

L-ARGININE AUGMENTS THE ANTIOXIDANT EFFECT OF GARLIC AGAINST ACETIC ACID -INDUCED ULCERATIVE COLITIS IN RATS

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ABSTRACT

Garlic contains many sulfhydryl compounds that act as antioxidants. However, the role of nitric oxide (NO) in inflammation is controversial. The aim of the present study is to investigate the possible protective effect of garlic against acetic acid-induced ulcerative colitis in rats, as well as the probable modulatory effect of L-arginine (NO precursor) on garlic activity. Intra-rectal inoculation of rats with 4% acetic acid for 3 consecutive days caused a significant increase in the colon weight and marked decrease in the colon length. In addition, acetic acid induced a significant increase in serum levels of nitrate as well as colonic tissue content of malondialdehyde (MDA). Moreover, colonic tissue contents of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were markedly reduced. On the other hand, pre-treatment of rats with garlic (0.25g/kgbw, orally) for 4 consecutive weeks and 3 days during induction of colitis significantly reduced the increase in the colon weight induced by acetic acid and ameliorated alterations in oxidant and antioxidant parameters. Interestingly, oral co-administration of garlic (0.25g/kgbw) and L-arginine (625mg/kgbw) for the same period of garlic administration mitigated the changes in both colon weight and length induced by acetic acid and increased garlic effect on colon tissue contents of MDA and GSH. In conclusion, L-arginine can augment the protective effect of garlic against ulcerative colitis; an effect that might be mainly attributed to its NO donating property resulting in enhancement of garlic antioxidant effect. Further studies will be needed to determine which one of the active ingredients of garlic has the main antioxidant effect to be used with L-arginine.

Keywords: Garlic; L-arginine; colitis, antioxidant.

INTRODUCTION

Ulcerative colitis is a chronic inflammatory disease of the colon and rectum. The exact cause remains unknown. Possible etiological factors, including genetic, immunological, reactive oxygen species (ROS) and environmental factors have been implicated (Kirsner and Shorter, 1982; Jewell and Patel, 1985; Millar *et al.*, 1996). The main sources of ROS in the inflamed mucosa are activated leukocytes and neutrophils which are capable of producing superoxide and cascade of various reactive species leading to the formation of reactive hydroxyl radicals and peroxides. These products cause impairment in cell membrane function via lipid peroxidation in inflammatory bowel disease. Those intermediate products of oxygen metabolism (superoxide, hydroxyl radicals and H₂O₂) are controlled by various cellular enzymes including superoxide dismutase (SOD), catalase (CAT) and non-enzymes as reduced glutathione (GSH) (Buffinton and Doe, 1995; Furrice *et al.*, 2004).

There is substantial evidence for the involvement of oxidative stress and profound alterations in the

biosynthesis of the labile free radical nitric oxide (NO) from L-arginine in the pathogenesis of colitis (Myers *et al.*, 1997; Aguilar-Nascimento *et al.*, 1999; Barbosa *et al.*, 2003; La *et al.*, 2003). NO is believed to participate in the regulation of the oxidation/reduction potential of various cells and may be involved in either the protection against or the induction of oxidative stress within various tissues depending on its concentration. Emerging evidence suggests that some diseases are related to either an inadequate or excessive production of NO (Moncada and Higgs, 1993).

The use of medicinal plants or their active components has become an increasingly attractive approach for the treatment of ulcerative colitis. Garlic as an antioxidant, has shown an inhibitory effect on lipid peroxidation and can enhance induction of endogenous antioxidants in rat kidney and liver (Banerjee *et al.*, 2001; Khanum *et al.*, 2004).

Hence, this work was designed to study the possible protective effect of garlic against ulcerative colitis induced by acetic acid in rats, as well as the probable modulatory effect of L-arginine (NO precursor) on garlic activity.

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MATERIALS AND METHODS

Chemicals

Acetic acid was obtained from Merck (Germany). L-arginine, reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), Ellman's reagent [5,5-dithio-bis(2-nitrobenzoic acid)], H₂O₂, thiobarbituric acid (TBA), NADPH, FAD, nitrate reductase, L-arginine, magnesium stearate and 1,1,3,3-tetraethoxy-propane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Avicel PH 200 was supplied from FMC Corporation, Philadelphia, USA. All other chemicals were of the highest available commercial grade. For the preparation of fresh garlic homogenate, garlic bulbs were purchased from a local supermarket.

Instruments and apparatus

1. Tablet Compressing Machine Carver Press, Carver Inc., Wabash (USA).
2. High Shear Blender: Hamilton Beach, Washington, NC, USA.
3. Beckman XL-70 ultracentrifuge, 100,000 rpm (USA).
4. Spectrophotometer Shimadzu (UV-1201 UV-VIS) (Japan).
5. Centrifuge Sigma 3K 20, up to 10,000g (Germany).
6. Homogenizer: Janke and Kunkel IKA 8,000-20.500 RPM (Germany).

Preparation of garlic formulation

The concept of "liquisolid" compacts is based on formulating liquid medications such as oily liquid drugs and solutions or suspensions of water-insoluble solid drugs carried in suitable nonvolatile solvent systems, termed the liquid vehicles, into acceptably flowing and compressible powders. Simple blending of such liquid medications with selected powder excipients, referred to as the carrier and coating materials can yield dry-looking, nonadherent, free flowing, and readily compressible powders. Various grades of cellulose, starch, lactose, etc., may be used as carriers, whereas very fine particle size silica powders may be used as coating materials. Utilizing a similar approach, Pather *et al.* (2001) introduced a solid dosage form based on a microemulsion adsorbed onto colloidal silicon dioxide and microcrystalline cellulose.

In solid solutions and "liquisolid" compacts, however, large quantities of silica often exceeding the limits were compressed into tablets; they presented significant "liquid-squeezing out" phenomena and unacceptably soft tablets, thereby hampering the industrial application of such systems (Spireas *et al.*, 1992).

Two hundred grams of peeled garlic bulbs were chopped into small pieces and small amount of water was added. The mixture was homogenized using a motor homogenizer till a fine and smooth paste was produced.

Avicel powder was added portion by portion to the paste with continued mixing till the garlic paste was converted into a solid powder. The flowability of the garlic powder formulation has been improved by addition of 3% magnesium stearate powder. In order to increase the density of the garlic formulation, it was compressed into tablets using tablets press and then the prepared tablets were crushed into small granules by milling.

Animals

Male Sprague-Dawley rats, weighing 220-250 g were obtained from National Institute of Cancer (Cairo) and housed (4/each cage) in the animal facility, Pharmacology & Toxicology Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt for 15 days before the experimentation. Animals were fed on standard diet pellets (El-Nasr Co., Abou-Zaabal, Egypt) and maintained under a 12 h light/ 12 h dark cycle at a constant temperature (22 ± 2°C), with food and water *ad libitum*.

Induction of ulcerative colitis and garlic and L-arginine treatment

Ulcerative colitis was induced in rats by intracolonic instillation of 2 ml/day of 4% acetic acid in saline for three days. Six cm of soft pediatric catheter size 6F was introduced into the anus of rats and acetic acid was carefully administered. Acetic acid was spread in the colon by injection of 2 ml air before taking out the catheter (Choudhary *et al.*, 2001). All rats were kept under light ether anaesthesia during the experiment. Garlic formulation was given orally in a dose of 0.25 g/kgbw (Pal *et al.*, 2006), while L-arginine was administered i.p. in a dose of 625 mg/kgbw (Nader *et al.*, 2007) for 4 weeks before induction of colitis and for 3 successive days during induction of colitis.

Experimental pharmacological protocol

Forty rats were randomly divided into 4 groups (8 animals in each group) as follows:

- Group 1: treated orally and intrarectally with saline and served as control group.
- Group 2: inoculated orally with saline and intrarectally with 4% acetic acid and served as acetic acid induced -colitis group.
- Group 3: treated orally with garlic formulation alone in a dose of 0.25g/kgbw for 4 weeks before induction of colitis and for 3 successive days during induction of colitis using acetic acid.
- Group 4: treated with garlic formulation, orally in a dose of 0.25g/kgbw and L-arginine, i.p., in a dose of 625 mg/kgbw, followed by acetic acid for the same duration period as mentioned before in group 3.

Table 1: Effect of garlic and garlic + L-arginine on acetic acid-induced changes in the colon weight and length

Parameters	Control	Colitis	Garlic	Garlic+ L-arginine
Colon weight (gm)	1.50 ± 0.08	2.31 ± 0.09 ^a	1.74 ± 0.07 ^b	1.60 ± 0.10 ^b
Colon length (cm)	17.2 ± 0.67	12.9 ± 0.72 ^a	15.00 ± 0.46	16.25 ± 0.62 ^b

Data are represented as means ± SEM of 8 rats per group.

a: Significantly different from control group; b: Significantly different from acetic acid-induced colitis group at $P \leq 0.05$ using one-way ANOVA, followed by Tukey-Kramer test.

Table 2: Effect of garlic and garlic + L-arginine on the colon contents of MDA, GSH, SOD & CAT and serum nitrate level in acetic acid-induced ulcerative colitis in rats

Parameters	Control	Colitis	Garlic	Garlic + L-arginine
MDA(nmol/mg protein)	8.55 +0.50	30.23 +1.52 ^a	11.83 +0.93 ^b	6.12 +0.32 ^{b,c}
Serum nitrate(μ mol/L)	16.48 +1.38	43.66 +2.28 ^a	13.82 +0.99 ^b	18.35 +1.33 ^{b,c}
GSH (umol/gm protein)	153.38 ± 5.59	36.19 ± 1.98 ^a	111.74 ± 5.21 ^{a,b}	136.73 ± 5.30 ^{b,c}
SOD (U/mg protein)	6.03 ± 0.91	1.03 ± 0.91 ^a	5.09 ± 0.31 ^b	6.66 ± 1.25 ^b
CAT (U/mg protein)	26.47 ± 2.12	10.33 ± 1.08 ^a	21.09 ± 1.50 ^b	27.30 ± 1.71 ^b

Data are represented as means ± SEM of 8 rats per group.

^a Significantly different versus control group at $P \leq 0.05$

^b Significantly different versus acetic acid-induced colitis group at $P \leq 0.05$

^c Significantly different versus acetic acid-induced colitis group treated with garlic alone at $P \leq 0.05$ using one-way ANOVA, followed by Tukey-Kramer test.

Blood samples

All rats were allowed to fast for overnight of the day before sampling. Blood samples were drawn from the retro-orbital veins, collected into centrifuge tubes and left to stand at room temperature for 10 min and then centrifuged at 3000 rpm for 10 min. The separated serum samples were stored at -20°C for determination of nitrate.

Tissue samples

Animals were sacrificed by cervical dislocation under deep ether anaesthesia 24 h after the last treatment. Abdomens were opened using a surgical scissor. Colon was removed via laparotomy, opened longitudinally and washed three times with cold saline solution and used for biochemical analysis. The colons weights and lengths were measured. Then, colons were homogenized with 10 ml of ice-cold 0.25 M sucrose using homogenizer and centrifuged at 14,000 rpm for 20 min for estimating the biochemical parameters (Cetinkaya *et al.*, 2005).

Biochemical studies

Determination of tissue protein concentration

The protein concentration of tissue samples was measured according to the method of Lowry *et al.* (1951).

Determination of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was determined as MDA by estimation of the tissue content of thiobarbituric acid reactive substances (TBARS) in the colon homogenates according to Mihara and Uchiyama (1978). A standard curve for

MDA was constructed using different concentrations of 1, 1, 3, 3-tetraethoxypropane in water. The experiment was performed by mixing tissue homogenate, thiobarbituric acid (TBA) and H_3PO_4 in a boiling water bath for 45 min. TBA adducts were extracted with n-butanol and measured spectrophotometrically at 535 nm.

Determination of reduced glutathione (GSH)

GSH contents in colon homogenates were determined as described by Ellman (1959). The method depends on reduction of Ellman's reagent by SH groups (GSH) to form 5, 5-dithio-2-nitrobenzoic acid (DTNB) in phosphate buffer, pH 8. The produced yellow color was measured spectrophotometrically at 412 nm. Results were expressed as μmol/g protein.

Determination of superoxide dismutase (SOD) activity

Estimation of SOD activity in the homogenate supernatant was based on inhibition of pyrogallol auto-oxidation as described by Marklund (1985). Samples were measured spectrophotometrically at absorbance 420 nm at 1 min interval for 5 min. SOD activity was determined from a standard curve. Data were expressed as U/mg protein.

Determination of Catalase activity

CAT activity was measured in the colon homogenate as described by Siveski-Iliskovic and Kaul (1994). The method is based on the measurement of the decrease in hydrogen peroxide concentration at 240 nm over a 5 min period using a spectrophotometer. The amount of CAT

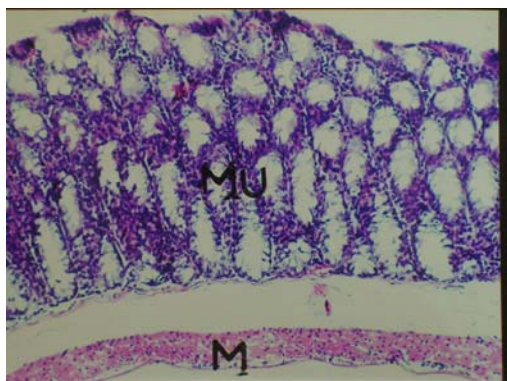


Fig. 1: Colon section of rats in control group showing the normal histological structure of the mucosal layer and *Lamina propria* (Mu) with underlying muscularis (M).
H & E x 40

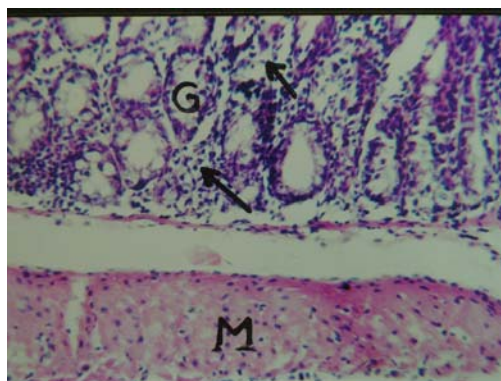


Fig. 2: Colon section of rats in acetic acid-induced colitis group showing diffuse goblet cells formation in mucosal epithelium (G) with oedema and diffuse inflammatory cells infiltration in *Lamina propria* (arrow) and hypertrophy in the muscularis (M).
H & E x 64

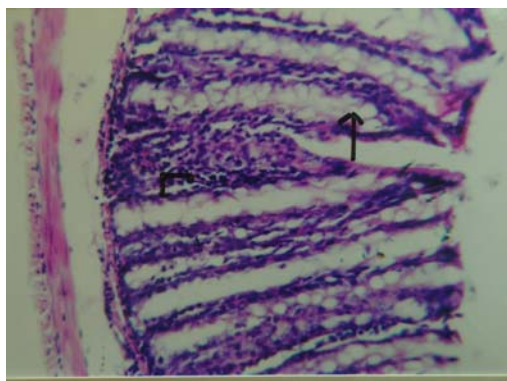


Fig. 3: Colon section of rats in acetic acid-induced colitis group pretreated with garlic showing goblet cells formation in the mucosal epithelium (arrow) with no apparent oedema.
H & E x 64

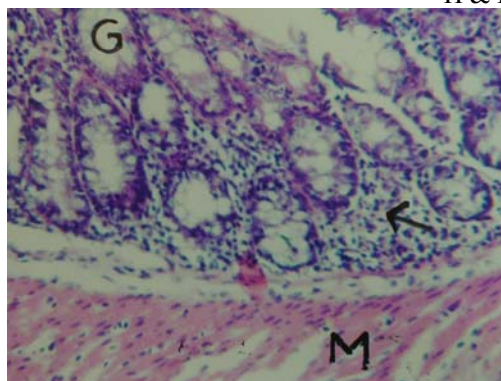


Fig. 4: Colon section of rats in acetic acid-induced colitis group pretreated with garlic and L-arginine showing inflammatory cells infiltration in *Lamina propria* (arrow) and muscular hypertrophy (M) with no apparent oedema.
H & E x 64

required to decompose 1 μmol of H_2O_2 per min at pH 7 at 25°C was defined as 1 unit of activity. Colon CAT activity was measured from a standard curve and enzyme activity was represented as U/mg protein.

Determination of serum nitrate level

An enzymatic end point method was used for determination of nitrate in serum using nitrate reductase method (Bories and Bories, 1995). NADPH reduces nitrate to nitrite and itself is oxidized to NADP. The decrease in NADPH is directly proportional to nitrate concentration when measured at 340 nm.

Histopathological examination

Tissue specimens were taken from the colons of each of the 4 groups of rats and then fixed in 10% formalin saline solution for 24 hours. Trimming was done on the fixed tissue specimens and washed in tap water for 12 hours. Serial alcohols (methyl, ethyl and absolute) were used for

dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 3 micron thickness by slide microtome. The obtained tissue sections were collected on glass slides and stained by heamatoxylin and eosin for histopathological examination by the light microscope (Banchrof *et al.*, 1996).

Statistical analysis of data

The data are expressed as means \pm SEM. Analysis of results was performed using one-way ANOVA, followed by Tukey-Kramer test for multiple comparisons. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Intra-rectal administration of 4 % acetic acid in rats significantly increased the colon weight and markedly decreased the colon length amounting to 54% and 25%,

respectively. On the other hand, administration of garlic formulation alone had only a decreasing effect on the increase in the colon weight induced by acetic acid recording 25%. However, co-administration of garlic+L-arginine resulted in both reduction of the increased colon weight and elevation in the decreased colon length induced by acetic acid amounting to 31% and 26%, respectively (table 1).

Interestingly, the rat colon content of MDA was markedly increased in acetic acid- treated group (254%) compared to the control normal group. On the other hand, there were significant decreases in MDA colon contents in groups treated with garlic alone by about 61 % and a combination of garlic + L-arginine by about 80 % as compared to acetic acid-induced colitis group (table 2).

In addition, serum nitrate level was significantly increased in acetic acid-induced colitis group (165%) when compared to the control group. The degrees of decreases in serum nitrate levels from the colitis group were 68 % and 58% in groups treated with garlic alone and garlic + L-arginine, respectively (table 2).

Moreover, there was a significant decrease in GSH colon content in colitis group (76%) compared to the control group. Pretreatment of rats with garlic alone and a combination of garlic and L-arginine significantly elevated GSH contents (209%&279%, respectively) as compared to the colitis group. Hence, GSH colon content in garlic +L-arginine treated group was significantly higher than that in the group treated with garlic alone (table 2).

On the other hand, SOD activity in colonic tissues was significantly decreased in acetic acid-induced colitis group (83%) compared to the control group. In contrast, there were significant elevations in SOD activities in groups treated with garlic (394%) and a combination of garlic + L-arginine (547%) compared to the colitis group (table 2).

Table 2 also indicated that acetic acid treatment significantly decreased CAT colonic activity by about 61% as compared to control group. On the other hand, garlic and a combination of garlic + L-arginine significantly increased CAT activities amounting to 104%&164%, respectively as compared to acetic acid -treated group.

The data illustrated in figs. 1 and 2 revealed that the colon section taken from acetic acid- treated rats showed diffuse goblet cells formation in mucosal epithelium with oedema and diffuse inflammatory cells infiltration in *Lamina propria* and hypertrophy in the muscularis. On the other hand, the colon section taken from acetic acid-induced colitis group pretreated with garlic formulation

demonstrated goblet cells formation in the mucosal epithelium with no apparent oedema. In addition, the colon section taken from acetic acid-induced colitis group pretreated with garlic and L-arginine showed inflammatory cells infiltration in *Lamina propria* and muscular hypertrophy with no apparent oedema formation (figs. 3 and 4).

DISCUSSION

The production of free radicals and alteration of antioxidant status is well established in ulcerative colitis (Ma *et al.*, 1998a&b). Our results revealed a significant increase in the colonic tissue content of MDA in acetic acid-induced colitis group compared to the control group and this is in accordance with the previous reports illustrating that treatment with acetic acid leads to increases in xanthine oxidase activity, platelet aggregating factor formation and ROS generation (Deliconstantinos and Villiotou, 2000, Galunskam *et al.*, 2002). In addition, there was a significant increase in serum NO₃ levels in ulcerative colitis group and this is in harmony with other studies revealing that up-regulation of inducible nitric oxide synthase occurs in ulcerative colitis associated with excessive generation of NO (Perner *et al.*, 2001). Similar studies indicated that a high nitrate level appears to be secondary to the magnitude of inflammation (Lundberg *et al.*, 1997).

Regarding the colon tissue content of GSH, the results of the present work are in accordance with the previous observations with significant reduced values of GSH in ulcerative colitis group (Hassan *et al.*, 1998). The tissue CAT activity was also decreased significantly in colitis group compared to the control group. These results cope with the finding of Galunskam *et al.* (2002). Moreover, our study showed that SOD activity significantly decreased in ulcerative colitis group and these data were in agreement with several observations demonstrating the decrease in SOD activity under the effect of different sorts of stress (Deliconstantinos and Villiotou, 2000).

Our study demonstrated that administration of garlic formulation alone or co-administration of garlic and L-arginine ameliorated alterations induced by acetic acid in serum nitrate as well as colon tissue contents of MDA, GSH, SOD and CAT, to reach the normal range of control group. These results are in agreement with a previous study which reported that garlic is able to inhibit lipids peroxidation (Kenjiro *et al.*, 2001) as garlic contains diallyl sulfides which significantly decrease MDA content and increase antioxidant enzymes activity (Tsao *et al.*, 2003; Kempaiah and Srinivasan, 2004; Vimal and Devaki, 2004). There are several studies revealing that allicin and ajoene, main constituents of garlic, inhibit platelets aggregation and migration and inhibit ROS generation (Apitz-Castro *et al.*, 1983 and 1991; Batirel *et al.*,

2002). Also, it was shown that Na-(1-deoxy-D-fructos-1-yl)-L-(Fru-Arg) was identified as a major antioxidant compound in garlic. The hydrogen peroxide scavenging activity of Fru-Arg was comparable to that of ascorbic acid, suggesting that it could contribute to the pharmacologic effects of garlic through its antioxidant properties (Kenjiro *et al.*, 2001). In addition, S-allyl-L-cysteine and S-allylmercapto-L-cysteine were proposed to be responsible for the antioxidative activity of garlic formulation (Ingrid and Jacques, 2006). Several other studies have reported that garlic contains a high amount of γ -glutamyl-S-alk(en)yl-L-cysteines and trace elements such as selenium (Se) which have antioxidant effects (Ingrid and Jacques, 2006). The major Se compounds in garlic are Se-methylselenocysteine and gamma-glutamyl-Se-methylselenocysteine (Auger *et al.*, 2004; Ogra and Suzuki, 2005).

Interestingly, our results revealed that co-administration of L-arginine with garlic causes significant augmentation in garlic effect on the colon MDA and GSH contents. These results are in agreement with other study reporting that L-arginine decreases plasma xanthine oxidase activity and inhibits lipid peroxidation (White *et al.*, 2004). The protective effect of L-arginine (NO precursor) can be explained by restoration/and or increase in NO production by quenching the release of superoxide anion from the endothelium, thus reducing oxidative stress (Jovanovic *et al.*, 1998; Jessup *et al.*, 1999; Jung *et al.*, 2000). NO is produced from L-arginine by nitric oxide synthase (NOS) enzyme (Mansour *et al.*, 2003). NO can protect the cells against the effect of ROS (Kelley *et al.*, 1999).

Histopathological investigation of colon section taken from acetic acid-treated rats showed diffuse goblet cells formation in mucosal epithelium with oedema and diffuse inflammatory cells infiltration in *Lamina propria* and hypertrophy in the muscularis and therefore, such study confirmed the biological evidence of oxidative stress induced by acetic acid. In addition, acetic acid-induced increase in the colon weight and reduction in the colon length were measures of inflammation in the colon. On the other hand, administration of garlic as well as garlic and L-arginine produced amelioration of histopathological changes induced by acetic acid confirming protection by such agents. In addition, pretreatment with garlic and L-arginine augmented garlic effect evidenced by reduction in the elevated colon weight as well as increase in the reduced colon length.

Finally, we concluded that garlic could have a valuable protective effect against ulcerative colitis due to its antioxidant constituents. In addition, L-arginine can augment the antioxidant effect of garlic against colitis, possibly due to its NO donating property. Further studies will be needed in future to explore the main active ingredient of garlic having a more antioxidant effect to be

used concomitantly with L-arginine as a prophylactic or preventive regimen against ulcerative colitis.

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