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Protective Effect of Dietary Antioxidants Curcumin, Vitamin C and Ginko Biloba on Oxidative Stress in Colonic Rats Induced by Butylated Hydroxyanisol

Fatma A.Khalil and Nahla H.Ali

Biochemistry and Nutrition Department, Women's College Ain Shams University, Cairo, Egypt.

Abstract: Butylated hydroxyanisole (BHA) has been shown to have positive and negative effects on the body.The present study aimed at to study the protective effect of dietary antioxidants curcumin, vitamin C and *Gingko biloba* on oxidative stress in colonic rats induced by BHA. Seventy rats were divided into seven groups each of 10 rats and treated for 6 weeks : group 1 served as normal control without any supplementation; group 2 fed on standard diet containing 2% BHA ; group 3 , 4 and 5 fed on BHA diet supplemented with dietary curcumin,, vitamin C, and *Gingko biloba* respectively; group 6 and 7 fed on BHA diet supplemented with, curcumin Plus vitamin C, *Ginko* plus vitamin C respectively. Results showed that administration of antioxidants caused a significant increase in reduced glutathione, total antioxidant, catalase activity as well as vitamin C while a significant decrease in malondialdehyde (MDA), nitric oxide, liver enzymes activities Moreover, a significant decrease in myeloperoxidase (MPO) activity and MDA in colonic rats. The histopathological features of BHA rats (untreated) included diffuse inflammatory cell infiltration in the mucosa. There was focal ulceration of the colonic mucosa extending through the muscularis mucosa in (Fig 1-B) but curcumin, vitamin C and *Ginko biloba* enhanced the tissue damage and produced the surface of mucosa without ulceration (Fig 1 –C, D and E). It was concluded that, the dietary antioxidants curcumin , vitamin C and *Ginko biloba* had beneficial effects on oxidative stress and toxicity which produced by BHA in rats . However, curcumin is the strongest antioxidant against toxicity and free radical in colon of rats.

Key words: Butylated hydroxyanisole, curcumin, vitamin C, *Ginko biloba,* oxidative stress, rats.

INTRODUCTION

Butylated hydroxyanisole (BHA) is a synthetic phenolic antioxidant that has been primarily used as a food preservative due to its chain – breaking action in the lipid preoxidiation (Tamura, 2010*)*. In contrast to its beneficial effects, BHA is also found to be toxic and even carcinogenic in some animal models. For example, oral administration of high doses of BHA has been shown to cause cytotoxicity and to increase the development of preneoplastic and neoplastic in rats, mice, hamsters and pigs (Yu, *et al.,* 2000).

Oxidative stress is well known as an inducer of cellular and tissue pathogenesis as well as contributor to the several diseases including cancer and inflammatory disorders carcinogensis and drug toxicity. Antioxidants can protect living organism from damage caused by the excessive production of free radicals and the concomitant lipid peroxidation (Ebrahimzadeh, *et al.,* 2010). Turmeric , commonly known as curcumin, *(Curcmma Longa)* is extensively used as a spice,food preservative and colouring material in India, China and South East Asia .It has been used in traditional medicine as a household remedy for various diseases (Chattopadhyay, *et al.,* 2004). The active constituents of turmeric rhizome are the curcuminoids (namely curcumin ,demethoxycurcmin, and bis–demethoxycurcumin) and volatile oils including turmerone sesquiphellanderene ,bisabolene and zingiberene.

Pharmacokinetic studies in animals demonstrate that 40-85 percent of an oral dose of curcumin passes through the gastrointesinal tract unchanged with the majority of the absorded flavonoid has being metabolized in the intestinal mucosa and liver (Sharma, *et al.,* 2001). The antioxidant activity of curcumin, it acts as a scavenger of oxygen free radicals like superoxide anions H_2O_2 and nitrite radical generation by activated macrophages, which play an important role in inflammation (Joe and Lokesh. 1994).

Vitamin C or ascorbic acid found in both animals and plants, vitamin C is the major water-soluble antioxidant within the body. The vitamin readily donates electrons to break the chain reaction of lipid peroxidation. The water-soluble properties of vitamin C allow for the quenching of free radicals before they reach the cellular membrane, it must be obtained from the diet . Moreover, a vitamin in cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protien disulfide isomerase. (Ortega, 2006).

Ginko biloba is one of the oldest herbal medicines that has been used as a *therapeutic* agent in modern pharmacology (Mustafa, *et al.,* 2006). Extracts of *Ginko* leaves contain both flavonoid and terpenoids constituents , which have anti-oxidants and anti –lipoperoxidative properties (Tesch, 2003) .*Ginko biloba* and its components such as quercetin and ginkolide may affect a number of cancers through many different

Corresponding Author: Fatma A.Khalil and Nahla H.AliBiochemistry and Nutrition Department,Women's College Ain Shams University, Cairo, Egypt.

pathways : increased antioxidant activity observed against cancer, inhibition of cell proliferation and induced cytotoxicity in liver cancer cells (Ye, *et al* ., 2007).

MATERIALS AND METHODS

Chemicals:

Butylated hydroxyanisole (BHA) tert-bytl-4-hydroxyanisal powder was purchased from Sigma Aldrich Company (ST Louis, Mo USA). Turmeric commonly knows as curcumin powder *(Curcuma Longa)* was obtained from the local market of Cairo, Egypt, vitamin C or ascorbic acid was obtained from the Epico Company of Egypt. *Ginko biloba* powder was purchased from pharmacy of Cairo, Egypt. All other chemicals used were for analytical pure grade.

Animals:

Seventy adult male Albino rats weighing about 140±9.2 g, were obtained from the breeding unit of the Egyptian organization for biological products and vaccines, Helwan, Egypt.

Diet:

Standard diet was prepared as nutrient requirement of laboratory animals (NRC, 1995). Composition of standard diet (g / kg diet) sucrose 500, (casein≥85% protein) 200, corn strach 150, corn oil 50, fiber source (cellulose type) 50, mineral mixture 35, vitamin mixture 10, DL methionine 3 and cholinne bitratrate 2.

Methods:

Experimental design:

All rats were housed in cages in a room at constant temperature 22± 1ºC with a relative humidity of 60±5 % and 12 h light / dark cycle. Animal housing conducted in accordance with the guide for the care and use of laboratory animals. The rats fed standard diet and water *ad libitum* for 7 days as an adaptation period before starting the experiment. Rats were divided into seven groups each of 10 rats and treated with 6 weeks.

Group (1): rats fed on standard diet without any supplementation (control group).

Group (2): rats fed on standard diet containing 2% BHA to induce oxidative damage and toxicity (Contoreggi, *et al.,* 1993).

Group (3): rats fed on standard diet containing 2% BHA and supplemented with 2% curcumin (Sharma, *et al.,* 2001).

Group (4): rats fed on standard diet containing 2% BHA and supplemented with 0.1 % vitamin C (LP, 1986).

Group (5): rats fed on standard diet containing 2% BHA and supplemented with 0.1 % *Ginko biloba* (Dias, *et al.,* 2008).

Group (6): rats fed on standard diet containing 2% BHA and supplemented with mixture of 2% curcumin plus 0.1 % vitamin C.

Group (7): rats fed on standard diet containing 2% BHA and supplemented with mixture of 0.1% *Ginko biloba* plus 0.1 % vitamin C.

After the end of the experimental period, all rats were fasted overnight and sacrificed using ether anesthesia. Blood samples were collected into two tubes, first tube contained anticoagulant as EDTA for determining the reduced glutathione (GSH) content in blood. The second tube contained no anticoagulant to obtain the serum and stored at - 80ºC for further biochemical analysis.

Biochemical analysis:

Reduced glutathione content (GSH) was measured in blood by the method of (Beutler, *et al.,* 1963), serum total antioxidants capacity (TAC) were measured by the method of described by Erel (2004), serum catalase (CAT) activity was measured by the method of (Goth., 1991),serum vitamin C was determined according to (Kyaw, 1978). Serum malodialdehyde (MDA) and nitric oxide (NO) levels were estimated by the methods of (Draper and Hadley, 1990; Miranda, *et al*. 2001), respectively. Serum aspartate amino transferase (AST) alanine amino transferase (ALT) and alkaline phosphatase (ALP) activities assayed by the methods of (Henry, *et al.,* 1960; Rosalki, *et al.,* 1993), respectively. Serum creatinine and urea were determined using the methods described by (Hinegard and Tiderstrom, 1973; Patton and Crouch, 1977), respectively.

Collection and examination of colon specimen:

Samples from distal colon were removed and opened longitudinally, after washing in ice –cold phosphate buffered saline (Pbs), they were placed in filter papers .The colon specimen were divided into 2 sections, the first section was stored at -80ºC for further biochemical analysis to determine myeloperoxidase (MPO) activity by the method of (Krawisz, *et al.,* 1984) , GSH content and MDA levels were determined in homogenate colon . The other section used for histopathological examination.

Histopathological examination:

Samples from distal colon fixed in 10% formal saline prior to wax embedding. Sectioning and staining with haematoxylin and eosin for histological evaluation of colonic damage by light microscopy (Drug and Wallington, 1980).

Statistical analysis:

All data expressed as mean ±standard deviation (SD) for ten rats per experimental group. Statistical analysis performed with SPSS11.0 one-way analysis of variance (ANOVA) was used to compare the mean values quantitative variables among the groups. Duncan´s multiple range tests was used to identify the significance of pair wise comparisons of mean values among the groups, The least significant difference (L.S.D) and $p < 0.05$ were considered to be statistically significant (levesque, 2007).

Results:

Results presented in table 1 showed a significant decrease in total antioxidants capacity (TAC) reduced glutathione (GSH), vitamin C and catalase activity (CAT) ,while a significant increase in malondialdehyde (MDA) and nitric oxide (NO) in rats were fed on BHA alone without treatment as compared to control group ($p< 0.05$) . Moreover, the dietary antioxidants curcumin, vitamin C and *Ginko biloba* for 42 days enhanced the antioxidant status and reduced the lipid peroxide (MDA).

The results in table 2 indicated that BHA at 2% in the diet caused oxidative stress and toxicity in liver and kidney evidenced by a significant increase in Serum AST, ALT and ALP activities as well as urea and creatinine levels in rats fed on BHA without treatment as compared to control group ($p< 0.05$). On the other hand, the dietary antioxidants curcmin, vitamin C and *Ginkgo biloba* for 42 days were significantly decreased liver enzyme activities and enhanced the kidney function. In table, 3 the results revealed that oxidative damage associated with an increase MPO activity as marker of neutrophilic in filtration accompanied by a significant increase of MDA level and decrease GSH concentration in colonic rats were fed on BHA alone as compared to control group. However, the dietary antioxidants protect the colonic tissue from oxidative damage and toxicity induced by BHA at 2%.

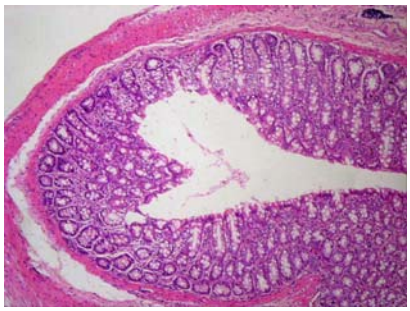
Table 1: Effect of curcumin, vitamin C and *Ginko biloba* on serum antioxidant status in toxicated rats by 2% BHA

Parameters Groups	Total antioxidant mM/L	GSH mg /dl	vitamin c mg/ L	catalase activity U/L	MDA nmol/ml	NO µ mol / l
Group (1)	2.3±0.08 ^{f}	24±1.6^{a}	57.5±0.95^{b}	877±10.31^{a}	1.62±0.09^{f}	4.5±0.2^{c}
Group (2)	1.6±0.03 ^{g}	8±0.81^{e}	12±0.81^{e}	375±12.9^{g}	4.5±0.08^{a}	14±0.81^{a}
Group (3)	2.76±0.04^{a}	20±1.7^{b}	30±1.4^{a}	775±12.9^{b}	2.5±0.08^{c}	5.7±0.35^{bc}
Group (4)	2.35±0.05^{e}	14.5±1.2^{c}	27±1.1^{b}	712.5±15^{c}	1.9±0.08^{e}	5±0.17^{c}
Group (5)	2.46±0.04^{d}	12±0.8^{d}	18±0.81^{c}	625±12.9^{d}	2.3±0.09^{d}	8±0.47^{b}
Group (6)	2.57±0.06^{c}	16±1.2^{c}	27.5±0.95^{b}	452.5±9.5^{e}	2±0.05^{e}	6±0.12^{b}
Group (7)	2.60±0.02^{b}	11.5±0.95^{d}	14.5±1.29^{d}	432.5±9.57^{f}	2.77±0.09^{f}	7.3±0.32^{b}
L.S.D	0.02	1.82	1.51	17.63	0.14	2.2

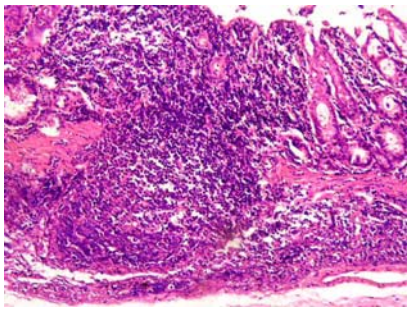
Values are mean ± SD for ten rats.
Means within the same column have a different superscript letters are significantly different ($p < 0.05$).

Histopathological results:

The histopathological features of BHA rats (untreated) included diffuse inflammatory cell infiltration in the mucosa. There was focal ulceration of the colonic mucosa extending through the muscularis mucosa in (Fig 1-B) but curcumin, vitamin C and *Ginko biloba* enhanced the tissue damage and produced the surface of mucosa without ulceration (Fig 1 –C, D and E).



(A)



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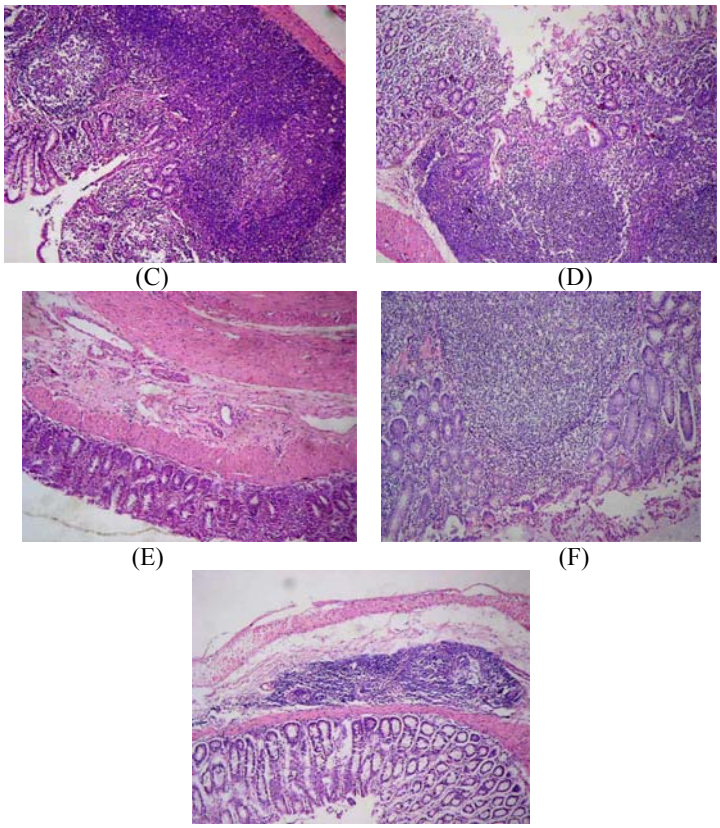

Fig. 1: (A) normal control group, (B) BHA group , (C,D and E) curcumin, vitamin C and *ginko biloba* treated groups respectively, (F,G), curcumin plus vitamin C, *ginko biloba* plus vitamin C treated groups, respectively (H&E ×100).

Table 2: Effect of curcumin, vitamin C and *Ginko biloba* on serum liver and kidney functions in toxicated rats by 2% BHA

Parameters Groups	AST IU/L	ALT IU/L	Al p IU/ L	Urea mg/ dl	Creatinine mg /dl
Group (1)	37.5 ±0.57^{f}	56±1.6^{f}	247.5 ±7.5^{d}	17.5±1.2^{e}	0.85±0.03^{e}
Group (2)	73.2±0.95^{a}	84±1.6^{a}	514±21.1^{a}	43.75 ±2.6^{a}	2.7 ±0.09^{a}
Group (3)	47.5±2^{e}	55±1.15^{f}	157.5±9.1^{f}	22.25 ±1.7^{d}	0.9±0.01^{de}
Group (4)	64.5±0.57^{b}	61.5±1.9^{d}	264 ±19.63^{d}	31.2±0.95^{b}	0.92 ± 0.02^{d}
Group (5)	64 ±1.4^{b}	71 ±1.15^{b}	347.7±11^{b}	33.5± 1.2^{b}	1.2 ±0.09^{c}
Group (6)	55.7±0.95^{d}	59±1.15^{e}	183 ±8.9^{e}	32 ±0.8^{b}	0.97 ±0.02^{d}
Group (7)	60.75±0.95^{c}	67.5±1.9^{c}	289.5 ±3 ^{c}	27.5±1.2^{e}	1.3 ±0.07^{b}
L.S.D	1.7	2.19	19.8	2.19	0.06

Values are mean ± SD for ten rats.
Means within the same column have a different superscript letters are significantly different ($p < 0.05$).

Table 3: Effect of curcumin, vitamin C and *Ginko biloba* on reduced glutathione (GSH), malondialdehyde (MDA) and myeloperoxidase activity (MPO) in toxicated rats by 2% BHA

Parameters Groups	GSH (content) mg/g tissue	MDA (level) n mol/mg tissue	MPO activity u/ g tissue
Group (1) (control)	92.5±4.11^{d}	15.5±0.57^{g}	16.3 ±0.41^{g}
Group (2) (BHA)	60±4.08 ^{f}	36.25 ±1.89^{a}	39 ± 2.1^{a}
Group (3) (BHA+curcumin)	138.7 ±8.5^{a}	20.75 ±0.95^{e}	22.2 ±0.8^{e}
Group (4) (BHA+vitamin C)	138.7 ±8.5^{a}	18.5 ±0.57^{f}	20.3 ± 0.9^{f}
Group (5) (BHA+*Ginko biloba*)	112.7 ± 3.2^{c}	26.75 ±0.95^{c}	24.5 ±1.5^{d}
Group (6) (BHA+curcumin+vitamin C)	86 ±4.7^{d}	23.5 ±0.57^{d}	26.2 ± 1.4^{c}
Group (7) (BHA+*Ginko* + vitamin C)	82 ±2.4^{e}	28.5 ± 0.57^{b}	28.3 ±0.7^{b}
L.S.D	7.17	1.43	1.6

Values are mean ± SD for ten rats.
Means within the same column have a different superscript letters are significantly different ($p < 0.05$).

Discussion:

Butylated hydroxyanisole (BHA), a commonly used food preservative, but BHA may exert toxic effect in some tissue of animals. (Tamura, 2010) reported that BHA induced to apoptosis appeared to be independed of formation of reactive intermediates, as evidenced by the lack of effects of antioxidants, direct incubation of BHA with isolated mitochondrial triggered cytochrome c release. (Schilderman, *et al.,* 1995) found that, BHA appeared to be a strong inducer of oxidative DNA damage in the epithelial cells in all of the tissue.

BHA was metabolized by cytochrome p 450 s or monooxygenase to tertiary butylhydroquinone (TBHq). Formation of TBHq initiates redo x – cycling resulting in the production of reactive oxygen species (Yu *et al.,* 1997). Rats' administration 500-600 mg / Kg bw of BHA for 10 weeks, resulted in decrease the growth rate and catalase activity (Madhavi *et al.,* 1996). BHA at levels 1 and 1.3% induced liver enlargement, proliferation of the smooth endoplasmic reticulum the formation of hepatic myelinoid bodies, and increase in hepatic enzyme activities (Contoreggi, *et al.,* 1993).

Alkaline phosphatase (ALP) is another enzyme, which has reported to be a sensitive biochemical marker of intestinal inflammation. Colonic inflammations that characterized by a significant oxidative stress and neutrophil infiltration resulted in an increase in ALP activity (Gonzalez, *et al.,* 2001).

The present investigation showed that the supplementation of dietary antioxidants significantly ameliorated the changes in biochemical and histopathological parameters in rats fed on BHA induced toxicity .The most changes pronounced in rats fed on BHA and supplemented with curcumin. Curcurmin (diferuloylmethane; {1,7 bis (4 hydroxy – 3 methoxyphenyl)- 1,6 heptadiene – 3,5 dione}) a major naturally occurring phenolic obtained from the plant *Curcuma longa.* As medicine, curcumin is shown to exhibit antioxidant , anti-inflammatory, antiviral , antibacterial ,antifungal and anticancer activities (Campbell and Collett, 2005) and thus has a potential to against various diseases including diabetes , asthma , allergies , arthritis , atheroscalerosis , neurodegenerative diseases and other chronic illnesses like cancers (Duvoix, *et al.,* 2005) . Curcumin is a stronger antioxidant inhibitor of lipid peroxidation than other flavonoide , which have a single phenolic hydroxyl group (Phan, *et al*., 2001).

Administration of curcumin reversed the changes induced by BHA supporting the hypothesis that plant products are effective antioxidative agent curcumin by scavenging or neutralizing free radical. Interacting with oxidative cascade,quenching oxygen, inhibiting oxidative enzymes like cytochrome p 450 and by cleating metal ions like Fe^{2+} inhibit peroxidation of membrane lipids and maintains cell membrane integrity and their function (Pulla and Lokesh, 1994).

Previous study has reported that curcumin is a potent inducer of detoxifying enzymes and thereby prevents the toxicity induced by chemicals carcinogen (Singletary *et al.,* 1998).

Dietary curcumin at level of 0.2 % could thus provide a useful component of dietary or pharmacological treatment and reduction of the incidence of mortality from gastrointestinal or colorectal cancer (Campbell and Collett, 2005).

Numerous studies have performed on the biotransformation of curcumin Lin *et al.* (2000) showed that metabolites of curcumin in mice was first biotransformed to dihydrocurcmin and tetrahydrocurcmin and that these compounds subsequently were converted to monoglucuronide conjugated. Thus curcumin glucuronide, dihydrocurcumin-glucuronide, tetrahydrocurcumin- glucronide are major metabolites of curcumin.

Devasena *et al.* (2002) examined the protective effect of a curcumin analog {bis -1,7 (2-hydroxyphenyl – hepta-1,6 diene 3,5 dione) }on hepatic lipid peroxidation and antioxidants status during 1,2 dimethyl hydrazine –induced colon carcinogensis in male wister rats . They observed curcumin analog modulating hepatic biotransformation enzymes and antioxidant status it may be due to the hydroxyl group in the aromatic ring is responsible for the protective effect rather than the methoxyphenyl group.

Vitamin C is an important antioxidant in extracellular fluid and inhibits the peroxidation of unsaturated lipids by scavenging or quenching free radical. Vitamin C may prevent certain type of oxidative damage produced by infiltrating macrophages and neutrophils within the inflamed colon (Zerin, *et al.,* 2010).

An extract of the leaves the *Ginko biloba* a mixture mainly composed of flavonoid glycoside and terpenoides (Ginkgolide and bilobalide) has showed to exhibit a variety of phamcological actions. The leaf extract acts as a scavenger of reactive oxygen species (pener, *et al.,* 2005).

The tissue damage produced by neutrophils and macrophages have been attributed to their ability to release ROS, nitrogen metabolites, cytotoxic proteins, lytic enzymes, and cytokines, as well as their negative effects on epithelial integrity. (Zerin, *et al*., 2010) .

In the present study, the tissue damage produced by high level of MDA, low content of GSH and increased the activity of myeloperoxidase (MPO) in colon rats fed on BHA without supplementation. Measurement of MPO activity has used as an indicator of neutrophil influx into inflamed gastrointesinal tissue (Mustafa, *et al.,* 2006).

Several investigators have demonstrated increased neutrolphil infiltration inflammatory might be regarded as a trigger of free radicals release which may exert toxic effects on fatty acid residuces in membrane lipid, increase in reactive oxygen species production and impaired antioxidant defense mechanism are postulated to be

causative factor in inflammatory diseases (Han and Meyhani, 2000). While, supplementation of rats with curcumin, vitamin C and *Ginko biloba* resulted in decrease MDA level and MPO activity and increase GSH level in the present study. Radical may explain this by scavenging effect of hydroxyl radical and superoxide anions other investigators have demonstrated that previous antioxidant stop lipoperoxidative by quenching the peroxyl radical (Dumont, *et al.,* 1992).

In this study, an inflammatory response characterized by mucosal ulceration and heavy neutrophil infiltration of the mucosa and sub mucosa (Fig 1–B). On the other hand treatment with curcumin, vitamin C and *ginko biloba* respectively resulted in (Fig 1–C,D,E) attenuation of tissue damage and reduction in cell infiltration. The results agreement with (Jian, *et al.,* 2005) studied that, 2% curcumin prevents and cures intestinal mucosal inflammation , the components of turmeric inhibit mediators of inflammation as NF-Kappa B, cyelooxgenase–2 (COX -2) lipooxygenase (LOX) and inducible nitric oxide synthase (iNos).

In conclusion, the results of the present study finding show that dietary antioxidants curcumin , vitamin C and *Ginko biloba* had beneficial effects on oxidative stress and toxicity which produced by BHA in rats . However, curcumin is the strongest antioxidant against toxicity and free radical in colon of rats.

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