

## Basic Study

# Phosalone-induced inflammation and oxidative stress in the colon: Evaluation and treatment

Seyedeh Farnaz Ghasemi-Niri, Faheem Maqbool, Maryam Baeeri, Mahdi Gholami, Mohammad Abdollahi

Seyedeh Farnaz Ghasemi-Niri, Faheem Maqbool, Maryam Baeeri, Mahdi Gholami, Mohammad Abdollahi, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Faheem Maqbool, Mohammad Abdollahi, International Campus, Tehran University of Medical Sciences, Tehran 1417614411, Iran

**Author contributions:** Ghasemi-Niri SF performed the majority of experiments and analyzed the data; Baeeri M performed the molecular investigations; Gholami M participated in treatment of animals; Ghasemi-Niri SF, Maqbool F and Abdollahi M designed, coordinated the research and wrote the paper; and Abdollahi M conceived the study.

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**Correspondence to:** Mohammad Abdollahi, PhD, Professor, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Enghelab Avenue, Tehran 1417614411, Iran. [mohammad@tums.ac.ir](mailto:mohammad@tums.ac.ir)  
Telephone: +98-21-66959104  
Fax: +98-21-66959104

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## Abstract

**AIM:** To investigate the side effects of phosalone on intestinal cells and to evaluate benefits of ellagic acid (EA) as a remedy.

**METHODS:** In order to conduct an *in vivo* study, a rat model was used. The rats were divided into ten groups based on the materials used in the experiment and their dosage. The first group was fed normally. The second group was administered EA through gavage. Next Four groups were given (1/3, 1/5, 1/10, 1/20) LD<sub>50</sub> phosalone; an organophosphorus compound. The last four groups received (1/3, 1/5, 1/10, 1/20) LD<sub>50</sub> phosalone and of EA. After one month, the rats were sacrificed and their colon cells were examined to evaluate the level of inflammation, proteins and oxidative stress markers.

**RESULTS:** The results of this research show that phosalone elevates oxidative stress and changes the level of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-

6 $\beta$  (IL-6 $\beta$ ) and nuclear factor (NF)- $\kappa$ B proteins. EA administration reduced phosalone toxicity and changed oxidative stress and inflammatory markers for all phosalone doses. Overall changes in reduction of TNF- $\alpha$  ( $230.47 \pm 16.55$  pg/mg protein *vs*  $546.43 \pm 45.24$  pg/mg protein,  $P < 0.001$ ), IL-6 $\beta$  ( $15.85 \pm 1.03$  pg/mg protein *vs*  $21.55 \pm 1.3$  pg/mg protein,  $P < 0.05$ ), and NF- $\kappa$ B ( $32.47 \pm 4.85$  pg/mg protein *vs*  $51.41 \pm 0.71$  pg/mg protein,  $P < 0.05$ ) manifest that the efficacy of EA is more viable for 1/3 LD<sub>50</sub> dose of phosalone. Furthermore, EA is effective to counteract the negative outcomes of oxidative stress. When EA was used to treat 1/3 LD<sub>50</sub> of phosalone's side effects, it improved the level of AChE activity ( $48.5\% \pm 6\%$  *vs*  $25\% \pm 7\%$ ,  $P < 0.05$ ), TTM ( $0.391 \pm 0.008$  mmol/L *vs*  $0.249 \pm 0.032$  mmol/L,  $P < 0.05$ ), FRAP ( $46.04 \pm 5.005$   $\mu$ mol/L *vs*  $18.22 \pm 1.9$   $\mu$ mol/L,  $P < 0.01$ ) and MPO ( $0.222 \pm 0.019$  U/mg protein *vs*  $0.387 \pm 0.04$  U/mg protein,  $P < 0.05$ ).

**CONCLUSION:** This research highlights that EA is effective to alleviate the side effects of phosalone by reducing the level of oxidative stress and inflammatory proteins.

**Key words:** Organophosphorus; Phosalone; Ellagic acid; Inflammation; Oxidative stress; Colon

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**Core tip:** This research uses a rat model to evaluate the colon related side effects of phosalone which is a member of the organophosphorus family. After feeding different dosages of phosalone to the rats for one month, the colon tissue of the rats were studied using oxidative stress and pathology tests. Both tests show that the higher doses of phosalone elevate reactive oxygen species (ROS), tumor necrosis factor- $\alpha$ , interleukin-6 $\beta$  and nuclear factor- $\kappa$ B proteins which result in more inflammation. In our study, ellagic acid (EA) which is a strong antioxidant reduced phosalone-induced side effects. The oxidative stress and pathology results concluded that EA helps reducing inflammation and ROS.

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## INTRODUCTION

Pesticides are substances used in agriculture to kill pests and also as a domestic insect killer<sup>[1]</sup>. Although they are significant in agriculture use but they may also enter human body through inhalation *via* air born

particles. Farmers may inhale such chemicals when they use them for pest control<sup>[2]</sup>. General public is prone to pesticide after eating agricultural products which are not washed properly. Over-usage of pesticides may cause plants to absorb them directly or indirectly through soil. In such case, washing may not completely cleanse the pesticides and their consumers are vulnerable to the resultant side effects<sup>[3,4]</sup>.

Phosalone [O,O-diethyl-S-(6-chloro-2-oxoben-zoxazolin-3-yl-methyl)-phosphorodithioate] is a member of the organophosphorus (OP) family, which is used extensively as a pesticide in agriculture and as a domestic insect killer<sup>[5]</sup>. As compared to Dicololor Di Three ethane (DDT), phosalone has less severe side effects on human and environment and because of this reason it has replaced DDT for pest control. Regardless of the fact, that phosalone is safer than DDT, but its toxicity has been one of the important research topics in toxicology. The most important known toxicity of phosalone is related to human nervous system. The mechanism of such damage is extremely toxic and phosalone can inhibit neural cholinesterase (ChE) activity, which elevates the level of acetylcholine thus therefore prevents neural signal pathway in the nervous system<sup>[6]</sup>. Furthermore, like the other members of the OP family, phosalone increases reactive oxygen species (ROS) in the human body tissues thus reduces the level and activity of anti-oxidant enzymes. Higher amount of ROS increases lipid peroxidation (LPO) in the membrane of cells, resulting in membrane damage and disturbance in the cell functional balance<sup>[7]</sup>. The final repercussions of ROS are faster cell aging and higher chances of DNA and RNA changes, subsequently leading toward cancer and gene mutations<sup>[8,9]</sup>.

The main route through which OP enters the body is mucosa in intestinal cells, where OP can pass through membrane barrier and enter blood. Human cardiovascular system distributes OP to other organs and results in nervous system and ROS related damages<sup>[10,11]</sup>. Furthermore, the effect of OP on micro flora in intestinal and gastrointestinal enzymes elevate neutrophil infiltration and pro-inflammatory proteins<sup>[12,13]</sup>. The consequence of such effects is the migration of several immune cells such as neutrophils, monocytes, lymphocytes, macrophages and chemokines then adhesion molecules move toward mucosal tissue. The final outcome of such damage is intestinal inflammation<sup>[14,15]</sup>.

This research elaborates ROS related side effects of phosalone and proposes a material to reduce and potentially eliminate such side effects. The proposed material should be able to offset free-radicals. This research shows that ellagic acid (EA) can be a remarkable candidate to considerably suppress the side effects of phosalone. EA is an important natural occurring substance, which has phenol components<sup>[16]</sup>. EA is present in numerous fruits and vegetables such

as grapes, nuts, strawberries, black currents, raspberries, green tea, pomegranates, and the stem and bark of *Eucalyptus globulus*, *Eucalyptus maculatu* and nuts. The international chemical name of EA is 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde] chromene-5,10-dione<sup>[17]</sup>.

The biological activities of EA has been investigated in several *in vivo* and *in vitro* studies and have shown that EA has anti-cancer, anti-inflammatory and anti-oxidant properties and in addition it has beneficial therapeutic effect on colon, skin, breast cancer and inflammatory bowel disease (IBD)<sup>[18]</sup>. EA can also improve mucosa production in goblet cells in colon; reduce pro-inflammatory proteins COX-2 and iNOS over expression and neutrophil infiltration<sup>[19]</sup>. The anti-oxidant effect of EA stem is clear from the fact, that EA can scavenge free radical, nitrogen reactive species, and ROS, including hydroxyl radicals, peroxy radicals, NO<sub>2</sub> radicals, and peroxy nitrite and therefore EA reduce DNA and cell damages<sup>[20]</sup>. Additionally, EA can potentially shield DNA and protect it from ROS, free radical and chelation of metal ions attack.

Regarding other effects of EA, some studies have reported that EA can affect cytochrome C in mitochondria which increases BAX/Bcl2, regulates cell division and apoptosis<sup>[21]</sup>. Also through stimulating the immune system, EA plays a positive role in intercellular complex signaling systems such as mitogen activated protein kinases (MAPKs) and/or the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B)<sup>[22]</sup>. An in-depth study of these effects is presented in this paper.

In our study, we evaluate effect of phosalone on inflammation and oxidative stress with four doses as well as subsequent effect of EA on colon cells.

## MATERIALS AND METHODS

### Chemicals

Acetylthiocholine iodide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) from Merck (Germany), trichloroacetic acid (TCA), Tris base, 1,1,3,3'-tetraethoxypropane (MDA), 2-thiobarbituric acid (TBA), *n*-butanol, 2,4,6-tripryridyl-s-triazine (TPTZ), *n*-butanol, acetic acid, FeCl<sub>3</sub>·6H<sub>2</sub>O, benzethonium chloride, 5,5'-Dithiobis(2-nitrobenzoic acid), Trizma® base, EA, o-Dianisidine dihydrochloride, phosphate buffer from Sigma-Aldrich (Germany), *n*-butanol, hexadecyl tri-methyl ammonium bromide (HETAB), ethylene diamine tetra acetic acid (EDTA), hydrochloric acid (HCL), acetic acid, sodium acetate, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), O-dianisidine hydrochloride, ferric chloride (FeCl<sub>3</sub>·6H<sub>2</sub>O), Coomassie reagent, bovine serum albumin (BSA), sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>), sulphuric acid (H<sub>2</sub>SO<sub>4</sub>), phosphoric acid (H<sub>3</sub>PO<sub>4</sub>), potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), potassium hydrogen diphosphate (K<sub>2</sub>HPO<sub>4</sub>), sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), cupric sulphate (CuSO<sub>4</sub>·5H<sub>2</sub>O) from Merck. Rat-specific tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and NF- $\kappa$ B ELISA kits from

(Bender MedSystems GmbH, Austria), analytical grade form of phosalone from local pesticide manufacturing companies (Agroxir) and were used in this study.

### Experimental animals

In our study, male Wistar rats weighing 180-200 g were selected according to the regulations of the ethics committee of Tehran University of Medical Sciences (TUMS) approved with code number of 93-02-45-26666. Animals were housed separately in standard polypropylene cages with a wire mesh top, kept under standard conditions, including temperature (23 °C  $\pm$  1 °C), relative humidity (55%  $\pm$  10%) and 12/12 h light/dark cycle, and fed a standard pellet diet and water ad libitum. All ethical themes of studies on animals were considered carefully.

### Experiment design

Animals were divided into ten groups based on the materials used in the experiment and their dosage, with six rats in each group. The first group was fed normally. The second group was administered EA (10 mL/kg) through gavage. Next Four groups were given different dosage of phosalone (1/3 LD<sub>50</sub>: 40 mg/kg, 1/5 LD<sub>50</sub>: 20 mg/kg, 1/10 LD<sub>50</sub>: 12 mg/kg and 1/20 LD<sub>50</sub>: 6 mg/kg), which is a member of organophosphorus family, through gavage. The last four groups received both phosalone (1/3 LD<sub>50</sub>: 40 mg/kg, 1/5 LD<sub>50</sub>: 20 mg/kg, 1/10 LD<sub>50</sub>: 12 mg/kg and 1/20 LD<sub>50</sub>: 6 mg/kg) and EA (10 mL/kg). After one month, the rats were sacrificed and their colon cells were examined to evaluate the level of oxidative stress factors.

### Sample preparation

After 30 d, all rats were anesthetized (40% Ketamine 1000, 25% Xylazine 2%, 0.1 mL/100 g body weight) and after that all of animals were humanly sacrificed and colonic tissues were immediately separated. Isolated segments were rinsed with normal saline and then placed in an ice bath throughout the procedure. Colonic tissue was divided into two pieces. The first piece was weighed and kept in 10 mL of formalin 10%, as a fixator for the purpose of histopathological evaluation. The second piece was weighed and homogenized in 10 volumes of ice cold potassium phosphate buffer (50 mmol, pH = 7.4) and then stored at -20 °C for 24 h. The sample was then sonicated and centrifuged for 30 min at 3500 *g*, and the supernatant was transferred to a micro tube. Then sample was kept at -80 °C until biomarker analyses.

### Determination of lethal dose of phosalone

An lethal dose (LD<sub>50</sub>) of phosalone is a standard measurement of toxicity that is stated in milligrams (mg) of phosalone per kilogram (kg) of body weight at which 50% of rats are killed. For finding the LD<sub>50</sub> of phosalone, we performed a study on Wistar rats.

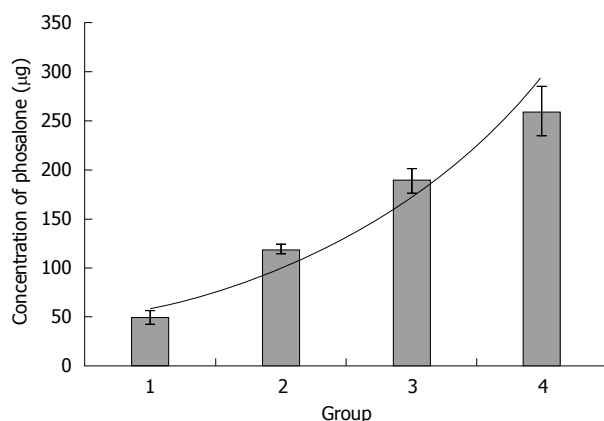


Figure 1 Determination of LD<sub>50</sub> of phosalone.

We divided five groups of rats and administrated with different doses of phosalone. One of group was control that didn't receive phosalone. But four groups received different doses of phosalone, like 50 mg/kg, 120 mg/kg, 190 mg/kg and 260 mg/kg. After two days we compared all groups and found LD<sub>50</sub> was between 120 mg/kg to 190 mg/kg. After that we analyzed all data and 120 mg/kg was LD<sub>50</sub>, used for phosalone in animal model in our study (Figure 1).

#### Assay of oxidative stress enzymes

**AChE activity:** AChE activity of erythrocytes was measured according to method of Ellman method using acetylthiocholine iodide as the substrate and 5-5-bis dithionitrobenzoic acid (DTNB). Briefly, 10 µL of sample was added to 3 mL of solution containing 25 mmol/L DTNB in 75 mmol/L phosphate buffer. Then 10 µL of 3 mmol/L acetylcholine iodide was added and absorbance changes were measured at 412 nm in a two-fold rays spectrophotometer<sup>[23]</sup>.

#### Myeloperoxidase activity assessment

MPO activity was determined by a dianisidine-H<sub>2</sub>O<sub>2</sub> method, modified for 96-well plates. Briefly, plasma samples (10 µg protein) were added in triplicate to 0.53 mmol/L o-dianisidine dihydrochloride (Sigma) and 0.15 mmol/L H<sub>2</sub>O<sub>2</sub> in 50 mmol/L potassium phosphate buffer (pH 6.0). After incubation for 5 min at room temperature, the reaction was stopped with 30% sodium azide. The absorbance was measured at 460 nm ( $\epsilon = 11300 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ) spectrophotometrically (Shimadzu 160A UV-VIS spectrophotometer). Results were expressed as units of MPO/mg protein, whereby 1 unit of MPO was defined as the amount of enzyme degrading 1 nmol H<sub>2</sub>O<sub>2</sub> per min at 25 °C<sup>[24]</sup>.

#### LPO measurement

To measure LPO, thiobarbituric acid-reaction substances (TBARS) were assessed in colon tissue. TBA reacts with lipid peroxides in the samples producing a measurable pink color that has absorbance at 532 nm

by a double beam spectrophotometer. Concentration of TBARS is recorded as µg<sup>[25]</sup>.

#### Assay of total thiols

To determine TTM in the control and test groups, 0.6 mL Tris-EDTA buffer (Tris base 0.25 mol/L, ethylene diamine tetra acetic acid 20 mmol/L, pH 8.2) was added to 0.2 mL of supernatant, and after quick vortex mixing, 40 µL 5-5'-dithiobis-2-nitrobenzoic acid (10 mmol/L in pure methanol) was added. The final volume of this mixture was made up to 4.0 mL by an extra addition of pure methanol. After 15 min incubation at room temperature, the samples were centrifuged at 3000 *g* for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. Data are shown as mmol/L<sup>[26]</sup>.

#### FRAP assay

Antioxidant power of plasma was evaluated by measuring its ability to reduce of Fe<sup>3+</sup> tripyridyltriazine (TPTZ) complex (colorless) to Fe<sup>2+</sup> TPTZ (blue colored) formed by the action of electron donating antioxidants at low pH. The ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mmol/L acetate buffer, 10 mL TPTZ in 40 mmol/L HCl and 20 mmol/L FeCl<sub>3</sub> in the proportion of 10:1:1 at 37 °C. 10 µL of the H<sub>2</sub>O diluted sample was then added to 300 mL freshly prepared reagent warmed at 37 °C. An intense blue color complex was formed when Fe<sup>3+</sup> TPTZ complex was reduced to Fe<sup>2+</sup> form. The complex between Fe<sup>2+</sup> and TPTZ gives a blue color with absorbance at 593 nm. Data are shown as µmol/L<sup>[27]</sup>.

#### Determination of TNF-α and IL-6β

A human specific ELISA kit (BenderMed System) was used to quantify TNF-α and IL-6 in the supernatant of colon tissue. To assess the amount of TNF-α, the absorbance of sample was measured in 450 nm as the primary wavelength and 620 nm as the reference wavelength by ELIZA reader as described in the kit brochure. TNF-α and IL-6β levels were expressed as pg/mg protein of tissue<sup>[28]</sup>.

#### Determination of NF-κB

The amount of NF-κB in colon cells extracts was measured by using NF-κB ELISA kits (BenderMed System) according to the manufacturer's instructions. The levels of NF-κB in nuclear extracts were calculated using the standard curve and expressed as pg/mg protein<sup>[29]</sup>.

#### Total protein assessment

The concentration of protein in the colon homogenate was measured by the Bradford method using BSA as the standard. The absorbance was measured by the spectrophotometer at 595 nm after 5 min. The bovine serum albumin was used as standard<sup>[30]</sup>.



**Statistical analysis**

At least four independent experiments in repetition were carried away. Data are presented as mean  $\pm$  SE. One-way ANOVA and Tukey's multi-comparison trials were held out by Stats-Direct 3.0.169 software to determine the statistical differences while the degree of significance had been set at ( $P < 0.05$ ).

**RESULTS****Pathology evaluation of the colon damage**

As shown in Figure 2, histopathological examination in normal group shows that there was no ulceration, no necrosis, no adhesions, no wall thickening and mucosal/submucosal polymorphonuclear (PMN) leukocyte infiltration. In EA group there was no blood and ulcer in mucosal/submucosal of the colon tissue. In the 1/3 LD<sub>50</sub> phosalone group, it was observed in some areas infiltration, adhesions, with no any overlying blood and serous adhesion.

The 1/3 LD<sub>50</sub> phosalone and EA group showed improvement in muscles and mucosa, a reduction inflammation in colon tissue and low lymphocytes infiltration in submucosal layer. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration and inflammation is less than 1/3 LD<sub>50</sub> phosalone group. Histological examination of 1/5 LD<sub>50</sub> phosalone and EA group showed improvement in mucosa with the reduction lymphocytes in submucosa region. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/5 LD<sub>50</sub> phosalone group. In the 1/10 LD<sub>50</sub> phosalone and 1/20 LD<sub>50</sub> phosalone with EA groups, the mild degeneration of mucosal muscle cells and muscle layers were observed. In 1/10 LD<sub>50</sub> phosalone and EA was seen a very mild inflammation due to lymphocytes infiltration between mucosal glands. But in 1/20 LD<sub>50</sub> phosalone and EA, there was no inflammation in different layers.

**AChE activity**

After pathological examination, the first step was the evaluation of EA through measurement of AChE activity. AChE activity was reduced in colon cells of groups receiving 1/3 and 1/5 LD<sub>50</sub> of phosalone in comparison to normal group ( $P < 0.01$ ). In two groups (1/3 and 1/5) LD<sub>50</sub> phosalone, AChE activity was significantly decreased in comparison to EA group ( $P < 0.05$ ). EA restored the activity of AChE which was suppressed by phosalone. Among different phosalone doses, such AChE activity retrieval was more significant for 1/3 LD<sub>50</sub> ( $P < 0.05$ ) (Figure 3).

**Myeloperoxidase activity**

Colonic myeloperoxidase (MPO) activity in 1/3 LD<sub>50</sub> phosalone group was noticeably higher than that of

the normal and EA groups ( $P < 0.01$ ). Data showed a remarkable difference between 1/5 LD<sub>50</sub> phosalone and EA group ( $P < 0.01$ ). Also, the group of animals which received EA and 1/3 LD<sub>50</sub> phosalone, showed a reduction of MPO activity (by 26%,  $P < 0.05$ ) in comparison to 1/3 LD<sub>50</sub> phosalone group (Figure 4).

**Oxidative-stress as TBARS**

Inflammation in colon referred as over-activity of oxidative stress was found high in 1/3 and 1/5 LD<sub>50</sub> phosalone groups as compared to normal and EA groups ( $P < 0.01$ ). Colonic lipid peroxidation in 1/10 LD<sub>50</sub> phosalone group was noticeably higher than that of the normal group ( $P < 0.01$ ). Although, EA decreased oxidative stress in all doses of phosalone, it down-regulated oxidant formation significantly in 1/5 LD<sub>50</sub> phosalone ( $P < 0.05$ ) (Figure 5).

**TTM**

An obvious reduction in TTM was observed in 1/3 LD<sub>50</sub> phosalone group as compared to normal and EA groups ( $P < 0.01$ ). 1/5 and 1/10 LD<sub>50</sub> phosalone groups significantly decreased TTM in comparison with normal group ( $P < 0.05$ ). EA restored significantly the TTM which was suppressed by 1/3 LD<sub>50</sub> phosalone (Figure 6).

**Anti-oxidant power as FRAP**

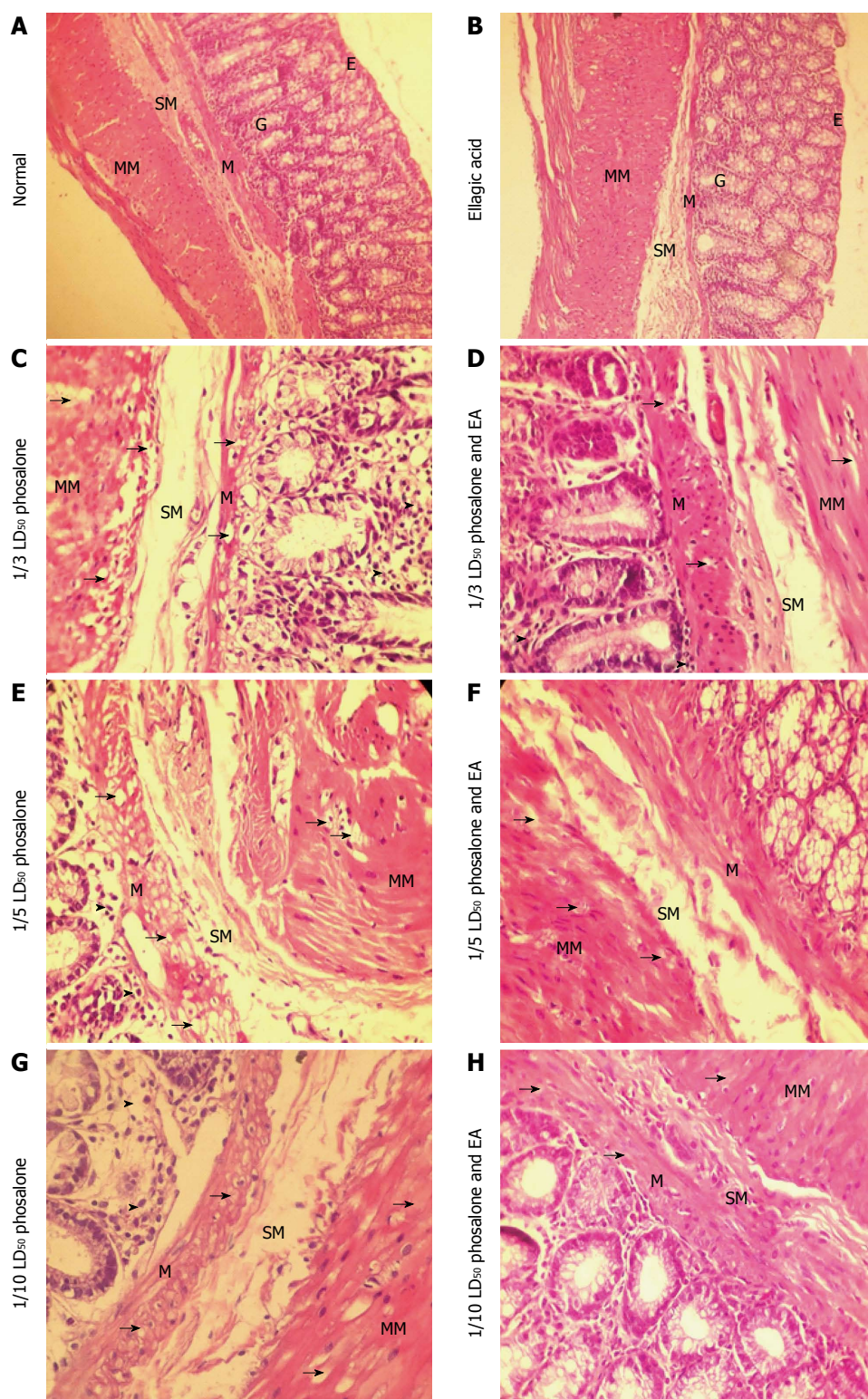
Less ability in overcoming the oxidative stress in all doses of phosalone groups was reported in contrast to normal and EA groups ( $P < 0.001$ ). FRAP value in 1/3 LD<sub>50</sub> phosalone was significantly less than EA and 1/3 LD<sub>50</sub> phosalone group ( $P < 0.01$ ). Amount of FRAP in 1/5 LD<sub>50</sub> phosalone was markedly lower than its normal content in EA and 1/5 LD<sub>50</sub> phosalone group ( $P < 0.001$ ). The amount of FRAP increased significantly in EA and 1/10 LD<sub>50</sub> phosalone group compared to 1/10 LD<sub>50</sub> phosalone group ( $P < 0.001$ ). A significant increase in FRAP was seen in EA and 1/20 LD<sub>50</sub> phosalone group when compared to 1/20 LD<sub>50</sub> phosalone ( $P < 0.01$ ) (Figure 7).

**TNF- $\alpha$  level**

An obvious rise in TNF- $\alpha$  level was observed in (1/3 and 1/5) LD<sub>50</sub> phosalone groups as compared to normal group ( $P < 0.01$ ). In (1/3 and 1/5) LD<sub>50</sub> phosalone groups showed a significant increase in TNF- $\alpha$  level in comparison with EA group ( $P < 0.05$ ). A noticeable improve in TNF- $\alpha$  content was seen in EA and 1/3 LD<sub>50</sub> phosalone group when compared with 1/3 LD<sub>50</sub> phosalone group ( $P < 0.001$ ). In EA and 1/5 LD<sub>50</sub> phosalone group as shown in Figure 8, EA prevented more secretion of TNF- $\alpha$  when compared to 1/5 LD<sub>50</sub> phosalone group ( $P < 0.05$ ) (Figure 8).

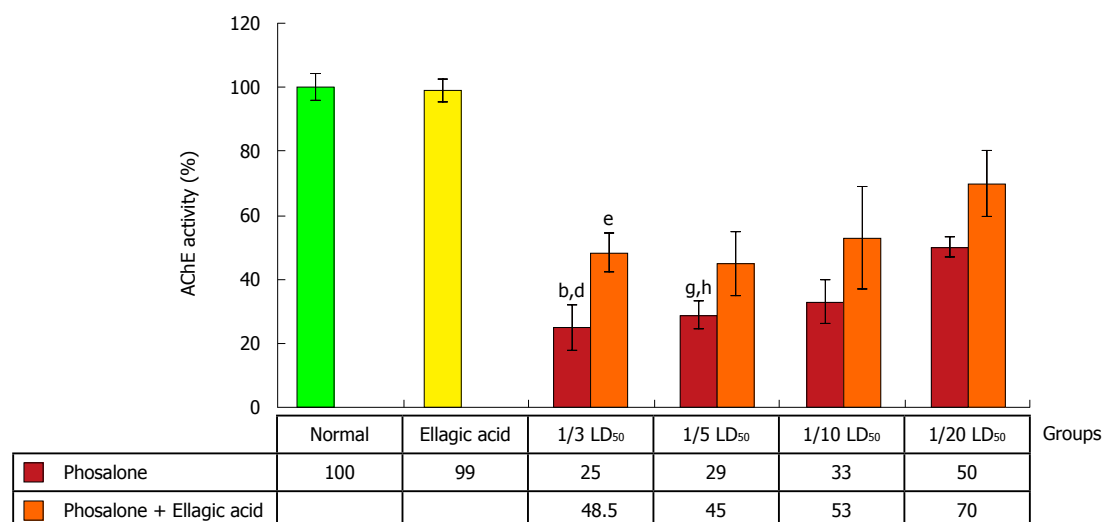
**IL-6 $\beta$  level**

All doses of phosalone groups showed a notable elevation in IL-6 $\beta$  level in comparison to normal group

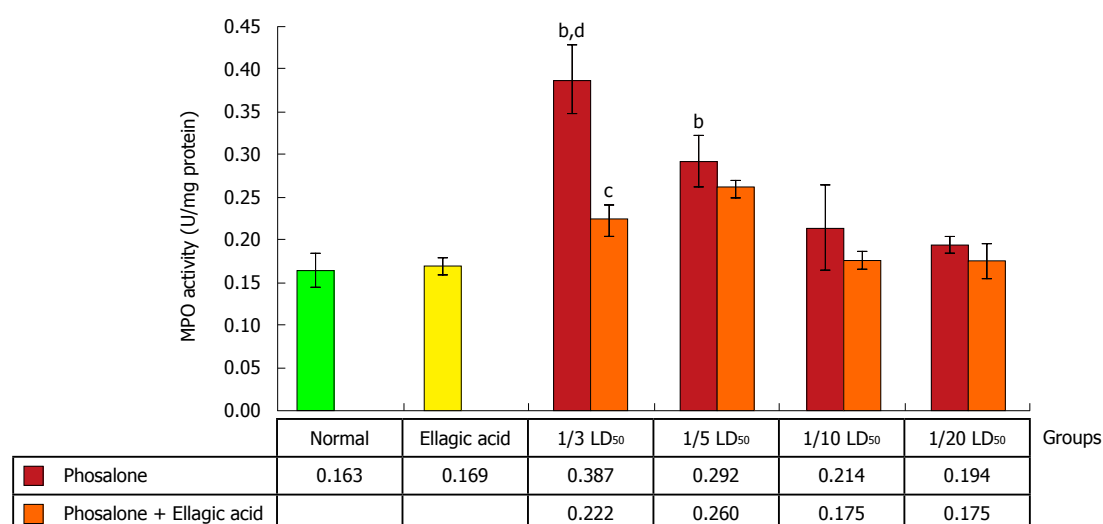


**Figure 2** Histological images of colon tissues from normal, ellagic acid and experimental groups. In control group, different parts of the colon tissue are healthy. There are no erosions or ulcers in epithelium. It cannot be seen any necroses and inflammation cells in mucus and mucosal glands in the lamina propria. The mucus thickness, the size of the glands, the muscle layer of mucosal and serous is normal. No degeneration, swelling and goblet cells are observable (A). In ellagic acid (EA) group, the following are normal, the mucosal, the goblet gland cells, epithelium, the mucosa thickness and the size of the glands. There are no ulcers, necrosis hyperplasia and inflammation cells such as neutrophils or lymphocytes. It cannot be observed any degeneration, swelling and goblet cells. The serous is normal without any adherent (B). In 1/3 LD<sub>50</sub> phosalone group, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but severe degeneration of mucosal muscle cells and muscle layers is observable. In addition to hyperemia, there is a significant infiltration of mononuclear inflammatory cells such as lymphocytes and plasma cells between the mucosal glands. There is no fibrosis and serous adhesion (C). In 1/3 LD<sub>50</sub> phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration and inflammation is less than 1/3 LD<sub>50</sub> phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (D). In 1/5 LD<sub>50</sub> phosalone group, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but relatively severe

degeneration of mucosal muscle cells and muscle layers is observable. In addition to hyperemia, there is a significant infiltration of mononuclear inflammatory cells such as lymphocytes and plasma cells between the mucosal glands. There is no fibrosis and serous adhesion (E). In 1/5 LD<sub>50</sub> phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/5 LD<sub>50</sub> phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (F). In 1/10 LD<sub>50</sub> phosalone group, no necrosis and ulcer is present in epithelial. The mucosal glands are normal but significant degeneration of muscle cells and mucosal layer is observable. In addition to hyperemia, there is a mild infiltration of mononuclear inflammatory cells such as lymphocytes between the mucosal glands. There is no fibrosis and serous adhesion (G). In 1/10 LD<sub>50</sub> phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/10 LD<sub>50</sub> phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (H). In 1/20 LD<sub>50</sub> phosalone group, there is no evidence of necroses, ulcer or inflammation in epithelium. Mucosal glands are normal but a mild degeneration is observable in muscle cells in mucosal layer. A mild diapedesis, hyperemia and inflammation in mucosal is visible. There is no fibrosis and serous adhesion (I). In 1/20 LD<sub>50</sub> phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/20 LD<sub>50</sub> phosalone group. There is no inflammation in different layers (J). ML: Muscular layer; SM: SubMucosa; M: Mucosa; G: Gland; E: Epithelium.



**Figure 3 Effect of phosalone and ellagic acid on AChE activity of colon cells.** Values are mean  $\pm$  SE. <sup>b</sup> $P < 0.001$  vs normal group; <sup>d</sup> $P < 0.001$  vs ellagic acid group; Ellagic acid significantly increased of AChE activity in 1/3 dose of phosalone group. <sup>e</sup> $P < 0.05$  vs (1/3 LD<sub>50</sub> phosalone) group; <sup>g</sup> $P < 0.05$  vs ellagic acid group, <sup>h</sup> $P < 0.01$  vs normal group.

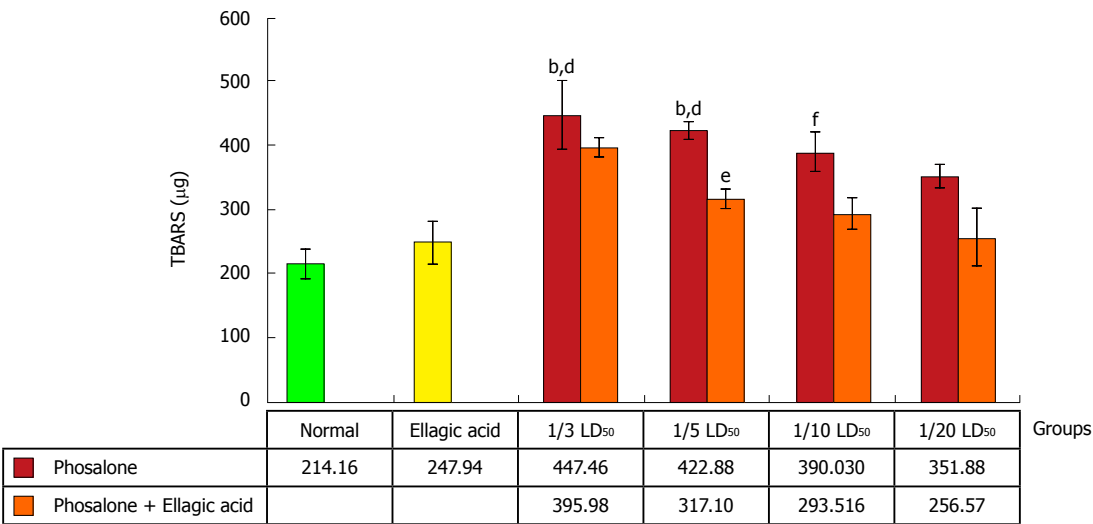


**Figure 4 Effect of phosalone and ellagic acid on myeloperoxidase activity of colon cells.** Ellagic acid significantly decreased of MPO in 1/3 dose of phosalone group. Values are mean  $\pm$  SE. <sup>b</sup> $P < 0.001$  vs normal group; <sup>c</sup> $P < 0.05$  vs (1/3 LD<sub>50</sub> phosalone) group; <sup>d</sup> $P < 0.01$  vs Ellagic acid group. MPO: Myeloperoxidase activity.

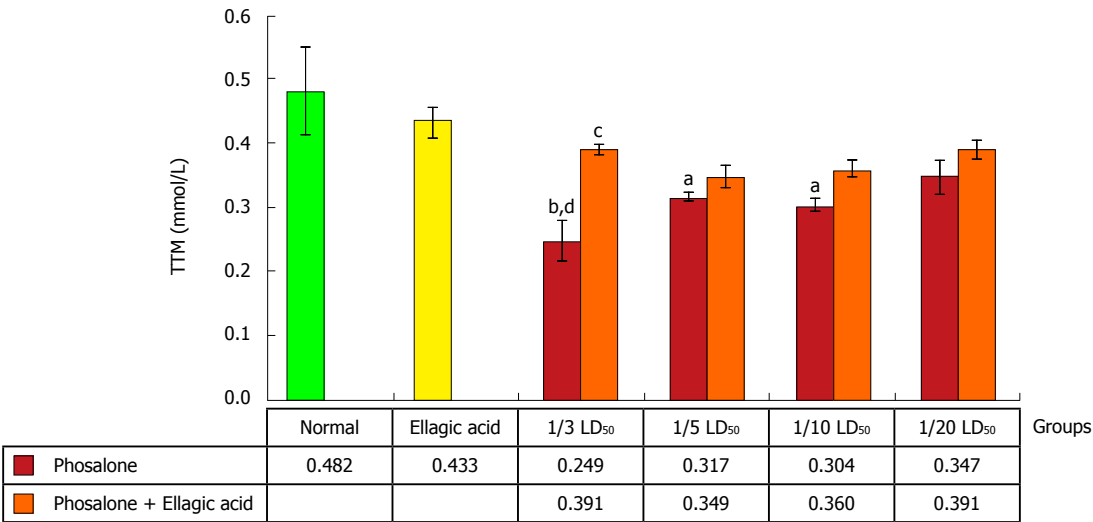
( $P < 0.001$ ). The EA and 1/3 dose of phosalone group differed from 1/3 LD<sub>50</sub> phosalone group remarkably ( $P < 0.05$ ). IL-6 $\beta$  level in 1/3 LD<sub>50</sub> phosalone group was noticeably higher than that of the EA group ( $P < 0.001$ ).

There was significant variation between EA, and EA and 1/5 LD<sub>50</sub> phosalone groups ( $P < 0.01$ ), while EA and 1/10 LD<sub>50</sub> phosalone group had a less potency in decreasing IL-6 $\beta$  level when compared to EA group ( $P$





**Figure 5** Effect of phosalone and ellagic acid on oxidative-stress as thiobarbituric acid-reaction substances of colon cells. Ellagic acid significantly decreased of thiobarbituric acid-reaction substances in 1/5 dose of phosalone group. Values are mean  $\pm$  SE. <sup>a</sup> $P < 0.001$  vs normal group; <sup>d</sup> $P < 0.01$  vs Ellagic acid group; <sup>e</sup> $P < 0.05$  vs (1/5 LD<sub>50</sub> phosalone) group; <sup>f</sup> $P < 0.01$  vs normal group.



**Figure 6** Effect of phosalone and ellagic acid on total thiol molecules activity of colon cells. Ellagic acid significantly increased of total thiol molecules in 1/3 dose of phosalone group. Values are mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs normal group; <sup>b</sup> $P < 0.01$  vs normal group; <sup>c</sup> $P < 0.05$  vs (1/3 LD<sub>50</sub> phosalone) group; <sup>d</sup> $P < 0.01$  vs Ellagic acid group.

< 0.05) (Figure 9).

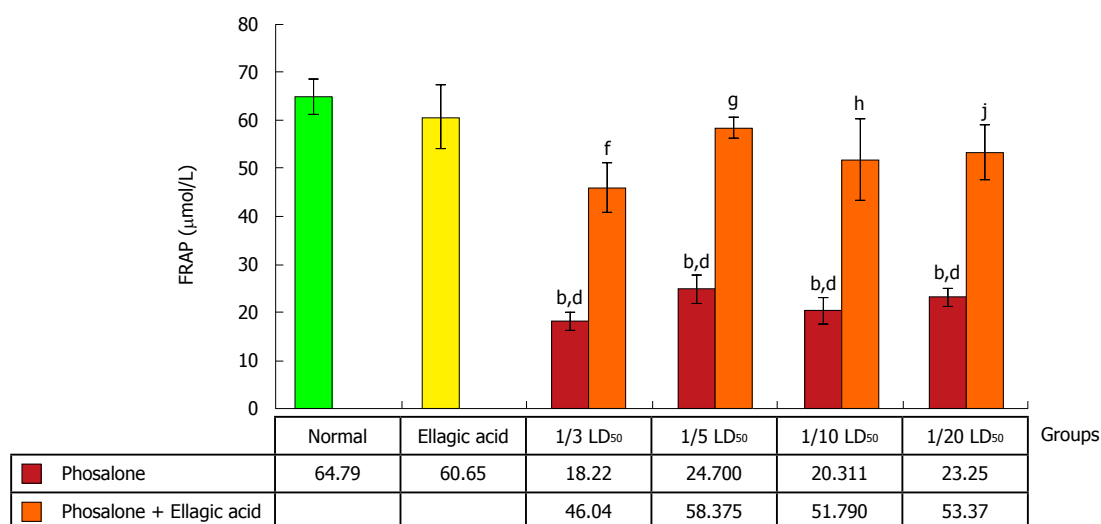
### NF- $\kappa$ B release

As seen in Figure 10, NF- $\kappa$ B production was significantly elevated in the (1/3 and 1/5) LD<sub>50</sub> phosalone groups when compared with normal group ( $P < 0.001$ ). A significant increase in NF- $\kappa$ B was seen in (1/10 and 1/20) LD<sub>50</sub> phosalone groups when compared with normal ( $P < 0.05$ ). The EA and 1/3 LD<sub>50</sub> phosalone group showed more reduction in NF- $\kappa$ B when compared with 1/3 LD<sub>50</sub> phosalone ( $P < 0.05$ ). The (1/3, 1/5 and 1/10) LD<sub>50</sub> phosalone groups which were treated with EA showed an apparent increase in NF- $\kappa$ B level when compared with EA group ( $P < 0.01$ ).

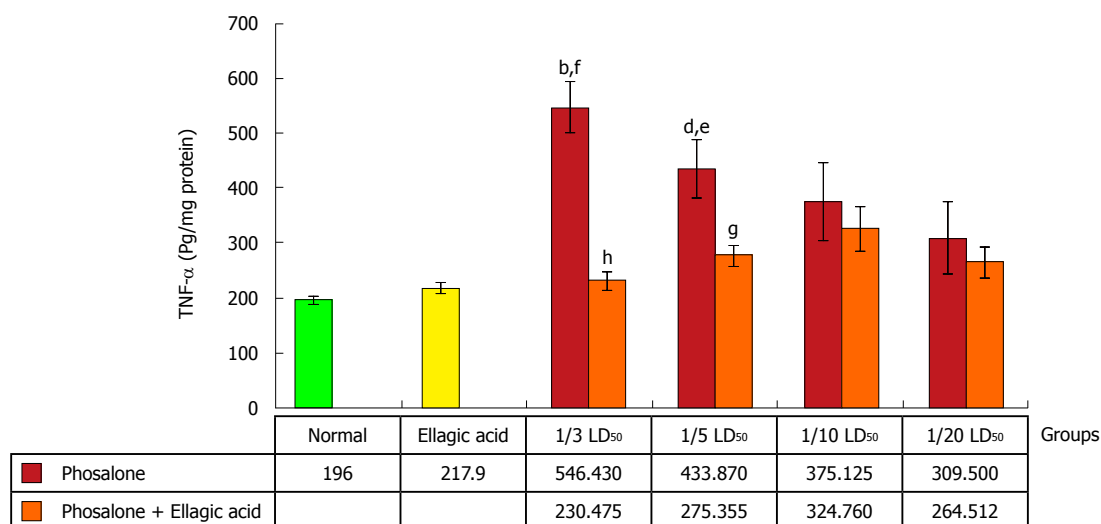
## DISCUSSION

In our study we succeeded to achieve our main hypothesis: to find phosalone toxicity in colonic tissues of rats as well as protective effects of EA, during subchronic exposure. Phosalone is type of OP pesticide that could affect different organs in daily and produce various toxicities<sup>[31]</sup>. As a result of phosalone exposure in rats, increase in oxidative stress and inflammatory markers were observed. In our experiment, EA was used to reduce colon injury induced by phosalone as a protective agent, which showed substantial decrease in oxidative stress and inflammatory markers. EA that is kind of polyphenol derived from different plants





**Figure 7 Effect of phosalone and ellagic acid on anti-oxidant power as (ferric reducing antioxidant power) of colon cells.** Ellagic acid significantly decreased of ferric reducing antioxidant power in all doses of phosalone groups. Values are mean  $\pm$  SE. <sup>b</sup> $P < 0.001$ , vs normal group; <sup>d</sup> $P < 0.001$  vs ellagic acid group; <sup>f</sup> $P < 0.01$  vs (1/3 LD<sub>50</sub> phosalone) group; <sup>g</sup> $P < 0.001$  vs 1/5 LD<sub>50</sub> phosalone; <sup>h</sup> $P < 0.001$  vs 1/10 LD<sub>50</sub> phosalone; <sup>j</sup> $P < 0.01$  vs 1/20 LD<sub>50</sub> phosalone.

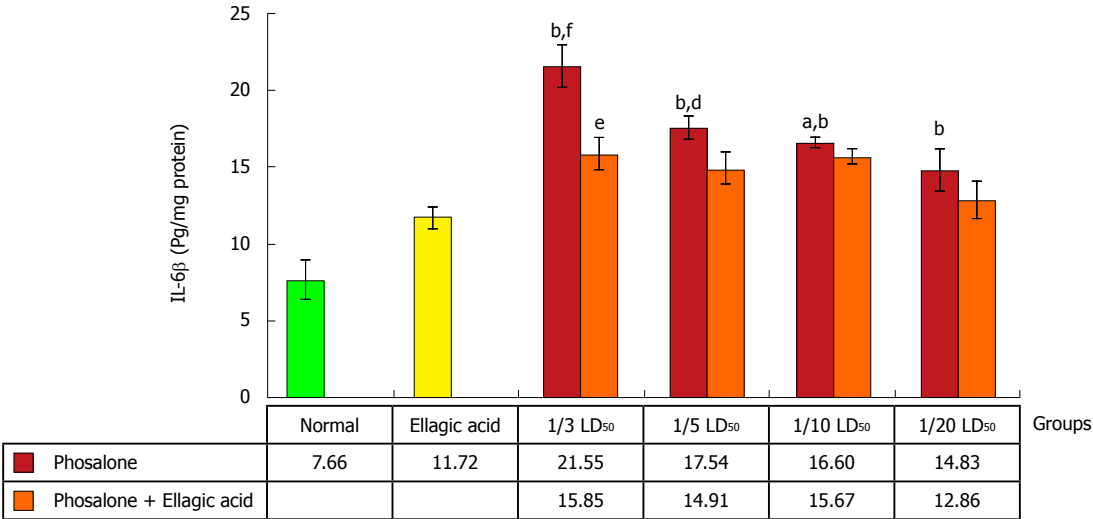


**Figure 8 Effect of phosalone and ellagic acid on tumor necrosis factor- $\alpha$  of colon cells.** Ellagic acid significantly decreased of tumor necrosis factor- $\alpha$  in 1/3 and 1/5 doses of phosalone groups. Values are mean  $\pm$  SE. <sup>b</sup> $P < 0.001$  vs normal group; <sup>d</sup> $P < 0.01$  vs normal group; <sup>e</sup> $P < 0.05$  vs ellagic acid group; <sup>f</sup> $P < 0.01$  vs ellagic acid group; <sup>g</sup> $P < 0.05$  vs (1/5 LD<sub>50</sub> phosalone) group; <sup>h</sup> $P < 0.001$  vs (1/3 LD<sub>50</sub> phosalone) group.

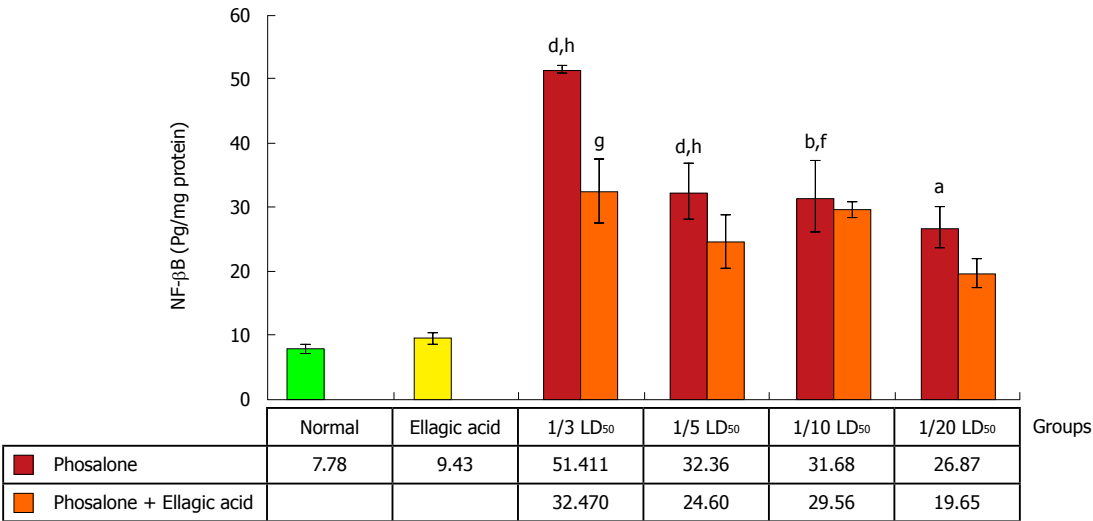
or fruits has already been reported to have sort of protective effects in different diseases<sup>[32,33]</sup>. As indicated in the present study, AChE activity was reduced with pronounced effect in colon cells of groups receiving (1/3 and 1/5) LD<sub>50</sub> phosalone in comparison to both normal group and EA group. In previous studies, the same inhibition of AChE was observed during behavioral studies in phosalone-treated rats brain cells<sup>[34,35]</sup>. AChE inhibition is among best indicator of toxicity induced by any xenobiotic or chemical that initiates other signaling pathways. Despite of little effect in other groups, EA considerably reversed the activity of AChE, suppressed by phosalone in group receiving 1/3 LD<sub>50</sub> phosalone. It has been already published that, by exposure of OPs elevated level of ACh *via* ChE inhibition could interfere with cholinergic receptors within hypothalamus and

potentiate release of adrenocorticotrophic hormone (ACTH)<sup>[36]</sup>. The present study proves that phosalone inhibits AChE activity that is associated with colon inflammation and EA could reverse its effect.

Increased production and decreased ability of ROS and antioxidant defense mechanism respectively can damage various signaling pathways, as well as cell constituents, including DNA, lipids and proteins. Induction of such oxidative impairment *via* redox signaling mechanisms can cause different human diseases<sup>[37]</sup>. In our experiment, biochemical assays showed that phosalone elevated oxidative stress *via* elevation of MPO activity and TBARS concentration, whereas in groups with combined EA administration; reduction in MPO activity and TBARS concentration has been observed. Irrespective of our study on phosalone,



**Figure 9** Effect of phosalone and ellagic acid on interleukin-6β of colon cells. Ellagic acid significantly decreased of tumor necrosis factor-α in 1/3 dose of phosalone group. Values are mean ± SE. <sup>a</sup>*P* < 0.05 vs ellagic acid group; <sup>b</sup>*P* < 0.001 vs normal group; <sup>d</sup>*P* < 0.01 vs ellagic acid group; <sup>e</sup>*P* < 0.05 vs (1/3 LD<sub>50</sub> phosalone) group; <sup>f</sup>*P* < 0.001 vs ellagic acid group.



**Figure 10** Effect of phosalone and ellagic acid on nuclear factor-κB of colon cells. Ellagic acid significantly decreased of nuclear factor-κB in 1/3 dose of phosalone group. Values are mean ± SE. <sup>a</sup>*P* < 0.05 vs normal group; <sup>b</sup>*P* < 0.01 vs normal group; <sup>d</sup>*P* < 0.001 vs normal group; <sup>e</sup>*P* < 0.01 vs ellagic acid group; <sup>f</sup>*P* < 0.001 vs ellagic acid group; Significantly different from at <sup>g</sup>*P* < 0.05 vs (1/3 LD<sub>50</sub> phosalone) group.

number of previous studies and literature demonstrate a close relation between exposure of pesticides and occurrence of various health problems *via* induction of oxidative stress<sup>[38,39]</sup>. A study conducted on humans *via in vitro* setup concluded that: oxidative stress and ROS were increased due to OPs exposure<sup>[40]</sup>. On other hand a pronounced effect of EA against free radical formation can be seen in groups receiving (1/3 and 1/5) LD<sub>50</sub> phosalone. However, it has been reported that both MPO and TBARS are indicators of oxidative stress and colon inflammation<sup>[41,42]</sup>. In addition to this, in our study, body's antioxidant defense mechanism was targeted by phosalone, which caused reduction in TTM and FRAP concentration as compared to normal and EA groups. A significant effect of EA as antioxidant has been observed in all groups receiving both EA and

phosalone in different doses. Protective and beneficial effects of EA in oxidative stress has been previously evidenced in many studies<sup>[43,44]</sup>. It can be derived from current biochemical tests that colon tissues of rats are prone to OPs like Phosalone and thus EA can better treat colon tissue damage *via* different mechanisms, while further studies can be conducted for treatment of IBD (inflammatory bowel disease). Oxidative stress and its balance is the most significant feature of normal physiology, in case of high toxicity it can initiate many signaling pathways that can lead to cell death. Our study shows that, EA play its role as protective agent in oxidative impairment, against free radical production and colitis due to phosalone exposure. So in colon inflammation induced by pesticides, EA can be used as anti-inflammatory, anticancer and antioxidant.

Furthermore we evaluated the effect of phosalone on different inflammatory markers along with protective effect of EA. Our concept regarding toxic mechanisms in colon inflammation is growing and general overview is that T cells secrete IL-2, IL-1B and Interferon- $\gamma$  (IFN- $\gamma$ ) that can excite macrophages to release extra TNF- $\alpha$  and IFN- $\gamma$ , ROS and other inflammatory mediators. Occurrence of TNF- $\alpha$ , IL-1B, ROS and some antigens target other signaling pathways which ultimately result in synthesis of cytokines<sup>[45]</sup>. TNF- $\alpha$  employs its action through elevating the synthesis of inflammatory mediators like IL-1 and IL-6<sup>[46]</sup>. Our recent study shows that phosalone increase TNF- $\alpha$  and EA well treat such condition in colon inflammation, which require further research regarding IBD. Phosalone caused increase in TNF- $\alpha$  level in all groups with significant change in group 1/3 LD<sub>50</sub> phosalone and 1/5 LD<sub>50</sub> phosalone as compared to normal and EA groups. EA showed prominent effect as protective agent to reduce TNF- $\alpha$  level in all groups with significant change in group 1/3 LD<sub>50</sub> phosalone and 1/5 LD<sub>50</sub> phosalone as compared to others.

In case of biomarker IL-6 $\beta$ , our study showed consistent finding and phosalone caused increase in IL-6 $\beta$  in all groups significantly as compared to normal and EA groups, whereas EA reversed its effect in all groups with significant change in group 1/3 LD<sub>50</sub> phosalone as compared to EA group. It is common belief that NF- $\kappa$ B shows its significant function in expression of various inflammatory mediators. NF- $\kappa$ B controls transcriptional activity involved in inflammatory and immune process *via* binding to specific DNA sequences in inflammatory genes<sup>[47]</sup>. In the same pattern, NF- $\kappa$ B was increased in all groups receiving phosalone with significant change. Contrary to this EA reduced its concentration in almost all groups with significant effect of group receiving 1/3 LD<sub>50</sub> phosalone. In parallel to our current study same effects of EA as anti-inflammatory agent has been observed in previous experiment<sup>[48]</sup>. It is clear from our results that how EA and phosalone target different biochemical pathways of toxicity. These distinct properties make NF- $\kappa$ B a promising target in novel treatment plans. There are new techniques that directly target NF- $\kappa$ B in inflammatory conditions including antioxidants, antisense DNA targeting, and proteasome inhibitors. Parallel to our study, a previous study also demonstrated that antioxidant effect may also give boost to anti-inflammatory actions<sup>[49]</sup>. However, EA's mechanism of actions to offset phosalone toxicity can be further studied regarding signaling pathways and gene expressions. Our research outcomes can give new directions, regarding novel treatment plans of colitis as well as awareness of phosalone toxicity in colon tissues.

Our data correlate well with the other studies and demonstrate that phosalone is among one of causative agents to induce colon inflammation and EA is an ideal antioxidant and anti-inflammatory compound in rat modeling studies which has extraordinary effects on

oxidant and inflammation systems. Anyhow, additional investigation for *in vivo* and human studies is required. It may indicate a new way toward the development of antioxidant therapy for colon inflammation.

## COMMENTS

### Background

Pesticides are chemical agents which are used to kill agricultural and domestic insects. Some of the pesticides are based on Organophosphorus (OP) compounds which are also harmful for human and can lead to early aging and cancer. Understanding the mechanism of action of OPs in human body is of prime importance in recent years. Such understanding will lead to finding the means to counteract the side effects resulted from OP exposure. Phosalone is an OP compound used in this study.

### Research frontiers

Prior researches have shown that OP exposure causes inflammation and oxidative stress in the body. The previous and on-going research efforts report serious damages to DNA, RNA and cell cycle due to OP agents.

### Innovations and breakthroughs

This research confirms the side effects of OP in colon cells in a rat model. Such side effects are the elevated level of inflammation and oxidative stress. The research results shows that among four dosages of phosalone, highest dosage leads to the most significant and serious level of inflammation and oxidative stress. To alleviate such deteriorative side effects, this research proposes utilizing ellagic acid (EA) which is a strong antioxidant. When rats were given EA along with phosalone, the level of inflammation and oxidative stress reduced significantly for the highest dose of phosalone.

### Applications

The results of this research can initiate appropriate warnings and precautions to all individuals including farmers who are exposed excessively to OP compounds. Such individuals can be directed to include EA in their diet through taking EA tablets or eating the fruits and vegetable which are rich source of antioxidants and EA like strawberries, grapes and green tea.

### Terminology

Reactive oxygen species (ROS) is a physiological process which happens when the body defense system gets triggered due to inflammation and oxidative stress. ROS leads to variety damages to DNA, RNA and cells.

### Peer-review

This study is very significant and interesting. The authors have done standard measurements of toxicity, and demonstrated EA can be used to reduce oxidative stress and regulate the level of inflammatory proteins. EA maybe a good candidate which can help treat and alleviate the side effects induced by OP compounds.

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