The ameliorative effect of Vitamin C in experimentally induced colon cancer in rats

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ABSTRACT

The present study was designed to investigate the ameliorative effect of vitamin C administration on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), Urea, Creatinine, Alpha-fetoprotein (AFP), Carcinoembryonic antigen (CEA) and carbohydrate antigen 19.9 (CA 19-9), in addition to tumor necrosis factor alpha (TNF-α), Cytochrome 4502E1 and Caspase-9 gene expression in colon cancer induced by 1,2-dimethylhydrazine (DMH) in rats. Forty white female albino rats were divided into four equal groups (10 each). Group I (control group): received no drugs. Group II: (DMH group): rats injected subcutaneously with DMH (35 mg/kg body weight), twice a week for 5 consecutive weeks for colon cancer induction. Group III: (protective group): rats administrated Vit C (200 mg kg-1 b. wt) orally and for 4 weeks before DMH injection and continued with Vit C administration daily till the end of the experiment. Group IV: (treatment group): rats injected subcutaneously with DMH then administrated Vit C for 6 weeks till the end of the experiment. Blood and colon tissue samples were collected from all animal groups at the end of the experiment. The obtained results showed that DMH injection to rats significantly increased serum (ALT, AST, Urea, Creatinine, AFP, CEA, CA 19-9) and colon tissue TNF-α and CYP2E1 while colon tissue caspase-9 gene was significantly decreased when compared to control. Administration of Vit C significantly decreased serum (ALT, AST, Urea, Creatinine, AFP, CEA, CA 19-9) in addition to TNF-α and CYP2E1 in colon tissue. How every, caspase-9 gene expression showed a significant increase. Histopathological examination of colon tissue edesquamation of the colon epithelium with heavy leukocytic infiltration in DMH group. While administration of vitamin C in colon cancer induced rats showed mild destruction of the lining epithelium with mild leukocytic infiltration in addition to mild malignancy of epithelium when compared to DMH group. These results indicated the protective effect of Vit C against DMH induced colon cancer.

Keywords: vitamin C, DMH, Colon cancer, Caspase-9 gene expression, Cytochrome 2E1, Tumor necrosis factor –alpha.
INTRODUCTION

Cancer is a disease characterized by the unchecked division and survival of abnormal cells. When this occurs in the colon or rectum, it is called colorectal cancer (CRC) (American Cancer Society, 2017). The risk factors for colon cancer are such as age, gender, physical activity, genetic factors, food habits, inflammatory bowel diseases and pro-carcinogens present in food supply chain and environment (Benson, 2007). But diet has main effect on gut related cancers; therefore those foods which positively affect colonic health by altering gut micro flora are considered useful strategy against colon cancer protection (Davis and Milner, 2009).

DMH is a toxic environmental pollutant, which was reported as a specific colon procarcinogen. The experimental colonic tumors induced by DMH in animals were of epithelial origin with a similar histology, morphology and anatomy to human colonic neoplasms. This pro-carcinogen could provide a sufficient model for studying colorectal cancer (Wang et al., 2004). DMH is considered to form active intermediates including azoxy methane and methyl azoxy methanol in the liver, which are moved subsequently into the colon via bile and blood. Methylazoxymethanol is decomposed to form methyl diazonium ions, which methylate cellular components. DMH also generates free radicals that induce oxidative DNA damage in the liver and colon. Damage to DNA from ROS is a consequence of oxidative stress and several oxidative DNA adducts, including 8-oxodG, have been implicated in the tumorigenic process (Aranganathan and Nalini, 2009). To induce colorectal tumors, the potency of DMH is the reason of inducing DNA methylation (Rowlatt et al., 2016), which was strongly associated with abnormal gene expression and tumorgenesis (Salehi et al., 2015).

Vitamin C is a water soluble ketolactone which can undergo intracellular oxidation to form ascorbate and dehydroascorbic acid (DHA). Humans lack the ability to synthesize vitamin C due to they lack the vital enzyme L-gulonolactone oxidase, which is the final step in the synthesis of vit C (Du et al., 2012). Vitamin C is abundant in fruits and vegetables which main source for dietary vitamin C intake. Vitamin C participant in many physiological processes and has a beneficial or therapeutic role in immune responses, cardiovascular disease and cancer (Deruelle and Baron, 2008). So, the aim of this study was to evaluate the protective effect of vitamin C administration against colon cancer induced in rats.

2. Materials and methods

2.1. Experimental animals

Forty white female albino rats of 6-8 weeks old and weighing 180-220g were used in this study. The rats were obtained from the laboratory animals research center, Faculty of Veterinary Medicine, Moshtohor, Benha University. The animals were housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of the experiment. Rats were fed on constant ration and fresh, clean drinking water was supplied ad-libitum. The animals were left for 14 days for acclimatization prior to the beginning of the experiment.

2.2 Drugs and Chemicals:

1) Dimethyl hydrazine was obtained from Sigma Aldrich Chemical Company. Which freshly dissolved in 1Mm EDTA/0.9% NaCl solution to ensure the stability of the chemical just prior to use. Colon cancer was induced by subcutaneous injection of DMH at a dose of
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35 mg/kg b.wt for 5 weeks (twice / week) (Moharib , 2016 and Hussein et al., 2013).

2) Vitamin C was obtained from El-Gomhouria Company, Cairo, Egypt. Vitamin C was administrated daily and orally to rats at dose of200 mg/kg b.wt (Aly et al., 2010).

2.3 Experimental Design:

Rats were randomly divided into four main equal groups (10 rats each), placed in individual cages and classified as follow: Group I: Control normal group: rats received no drugs. Group II: DMH group: rats injected with DMH at dose of 35 mg/kg b. wt, s.c twice a week for 5 consecutive weeks. Group III: (Protective group): rats were received vitamin C at dose of200 mg/kg b.wt /daily, orally for 4 weeks before DMH injection and continued with vitamin C administration daily till the end of the experiment. Group IV : (Treatment group):rats injected subcutaneously with DMH after 4 weeks of the experiment then administrated vitamin C for 6 weeks till the end of the experiment(15th week).

2.4 Sampling:

Blood and colon tissue samples were collected from all animal groups (control and experimental groups) at the end of experiment (15th week).

2.4.1 Serum samples:

Blood samples for serum separation were collected by ocular vein puncture in dry, clean and screw-capped tubes after overnight fasting. Serum was separated by centrifugation at 3000 r.p.m. for 15 minutes. The clean, clear serum was taken by automatic micropipettes and received in dry sterile eppendorf’s tubes and kept in a deep freeze at -20ºC until used for subsequent biochemical analysis. All sera were analyzed for (ALT, AST, Urea, Creatinine, AFP, CEA and CA 19-9).

2.4.2 Colon samples:

Rats were sacrificed bycervical decapitation. The abdomen was opened and the colon specimen was quickly removed and opened gently using a scraper, cleaned by rinsing with ice-cold isotonic saline to remove any blood cells and clots then tissue samples were put in 2 ml Eppendorf tube and were immediately kept in liquid nitrogen and stored at -80ºC till RNA extraction.All colon tissue samples were analyzed for determination of: TNF-α, Caspase- 9 gene and CYP450 2E1.

2.4.3 For Histopathological Examination:

Colon of rats was carefully examined by naked eyes for detection of any abnormalities. Small colon samples were taken from different parts. The specimens were preserved in 10% neutral buffered formalin solution and subjected for histopathological staining and examination according to the technique described by Bancroft and Stevens (1996).

2.5 Biochemical analysis:

Serum was used for determination of ALT and AST (IFCC,1986),Urea (Fawcet and Scott,1960), Creatinine (Muarry et al.,1984),AFP(Sell, 1990), CEA (Begent and Rustin, 1989), CA19.9 (Minamide et al., 2000), colon tissue used for determination of TNF-α, Caspase- 9 gene and CYP4502E1 (Livak and Schmittgen, 2001).

2.6 Statistical analysis:

The obtained data were statistically analysed by one-way analysis of variance (ANOVA) followed by the Duncan’s multiple test. All analyses were performed using the statistical package for social science (SPSS, 18.0 software, 2011). Values at 0.05 was considered to be significant.

3. RESULTS

The obtained results demonstrated in (Table 1) revealed that, administration of DMH induced colon cancer in rats exhibited a significant increase in serum AFP, CEA and CA 19.9 concentrations when compared with normal control group. Treatment with vitamin Cto DMH induced colon cancer in rats
significantly reduced elevated serum AFP, CEA and CA 19.9 concentrations when compared with non-treated colon cancer group. Administration of vitamin C (before, during and after) injection DMH resulted in significant decrease in serum AFP, CEA and CA 19.9 concentrations when compared with vitamin C treated group.

The obtained data presented in Table 2 revealed that, administration of DMH induced colon cancer in rats caused significant increase in serum ALT, AST, Urea, Creatinine and TNF-α and CYP2E1 in colon tissues. However, Caspase-9 gene expression in colon tissues were significantly decreased when compared with normal control group. Vitamin C treatment to colon cancer rats’ significantly decreased ALT and AST activities and decreased serum levels of Urea and Creatinine. Also decreased TNF-α and CYP2E1 in colon tissues when compared with DMH induced colon cancer non-treated group. On the other hand, vitamin C treatment increased Caspase-9 gene expression in colon tissues when compared with DMH induced colon cancer non-treated group. Administration of vitamin C (before, during and after) injection DMH resulted in significant decrease in serum ALT, AST, Urea, Creatinine and TNF-α and CYP2E1 in colon tissues and significant increase Caspase-9 gene expression in colon tissues when compared with vitamin C treated group.

**Histopathological Results:**

The colon of control normal group showed normal histological structure (Fig.1). Colon tissue of DMH group showed severe destruction and desquamation of the colon epithelium with heavy leukocytic infiltration were seen (Fig.2). Colon tissue of Vitamin C protected group showed mild destruction of the lining epithelium in comparison to DMH group (Fig.3) with mild mucosal leukocytic infiltration in addition to mild malignancy of epithelium (Fig.4). Colon tissue of from this group Vitamin C treated group showed destruction of the lining epithelium in comparison to DMH group (Fig. 5). Also, it showed leukocytic infiltration (Fig.6).

**Table (1): Effect of vitamin C administration on serum AFP, CEA and CA 19.9 concentrations in 1,2dimethylhydrazine-induced colon cancer in rats.**

<table>
<thead>
<tr>
<th>parameters</th>
<th>Animals groups</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Group1</td>
<td>Group2</td>
<td>Group3</td>
<td>Group4</td>
<td></td>
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<tr>
<td>AFP(ng/ml)</td>
<td>3.75±0.18d</td>
<td>50.88±0.27a</td>
<td>31.22±0.54c</td>
<td>42.54±0.55b</td>
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<tr>
<td>CEA(ng/ml)</td>
<td>12.28±0.33d</td>
<td>64.74±0.74a</td>
<td>47.93±0.93c</td>
<td>59.46±0.53b</td>
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<tr>
<td>CA19.9(U/ml)</td>
<td>1.68±0.08d</td>
<td>7.84±0.26a</td>
<td>4.16±0.29c</td>
<td>6.78±0.28b</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05). Group 1: Control group, Group 2: colon cancer, Group3: Vit C protected group and Group 4: Vit C treated group.
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Table (2): Effect of vitamin C administration on serum (ALT, AST, Urea, Creatinine) and TNF-α, Caspase-9 gene expression, CYP2E1 in colon tissue of 1,2dimethylhydrazine-induced colon cancer in rats.

<table>
<thead>
<tr>
<th>parameters</th>
<th>animals groups</th>
<th>Group1</th>
<th>Group2</th>
<th>Group3</th>
<th>Group4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT(U/L)</td>
<td></td>
<td>71.07±0.43d</td>
<td>185.88±1.74a</td>
<td>137.10±2.99c</td>
<td>164.50±2.47b</td>
</tr>
<tr>
<td>AST(U/L)</td>
<td></td>
<td>120.35±2.08d</td>
<td>215.57±2.91a</td>
<td>170.74±1.74c</td>
<td>193.94±2.41b</td>
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<tr>
<td>Urea(mg/dl)</td>
<td></td>
<td>24.94±0.37d</td>
<td>79.58±0.84a</td>
<td>40.22±0.89c</td>
<td>57.87±0.74b</td>
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<tr>
<td>Creatinine(mg/dl)</td>
<td></td>
<td>0.85±0.05d</td>
<td>3.58±0.2a</td>
<td>1.69±0.06c</td>
<td>2.46±0.08b</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td>1±0.06d</td>
<td>13.27±0.42a</td>
<td>6.73±0.26c</td>
<td>10.34±0.28b</td>
</tr>
<tr>
<td>Caspase-9 gene</td>
<td></td>
<td>1±0.06a</td>
<td>0.07±0.001d</td>
<td>0.51±0.02b</td>
<td>0.19±0.01c</td>
</tr>
<tr>
<td>CYP2E1</td>
<td></td>
<td>1±0.06d</td>
<td>11.88±0.35a</td>
<td>5.43±0.22c</td>
<td>9.45±0.29b</td>
</tr>
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</table>

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05). Group 1: Control group, Group 2: Colon cancer, Group3: Vit C protected group, and Group 4: Vit C treated group.

**Fig. 1** Photomicrograph of colon from normal control group showing normal histological feature. (H&E, scale bar =200 µm).
Fig. 2 Photomicrograph of colon from DMH group showing destruction and desquamation of the colon epithelium with heavy leukocytic infiltration. (H&E, scale bar =200 µm).

Fig. 3 Photomicrograph of colon from vitamin C protected group showing mild destruction of the lining epithelium in comparison to positive control group. (H&E, scale bar =200 µm).

Fig. 4 High magnification of Fig.3 showing mild leukocytic infiltration in the mucosa in addition to mild feature of malignancy in the lining epithelium of the colon in comparison to control positive group. (H&E, scale bar =50 µm).
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**Fig. 5** Photomicrograph of colon from vitamin C treated group showing destruction of the lining epithelium in comparison to DMH group. (H&E, scale bar =50 µm).

**Fig. 6** Photomicrograph of colon from vitamin C treated group showing leukocytic infiltration in comparison to DMH group. (H&E, scale bar =50 µm).

4. DISCUSSION

A significant increase in serum AFP, CEA and CA19.9 levels were observed in DMH group when compared to normal control. The results are in agreement with Umesalma and Sudhandiran, (2010) who reported that, elevation in serum CEA concentration was observed in DMH-induced colon cancer in rats, 1, 2- dimethylhydrazine, a potent carcinogen administered, induced reactive oxygen species (ROS) damage to colon that causes instability of colon cell metabolism, which leads to different changes in tumor markers (CEA and AFP) are representatives of colon function. In addition to, serum CEA was elevated in 20% of patients at primary diagnosis of colon cancer and in 46.6% of patients at reappearance (Chang et al., 2012). Both elevated serum CEA and CA 19-9 levels were associated with the presence CRC. Elevated serum CEA and CA 19-9 levels were significantly correlated with larger lesion size and multiplicity of adenomas (Kim et al., 2017).

Treatment of vitamin C to DMH-induced colon cancer in rats significantly reduced elevated serum AFP &CEA and CA 19-9 levels when compared with DMH non-treated group. Serum levels of CEA decreased in colon cancer patients after the post
A treatment of high dose intravenous ascorbic acid (Mikirova et al., 2012). Furthermore, the level of AFP was significantly decreased when pretreated and post-treatment by vitamin C in albino rats (Ahmed et al., 2008).

Potential mechanisms of action of vitamin C on cancer cells remains still uncertain, but recent experimental data suggest some possible mechanisms. First, vitamin C has a pro-oxidant effect at higher concentrations. Which high-dose of vitamin C generated hydrogen peroxide ($H_2O_2$) in the extracellular fluid, which then entered into cells (Chen et al., 2007). $H_2O_2$ is able to accelerate the production of additional reactive oxygen species (ROS) such as aldehydes. These ROS are capable of several effects, including DNA damage, cell membrane dysfunction, and cellular adenosine triphosphate depletion in cancer cells due to reduced levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase. These processes are not found in normal cells and may lead to death of cancer cells (González, 1992 and Sun et al., 1993). Second, vitamin C may modulate inflammation resulting in increase of host resistance to cancer. Which highly concentration of vitamin C decreased the production of interleukin-18, which is related to tumor cell growth, proliferation, and migration (Cho et al., 2003).

The presented result in Table (2) revealed that, administration of DMH induced colon cancer in rats exhibited significant increase in ALT and AST activities when compared with normal control group. Severe increase in serum ALT, AST and ALP activities were observed in DMH-untreated rats when compared with control rats might be due to the loss of cellular functional integrity of hepatocytes membrane resulted from highly reactive electrophiles i.e., carbonium ions and alkyl free radicals which severely damage the liver causing necrosis and fatty infiltration methylate nucleobases and upset the polysomal assembly and enzymes are located in liver cells leak out and make their way into the general circulation (Abdelmoneim et al., 2013).

Vitamin C (Protection and Treated) groups revealed that, a significant decrease in serum AST and ALT activities when compared with DMH non-treated group. Pretreatment with vitamin C showed significant decreasing in liver marker enzymes ALT and AST. Vitamin C was able to weaken hepatic damage induced by some chemical agents, especially in animals. Vitamin C normalized levels of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. These may be ascribe mainly to the ameliorative effect of vitamin C against the oxidative stress induced by malathion as vitamin C has antioxidant activity (Abu-El-Zahab et al., 2016). The mechanism by which vitamin C decrease the hepatotoxicity is by decreasing lipid peroxidation and altering antioxidant defense system or by denoting electrons to free radicals and overcome their reactivity (El-Gendy et al., 2010; Ahmad and Al-Jawary, 2012).

DMH exhibited significant increase in serum urea and creatinine in DMH non-treated group (Table 2). The urea cycle was at higher levels after hydrazine treatment when compared with control group including citrulline and ornithine in urine or plasma at 24 h post-dosing. Accordingly, the levels of associate metabolites, namely fumarate and urea, also increased. Biosynthesis of amino acids was up-regulated by hydrazine treatment, glutathione metabolism, and lysine metabolism, so the urea cycle might be expected to be up-regulated in order to remove excess ammonia derived from
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Elevated amino acids. In addition, the amino groups of amino acids that have been used as metabolic fuel are converted into urea through the urea cycle (Bando et al., 2011). Maleichydrazide caused significant increase in urea and creatinine levels in blood samples which had toxic effects and act as an inhibitor of the synthesis of nucleic acids and proteins and had carcinogenic effects in mice and rats (Yazar and Baydan, 2008). Raised creatinine concentration is an index of kidney dysfunction (Garba et al., 2007).

But administration of vitamin C in (Protection and Treated) groups revealed that, a significant decrease in serum Urea and Creatinine levels when compared with DMH non-treated group. These results are agreement with those reported by Waheed et al., (2011) who demonstrated that the levels of urea and creatinine in serum of rats treated by Vit C were lower than that of positive control group. The improvement marked in the level of serum urea and creatinine in group treated by Vit C due to soluble water antioxidant Vit C- The protective effect of vitamin C because reverse action on free radicals by its antioxidant nature. Vitamin C may be recommended as a supplementary therapy with certain anticancer. Vitamin C has Protective effect against chemically-induced damage in various rodent organs (Zaidi and Banu, 2004 and Ajith et al., 2007). Vitamin C was found to be effectively protecting chemically-induced oxidative renal damage in animals. The protective effects may be partially mediated by preventing the renal antioxidant status (Naziroglu et al., 2004 and Abraham, 2005).

Administration of DMH induced colon cancer in rats exhibited significant decrease in Caspase-9 gene expression in colon tissues (Table 2). These results are nearly similar to those reported by (Hussein et al., 2013) which revealed a significant decrease in the colon tissue caspase-9 gene activity and DNA fragmentation percent in DMH-induced colon cancer group. DMH is an alkylating agent which damages cellular DNA by forming adduct. Apoptosis is a tightly regulated state of programmed cell death (Goncu and Parlak, 2008) and caspases play a key role for initiation and execution of cell death (Ghavami et al., 2009). Due to DMH-induced colorectal rodent tumors exhibit k-ras mutations following essential activation of PI3K/Akt pathway (Camacho et al., 2010). PI3K activates the downstream target Akt to
intermediate several biological effects (Vivanco and Sawyers, 2002). After activation Akt inactivates several downstream targets including B cell lymphoma-2 (Bcl-2) family members, caspase-9 by blocking apoptosis (Bandres et al., 2004).

Administration of vitamin C to DMH in (Protection and Treated) groups showed significant increase in Caspase-9 gene expression in colon tissues. The results are in agreement with (Shinozaki et al., 2011) who recorded a significant increase in caspase-9 activity in human leukemia HL60 cells which treatment by ascorbic acid. Ascorbic acid caused induction of apoptosis activates caspases-3, caspase-8, and caspase-9.

Vitamin C protects DNA and mitochondrial proteins of the normal cell from the damage caused by ROS. This role of vitamin C in the prevention of cell damage by the scavenging of ROS reflected the first line of defense against cancer (Li et al., 2001). Vitamin C treatment at a concentration of 2 mM resulted in the induction of apoptosis by increasing calcium influx into the endoplasmic reticulum, and upregulation of the expression of the pro-apoptotic factor Bax after 12 hours of the treatment (Kim et al., 2012). Similarly, when treated with vitamin C made inflection of p53, p21, Bcl-2 and Bax in adult T-cell leukemia cell lines resulted in the induction of cancer cell death by apoptosis in a dose dependent manner (Harakeh et al., 2007).

Administration of DMH induced colon cancer in rats exhibited significant increase in cytochrome P4502E1 colon tissues when compared to normal control (Table 2). Similarly Gunasekaran et al., (2014) observed that, DMH-induced activation of P450 enzymes in the rat colonic mucosa. DMH is a potent inducer of CYP 2E1 in rat liver tissue (Arikawa and Gallaher, 2008). Furthermore, CYP2E1 is one of the enzymes catalyzing the conversion of metabolites of DMH are, azoxymethane and methylazoxymethanol, which to DNA alkylating species capable of causing initiation of colon cancer (Sohn et al., 2001). DMH produced free radicals might be due to P-450-dependent enzymes which increase oxidative stress by the formation of H2O2 and O2* (Farber and Gerson, 1984). On the other side, administration of vitamin C to DMH in (Protection and Treated) groups caused significant decrease in cytochrome P4502E1 in colon tissues when compared with DMH untreated group. Due to vitamin C plays an important role in collecting reactive oxygen species, acting as an antioxidant for maintaining the intracellular redox balance and minimizing the oxidative damage caused by these free radicals so vitamin C can neutralize reactive oxygen species, derived from the imbalance between antioxidant defenses and oxidative stress caused by diseases such as cancer or its treatment (Rahal et al., 2014). Control negative group showed normal histological feature of colon like finding of Venkatachalam et al., (2012). In positive control group, administration of DMH causes coloncancer with rapid cellular proliferation (Habib et al., 2016). Colon of this group showed elongated to oval hyperchromatic nuclei of the epithelium and glands with destruction of basement membrane that was similar to Ojep and Seng (2010) and Venkatachalam et al., (2012). Destruction of the lining epithelium with leukocytic infiltration were similar to finding of Hussein et al. (2013).

5. CONCLUSION

The findings of the present study showed that administration vitamin C provided an effective protection against colon cancer induced by DMH in rats, since vitamin C were able to ameliorate serum biochemical
parameters. Also, vitamin C treated increase the apoptotic marker caspase-9-gene expression. The obtained results suggest the potential effective of vitamin C as an addition chemopreventive agent in treatment of colon cancer. So, we recommended that, supplementation of diet rich in the natural ascorbic acid is very important for protection of different body organs from cancer. In addition, we strongly support the use of ascorbic acid as pure active ingredients in pharmacological industry for production of new drugs used as therapeutics for cancer treatment especially colon cancer.

6. REFERENCES


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