stress were evaluated in colon. Additionally as NADPH is required for regeneration of reduced glutathione (GSH), isocitrate dehydrogenase (IDH2) and glucose-6-phosphate dehydrogenase (G6PDH) activities were also evaluated.

Results: Normal colonic mucosa and crypt morphology was observed in control group while mild inflammation was seen in all other groups. There was an increase in lipid peroxidation on DMH treatment and decrease in superoxide dismutase (SOD) activity. Concomitantly there was an increase in IDH2 activity suggestive of increased requirement of NADPH. Extract+DMH treatment led to further increase in lipid peroxidation and decrease in GSH though SOD and catalase (CAT) activities were elevated. Administration of extract alone also augmented oxidative stress by increased lipid peroxidation and decreased antioxidant enzymes. Administration of extract either alone or with DMH led to a decrease in IDH2 activity. However, G6PDH activity was increased only on extract treatment.

Discussion and Conclusion: The results obtained herein did not show any protective effect of *Ocimum gratissimum* extract in DMH induced colonic injury, rather it suggests that its administration potentiates oxidative stress in DMH induced colonic toxicity.

### INTRODUCTION

*Ocimum* (Lamiaceae), also known as holy basil or tulsi is a herb, popular for its medicinal properties since ancient times. *Ocimum* is known for its antioxidant, anti-carcinogenic and other pharmacological properties<sup>1</sup>. Anti-cancerous potential of aqueous extract of *Ocimum gratissimum* has been reported<sup>2</sup> but the effect of ethanolic *Ocimum gratissimum* extract has not been elucidated yet. DMH is a potent colon carcinogen, inducing colorectal tumors in experimental animals and is the most widely used model of chemically induced colon carcinogenesis. DMH is metabolized in liver to form azoxymethane and methylazoxy methanol which is further transported to colon via bile or blood to generate its ultimate carcinogenic metabolite, diazonium ion which elicits an oxidative stress by methylating biomolecules of colonic epithelial cells. Therefore, the present study was designed to evaluate the effects of ethanolic extract of *Ocimum gratissimum* in DMH induced colon toxicity by monitoring its influence on oxidative stress.

Oxidative stress induced by any compound is implicated as a mechanism of toxicity in numerous tissues and organ systems. Oxidative stress is caused when the production of reactive oxygen species (ROS) overrides the antioxidant capacity of a cell or tissue<sup>3</sup>. Formation of ROS causes

damage to the critical cellular macromolecules as DNA, lipids and proteins resulting in the transformation of normal cells to malignant cells<sup>4</sup>. Catalase, superoxide dismutase (SOD), and glutathione peroxidase are some of the enzymes that help decrease oxidative tension during aerobic respiration. However, the effectiveness of these proteins as the scavengers of ROS depends on the availability of NADPH. Nicotinamide adenine dinucleotide phosphate (NADPH) provides the reductive environment that enables the cells to nullify the reactive oxygen species (ROS). Glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH) are some of the important enzymes that enable aerobic cells to fulfill their requirement for NADPH.

Isocitrate dehydrogenase is an important enzyme for the generation of NADPH. This enzyme IDH exists in three isoforms. Of these IDH1 and IDH2 are NADP linked cytoplasmic and mitochondrial isoenzymes respectively. IDH2 has been implicated in NADPH generation in mitochondria<sup>5</sup>.

Glucose-6-phosphate dehydrogenase (G6PDH) catalyses the oxidation of glucose 6-phosphate to 6-phosphogluconolactone and generation of NADPH. NADPH produced helps in maintaining the sulfhydryl

Table 1: Effect of *Ocimum gratissimum* on oxidative stress parameters

Variable	Group1:Control	Group2: DMH	Group3:DMH+ extract	Group4:Extract
Lipid peroxidation (nmoles of MDA/min/mg protein)	0.62 ± 0.18	2.17 ± 0.70 <sup>a</sup>	3.5 ± 0.88 <sup>b,c</sup>	4.28 ± 0.25 <sup>b,d</sup>
SOD (units/mg of protein)	227.90 ± 83.58	119.80 ± 41.02 <sup>e</sup>	533.23 ± 99.79 <sup>b,d</sup>	183.50 ± 25.15 <sup>f</sup>
Catalase (units/mg of protein)	1.17 ± 0.37	1.19 ± 0.43	1.40 ± 0.33	0.61 ± 0.12 <sup>a,c,f</sup>
Reduced Glutathione(μmoles/mg of protein)	15.59 ± 2.41	17.04 ± 1.55	10.45 ± 0.91 <sup>b,d</sup>	23.05 ± 2.34 <sup>b,d,f</sup>
Glutathione-S-transferase(μmole ofGSH-CDNB conjugateformed/min/mg protein)	2.27 ± 0.85	3.44 ± 0.66 <sup>e</sup>	4.66 ± 1.1 <sup>b,d</sup>	10.41 ± 0.63 <sup>b,d,f</sup>

Values are mean ± S.D. of 5-7 observations in each group. <sup>a</sup> p<0.01 w.r.t. group 1, <sup>b</sup> p<0.001 w.r.t. group 1 and <sup>c</sup> p<0.01 w.r.t. group 2, <sup>d</sup> p<0.001 w.r.t group 2, <sup>e</sup> p<0.05 w.r.t. group 1, <sup>f</sup> p<0.001 w.r.t. group 3

groups of cellular proteins required in antioxidant process in the reduced state<sup>6</sup>.

Natural products are of particular interest as a protective agent against various diseases because of their potentially low toxicity profiles and potential effectiveness. Therefore, the present study was designed to monitor the effect of ethanolic extract of *Ocimum gratissimum* on DMH induced toxicity by histopathological examination of colon and role of oxidative stress.

## MATERIALS AND METHODS

### Chemicals

All the chemicals used in the study were obtained from Himedia Laboratories, Sisco Research Laboratories and Sigma Chemicals Company. N,N-dimethyl hydrazine dihydro chloride (DMH) analytical grade was obtained from Sigma Chemicals Company.

### Animals

Male Wistar rats (150-250g) were procured from Central Animal House, Panjab University, Chandigarh. The rats were acclimatized for a week prior to their inclusion in the study. The studies were performed with approval of the Institutional Animal Ethical Committee, and the care of the animals was in accordance with institutional guidelines.

### Study Design

Animals were divided into the following groups:

**Control:** These animals were treated with intraperitoneal (ip) injection of EDTA (1mM) twice a week for a period of two weeks.

**DMH treated:** Animals of this group were administered with DMH (40mg/kg body weight, pH6.5); twice a week for a period of 2 weeks through intraperitoneal route<sup>7</sup>.

**Extract+DMH:** Animals were treated with ethanolic extract of *Ocimum gratissimum* (300mg/kg body weight) orally for 5 days<sup>8</sup>, followed by intraperitoneal injection of DMH (40mg/kg body weight, pH6.5) twice a week for a period of 2 weeks.

**Extract:** Animals of this group were treated with ethanolic extract of *Ocimum gratissimum* (300mg/kg body weight) orally for a period of five days.

All the animals were maintained for 10 weeks on standard pellet diet and subsequently sacrificed by cervical dislocation.

**Extract Preparation:** Preparation of extract was carried out by method of Manikandan *et al.*,<sup>8</sup>. *Ocimum gratissimum* leaves were obtained from pharmaceutical garden of University Institute of Pharmaceutical sciences, Panjab University, Chandigarh. The plants were identified by Department of Botany, Panjab University, Chandigarh, based on the shape and colour of leaves, stem and inflorescence. The leaves were washed properly with water and dried in oven at 60°C. Leaves were then grinded using mixer grinder to obtain powder. This powder was dissolved in 70% of ethanol and left for 36 hours. The mixture was filtered and the filtrate was concentrated in rota vapor under reduced pressure to prepare the extract. The residue obtained was a thick paste, green in color and gummaceous in nature. The extract was dissolved in normal saline to obtain a final concentration of 3%.

**Preparation of post mitochondrial supernatant:** The colon was cut longitudinally to expose lumen and rinsed with ice cold physiological saline. 10% Homogenate was prepared in ice-cold 20mM Tris-HCl (pH 7.4) by gentle homogenization. Colon tissue homogenate was then centrifuged at 10,000g for 20min at 4°C using Eppendorf cold centrifuge. The supernatant was termed as post mitochondrial supernatant and was used further for biochemical estimations.

### Preparation of mitochondrial suspension

Mitochondrial suspension was prepared according to method of Arnaud *et al.*,<sup>9</sup>. 10% colon homogenate was prepared in ice-cold 0.25 M sucrose solution and filtered through a single layer of muslin cloth. Further the filtrate was centrifuged at 500×g for 10 min at 4°C. The pellet was resuspended in isolation buffer and centrifuged at 11,300×g for 10 min. The pellet obtained was again resuspended in isolation buffer and was used as mitochondrial suspension for biochemical estimations.

### Histopathological Examination

The colon tissue was resected and fixed in the 10% formalin. The sections of the tissue were prepared using

Table 2: Effect of *Ocimum gratissimum* on NADPH synthesizing enzymes

Variable	Group1:Control	Group2: DMH	Group3:DMH+ extract	Group4:Extract extract
IDH2(μmoles of citrate oxidized/min/mg protein)	3.7±1.07	5.1±0.60 <sup>b</sup>	1.5±0.10 <sup>a,c</sup>	1.2±0.53 <sup>a,c</sup>
G6PDH(μmoles of D-glucose-6-phosphate oxidized/min/mg protein)	4.06±0.86	4.96±1.25	4.43±1.20	7.13±1.84 <sup>a,d,e</sup>

Values are mean±SEM of 4-8 observations in each group. <sup>a</sup>p<0.001 w.r.t. group1, <sup>b</sup>p<0.01 w.r.t. group1, <sup>c</sup>p<0.001 w.r.t. group2, <sup>d</sup>p<0.05 w.r.t group2, <sup>e</sup>p<0.01 w.r.t. 3.

routine procedure and stained with hematoxylin and eosin. The slides were evaluated by a histopathologist without the prior knowledge of the experimental design.

#### Estimation of oxidative stress

MDA was assayed according to method of Wills<sup>10</sup>. SOD activity was estimated with method of Kono<sup>11</sup>. Estimation of Catalase activity was carried out by method of Luck<sup>12</sup>. Glutathione estimation was done by method of Ellman<sup>13</sup>. The activity of GST was estimated according to method of Habiget *et al.*,<sup>14</sup>.

#### Estimation of NADPH synthesising enzymes

##### Isocitrate dehydrogenase (IDH)

Mitochondrial IDH2 was assayed by the method of Cleland *et al.*,<sup>15</sup>. The enzyme activity was measured by following the increase in extinction of NADP at 340nm for 3 minutes as NADP is reduced to NADPH.

##### Glucose-6-phosphate dehydrogenase (G6PDH)

G6PDH was assayed in homogenate by method of Lohret *et al.*,<sup>16</sup>. The enzyme activity was assayed by following the increase in extinction of NADP at 340nm for 3 min as NADP is reduced to NADPH.

##### Protein estimation

The protein in the samples was estimated by the method of Lowry *et al.*,<sup>17</sup>.

##### Statistical analysis

The results were expressed as Mean±SD. The differences between groups were analysed by ANOVA using SPSS software package for windows. Post hoc testing was performed for inter group comparisons using the least significance difference test. A value of p <0.05 was considered to indicate a significant difference between groups

## RESULTS

### Histopathological Examination

On histopathological examination, normal colonic mucosa and crypts were observed in the control group. (Figure 1a). Mild inflammation was observed in group 2 (Figure 1b), group 3 (Figure 1c) and group 4 (Figure 1d) animals.

### Oxidative stress

#### Lipid Peroxidation

Lipid peroxidation levels are represented in table1. A significant increase in MDA levels was observed in DMH treated group. The animals treated with extract in the presence or absence of DMH also exhibited even higher levels of MDA.

#### Superoxide dismutase (SOD)

SOD activity decreased significantly in animals treated with DMH and extract only group. Whereas, a significant

increase in SOD activity was observed on pretreatment with extract in DMH treated group as compared to DMH treated group. (Table1)

#### Catalase (CAT)

CAT activity showed no significant alteration on DMH treatment in presence or absence of extract. However, the activity of CAT was significantly decreased in animals treated with extract only as compared to the other three groups. (Table1)

#### Reduced Glutathione (GSH)

No significant alteration was observed in GSH levels on DMH treatment. However, GSH level decreased significantly in animals treated with both extract and DMH as compared to DMH treated animals. The animals treated with extract only depicted a significant increase in GSH level as compared to all other groups. (Table 1)

#### Glutathione-S-transferase (GST)

On DMH treatment, GST activity increased significantly in comparison to control group. The animals treated with extract in presence or absence of DMH also exhibited a significance increase in GST activity with maximum levels seen on treatment with extract only. (Table 1)

#### Assessment of NADPH synthesising enzymes:

##### Isocitrate dehydrogenase (IDH)

On treatment with DMH, a significant increase in IDH2 activity was observed. However, treatment with extract in presence or absence of DMH led to a significant decrease in IDH activity as compared to control and DMH treated animals. (Table 2)

##### Glucose-6-phosphate dehydrogenase (G6PDH)

G6PDH activity showed no significant alteration in DMH group and extract+DMH group. However, the activity of G6PDH was significantly increased on administration of extract only as compared to all other groups. (Table 2)

## DISCUSSION

The present study was designed to evaluate the effect of ethanolic extract of *Ocimum gratissimum* on DMH induced colonic injury. The results did not show any protective effect of *Ocimum gratissimum* on DMH induced toxicity rather these were suggestive of toxic properties of ethanolic extract.

The histopathological examination was done to evaluate the effect of *Ocimum gratissimum* ethanolic extract on colon tissue. The colon tissue of control animals showed a normal mucosa and crypt morphology on histopathological examination (Figure 1a). However, a mild inflammation was observed in DMH group (Figure 1b), extract+DMH group (Figure 1c) and extract group

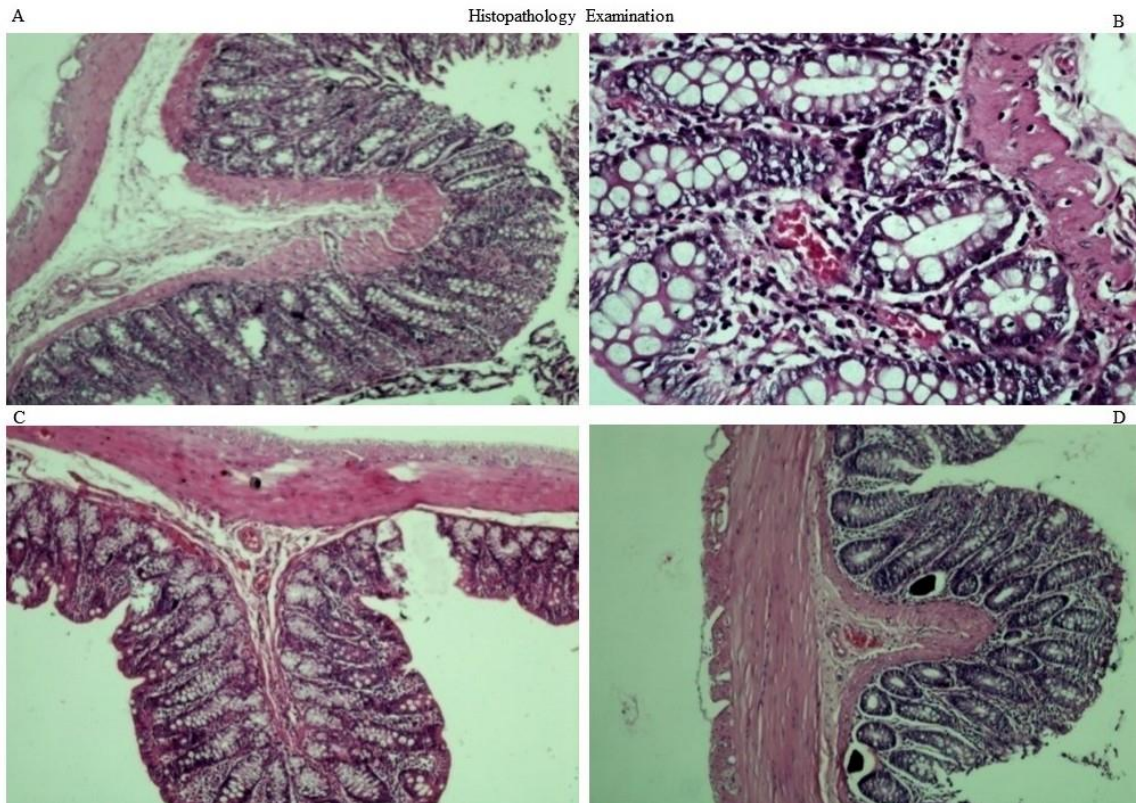


Figure 1: Effect of *Ocimum gratissimum* on Histopathology of rat colon  
Photomicrograph of colonic histopathology. A) represents the normal colonic mucosa and crypt morphology of control group(X 40).B-D)depicts theinflammatory cells infiltration in colonic tissue of DMHgroup (X 100), DMH+Extract(X 40), Extract(X 40) respectively.

(Figure 1d). Overall, there was no major changes histologically in all other groups. Earlier studies have reported a dose dependent toxic effect of aqueous extract of *Ocimum gratissimum* on liver, kidney and intestine<sup>18-21</sup>. Absence of prominent histopathological changes in present study suggest that the dosage given and time period of dose were not sufficient to instigate histopathological changes.

The membrane peroxidation triggered by increased oxidative stress is well associated with carcinogenesis<sup>22</sup> and toxicity<sup>23,24,4</sup>. Uncontrolled increase in ROS can lead to direct damage of protein, lipid and DNA. Lipid peroxidation or MDA formation is one of the important and relevant markers of oxidative damage and in the present study, these levels were elevated after treatment with DMH (Table 1). These results are consistent with other reports showing remarkable increase in MDA level after DMH treatment<sup>22,25</sup>. Pretreatment with ethanolic extract of *Ocimum gratissimum* in presence or in absence of DMH also exhibited elevated MDA level. A significant elevation in MDA levels on treatment with only *Ocimum gratissimum* extract are suggestive of toxic effects of ethanolic extract. Several reports have also shown the toxic effects of *Ocimum gratissimum* on various organs including liver, intestine, lung<sup>18-21</sup>. Therefore, this elevation in MDA level might be due to the toxicity of ethanolic extract of *Ocimum gratissimum* on intestine.

SOD is an important antioxidant enzyme that catalyzes the dismutation of  $O_2^{\cdot}$  radical to  $H_2O_2$  which in turn is acted

upon by CAT and converted to  $H_2O$ . Thus SOD and CAT are mutually supportive enzymes, which provide protection against ROS. According to the results of the present study, the activity of SOD decreased in DMH treated animals with no significant change in CAT activity. Decrease in SOD activity may be due to the saturation of SOD with superoxide radicals or due to decreased expression of SOD. A decrease in SOD activity will make the colonic mucosa more susceptible to damage by ROS. As CAT decomposes  $H_2O_2$  generated by SOD, no significant change in CAT activity was observed due to decrease in substrate levels. Pretreatment with ethanolic extract followed by DMH showed elevated activity of SOD and CAT. As increase in SOD activity lead to increased  $H_2O_2$  production and thereby increase CAT activity. An elevated SOD and CAT activity may be in response to the toxic effect of the extract. This may be an adaptive response to combat the increased oxidative stress caused by DMH and the extract. Several studies have reported induction of antioxidant enzymes on toxic insult<sup>26</sup>. The increase in SOD and CAT activity in DMH + extract treated animals suggest the potentiation of the toxic effect of two agents. SOD and CAT activity was significantly lower in animals treated with the extract only as compared to control animals. Decreased activity of SOD and CAT would lead to an increase in oxidative stress and these results are corroborated by an observed increase in MDA levels in this group.

GSH and its oxidized counterpart represent a major redox buffer system of the cell. GSH can either act as a non-enzymatic antioxidant by direct interaction of -SH group with ROS or it can be implicated in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme for GST. Therefore, the level of GSH serves as an important index for the capability of the cell to counteract the oxidative stress. GST catalyses the conjugation of GSH and xenobiotics and is an important phase II metabolizing enzyme. According to the results of the present study, no significant change were observed in the GSH and GST levels in DMH treated animals as compared to control animals. This suggests that GSH and its corresponding enzymes may not play a significant role in DMH induced colon toxicity in the initial stages. The animals treated with ethanolic extract followed by DMH showed decreased GSH level and increase in GST activity. This indicates an increased oxidative burden in this group as GSH levels are decreased. An increased GST activity suggests the cellular response for the clearance of the toxic intermediates / metabolites by the colonic tissue. In animals treated with extract only, there was an increase in GSH and GST levels which might be an adaptive response to the toxic insult by extract.

NADPH is an important coenzyme which is required for reduced biosynthesis and also to nullify the effect of oxidative stress by maintaining the level of reduced glutathione<sup>27,5</sup>. To study the effect of *Ocimum gratissimum* on NADPH generation, mitochondrial IDH2 activity was studied. As the inner membrane of mitochondria is impermeable to NADPH, IDH2 derived NADPH has an essential role in protection against ROS produced by the electron transport chain. In addition, IDH2 is also inactivated on oxidative damage by lipid peroxidation, singlet oxygen species, ROS etc<sup>5</sup>. An increase in NADP linked mitochondrial IDH2 activity on DMH treatment observed in the present study, indicates the increased requirement of NADPH to combat the mitochondrial oxidative stress generated due to increased requirement of ATP. Administration of ethanolic extract of *Ocimum gratissimum* in presence or absence of DMH led to a significant decrease in IDH2 activity. This may be because of the increased oxidative stress due to ethanolic extract which is leading to an oxidative insult and decreasing the enzyme activity. Similar decline in IDH2 activity has been noticed in cadmium induced toxicity by Ivania et al.,<sup>28</sup>.

G6PDH, regulatory enzyme of HMP shunt, also plays a significant role in NADPH generation. G6PDH activity did not show any significant change in DMH and DMH + extract treated animals. Since NADPH is utilized for regeneration of reduced glutathione, the results corroborate the findings of glutathione levels in these groups. A significant increase was observed in G6PDH activity on administration of *Ocimum gratissimum* extract only. This may be an adaptive change due to increased requirement of NADPH for providing a reductive environment to neutralize ROS generated by *Ocimum gratissimum* extract and a similar change is also reflected in the glutathione levels.

Therefore, the results of the present study suggest that administration of ethanolic extract of *Ocimum gratissimum* did not show any protective effect in experimentally induced colon toxicity and, in fact led to the potentiation of the injury. The results also suggest that *Ocimum gratissimum* extract may have a damaging effect on colonic mucosa by modulation of oxidative stress.

## CONCLUSION

To conclude, no protective effect of ethanolic extract of *Ocimum gratissimum* was observed in DMH induced toxicity, rather the present study suggests that administration of ethanolic extract can lead to oxidative insult and may have toxic effects on colon tissue.

## DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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