Crocus sativus L. protects against SDS-induced intestinal damage and extends lifespan in *Drosophila melanogaster*

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Abstract. Medicinal plants are important sources of potentially therapeutic biochemical drugs. *Crocus sativus* L. has been used to treat various diseases in China, the Republic of Korea and Japan. The present study investigated the protective effect of *C. sativus* L. extract in *Drosophila melanogaster* intestinal immunity. Wild-type flies were fed standard cornmeal-yeast medium and used as controls, and flies supplemented with 1% *C. sativus* L. aqueous extract in standard medium were used as the experimental group. Following the ingestion of the various toxic compounds, the survival rate of the flies was determined. Cell viability and levels of reactive oxygen species (ROS) were detected using 7-amino-actinomycin D and dihydroethidium staining, respectively. The present study demonstrated that aqueous extracts of *C. sativus* L. may significantly increase the lifespan and survival rate of adult flies. Additionally, *C.* sativus L. may decrease epithelial cell death and ROS levels, resulting in improved intestinal morphology. These findings indicated that *C. sativus* L. had a protective effect against intestinal injury and may extend the lifespan of *Drosophila*. Therefore, the findings of the present study may improve the understanding of clinical researchers on the complex effects of *C. sativus* L. in intestinal disorders.

Introduction

The intestinal epithelium is frequently exposed to various pathogens and toxic compounds. Therefore, an efficient and powerful immune system is required (1). The intestinal tract of *Drosophila melanogaster* is structurally and functionally similar to that of mammals (2). Signaling mechanisms that control epithelial regeneration, innate immunity and inflammation are evolutionarily conserved. *Drosophila* is a simple and powerful model that is frequently used to investigate the signaling events of intestinal homeostasis. The *Drosophila* midgut maintains a balance between immune suppression against indigenous intestinal flora and a robust immune response against invading microbes (3). The generation of antimicrobial peptides (AMPs) and production of reactive oxygen species (ROS) are two important microbicidal systems that may inhibit infection by external pathogens (4,5). The local production of AMPs is important for the inducible defense mechanisms of intestinal immunity, which are triggered by the immune deficiency pathway (6,7). The production of ROS by the NADPH oxidase and dual oxidase 1 is a complementary mechanism for host defense against pathogens (7). ROS are able to disrupt DNA, RNA and proteins of external pathogens, and of degrading lipids in their cell membrane. Furthermore, ROS may stimulate intestinal stem cell (ISC) proliferation (8). However, excessive levels of ROS may lead to cytotoxicity, cancer, degenerative diseases of aging, and damage the host intestinal epithelial cells. Therefore, a balance between the production and removal of ROS is essential for host health (5). ISCs equivalent to those in the mammalian intestine have been identified in the *Drosophila* midgut (9). In order to maintain intestinal homeostasis, the rate of stem cell division is increased in response to stressful conditions, which may be induced by pathogens, toxic compounds or aging (10,11). Dysfunctional intestinal cell turnover may lead to compromised tissue integrity or cancer (12). Additionally, various toxic compounds, such as sodium dodecyl sulfate (SDS), dextran sulfate sodium (DSS) and paraquat, may affect intestinal homeostasis by inducing a stress response that may lead to damage and apoptosis of epithelial cells (8,13).

Medicinal plants are an important source of potentially therapeutic chemical substances. *C. sativus* L. is a traditional medicine, also termed as saffron, that consists of crocin, croetin, safranal and picrocrocin (14). It has been previously used in traditional Chinese, Korean and Japanese medicine to treat spasms, bronchospasm, liver disease, pain, insomnia and digestive ailments. It has also been used as a stimulant and for supportive treatment of cancer, including lung cancer (15). Previous studies have identified the anti-inflammatory (16), anti-nociceptive (17), antimicrobial (18), antioxidant (19) and immunomodulatory effects of *C. sativus* L. extract with high efficacy and low toxicity (20). However, the protective effect of *C. sativus* L. against intestinal damage, and the underlying mechanism of action, remain to be elucidated.

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The present study investigated the protective effect of *C. sativus* L. extract against intestinal damage by using *Drosophila* as a model system to analyze the lifespan and intestinal homeostasis following the ingestion of toxic compounds. It was demonstrated that *C. sativus* L. may significantly increase the lifespan of *Drosophila*. In addition, aqueous extracts of *C. sativus* L. may significantly increase the survival rate of *Drosophila* following treatment with SDS, DSS and paraquat. Normal adult intestinal morphology was observed in the *C. sativus* L.-fed group following exposure to SDS. This may be due to decreased levels of ROS and reduced epithelial cell death. These findings may improve the understanding of clinical researchers on the complex function of *C. sativus* L. in intestinal disorders. Further investigation is required to determine which components of *C. sativus* L. exhibit the protective effects observed in the present study.

Materials and methods

Drosophila strains. Wild-type w^{1118} flies (*Drosophila melanogaster*) were obtained from the Bloomington Drosophila Stock Centre (Bloomington, IN, USA), and reared on a standard cornmeal-yeast medium at 25˚C and 60% humidity under a 12/12-h light/dark cycle.

Aqueous extracts of C. sativus L. and growth medium of Drosophila. The dried stigmas of *C. sativus* L. were obtained from the Qinghai-Tibet plateau of China in 2014 and were identified by Professor Xiuhua Wang (Northeast Forestry University, China). Aqueous extracts were obtained as previously described (21). *C. sativus* L. (2 g) were immersed in 100 ml deionized water overnight at 25˚C. The aqueous extraction was boiled for 3 h, and the extraction process was repeated twice. Total extracts were mixed and concentrated to 50 ml. *Drosophila* that were fed a standard cornmeal-yeast medium were used as the control group, and those fed the standard medium containing 1% extract of *C. sativus* L. were used as the experimental group.

Quantitative analysis of Crocin I. Crocin I (Chengdu Must Bio-Technology Co., Ltd., Chengdu, China) was used as a reference standard for the quality control of the *C. sativus* L. extracts. High-performance liquid chromatography (HPLC) analysis was performed as previously described (22). Briefly, the HPLC system (LC-20AT, Shimadzu Corporation, Kyoto, Japan) consisted of a quaternary pump, C_{18} gravity column (Alltima C_{18}; 250x4.6 mm, 5 μm) and HPD-20A detector. The mobile phase was 15% methanol in water at a flow rate of 1.0 ml/min and detector wavelength of 275 nm.

Lifespan analysis. The lifespan of the *Drosophila* was determined at 25˚C and 60% humidity with a 12/12-h light/dark cycle. Newly enclosed adult flies were collected within a 24-h period, and the flies were allowed to mate for 48 h. A total of 30 adult flies were cultured and transferred to new vials containing fresh food every 2-3 days. The surviving flies were counted every day, and each experiment was repeated three times.

Survival experiments. The 3-to 5-day old adult flies (15 males and 15 females) were starved for 2 h prior to being transferred to a vial containing 5 layers of filter paper hydrated with 5% sucrose (w/v) with toxic compounds, including 0.6% SDS (w/v; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 6 mM paraquat (Sigma-Aldrich; Merck Millipore) or 4% DSS (w/v; MP Biomedicals, LLC, Santa Ana, CA, USA). Sucrose (5%) solution with no additives was used as the positive control for all experiments. Filter papers were changed every day, and the survival rate was monitored daily, as mentioned above.

7-Amino-actinomycin D (7-AAD) staining. Adult Drosophila were orally exposed to 0.6% SDS and incubated at 25˚C for 96 h. A total of 10-15 dissected fly intestines were stained with 7-AAD (5 μg/ml in PBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min at room temperature, fixed for 30 min in PBS with 4% paraformaldehyde, and then stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, as previously described (21). The intestines were mounted using mounting medium [70% glycerol in PBS containing 2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO); Sigma-Aldrich; Merck Millipore), and images were captured using an Axioskop 2 Plus microscope (Carl Zeiss AG, Oberkochen, Germany). The presented data are from three independent experiments.

ROS detection. Adult females were orally exposed to 1% SDS and incubated at 25˚C for 48 h. A total of 10-15 intestines were dissected in ice-cold PBS and incubated in dihydroethidium (DHE; 5 μM in PBS; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature, then rinsed 4 times in PBS for 5 min each, fixed in PBS with 4% paraformaldehyde for 20 min, and then stained with DAPI for 10 min. Intestines were then rinsed 4 times with PBS and mounted using mounting medium (70% glycerol in PBS containing 2.5% DABCO; Sigma-Aldrich, Merck Millipore), then analyzed with an Axioskop 2 Plus microscope (Carl Zeiss AG). The data presented are from three independent experiments.

Statistical analysis. Statistical analysis was performed using a two-tailed unpaired Student's t-test with Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.001$ was considered to indicate a statistically significant difference. Data are presented as the mean ± standard error.

Results

Identification of Crocin I in C. sativus L. aqueous extracts using HPLC. HPLC analysis was used for the identification and quantification of Crocin I in the previously collected *C. sativus* L. aqueous extracts. The chromatogram of the standard was eluted at a retention time of 9.3 min, and it was determined that the *C. sativus* L. aqueous extracts that were collected previously contained 0.181% Crocin I (Fig. 1)

C. sativus L. extends the lifespan of adult flies. Aging is a multifaceted process associated with a gradual decline in physiological function, which leads to serious diseases, including cancer and diabetes (23). A previous study demonstrated that various traditional plants and their extracts contain high levels of phytochemicals, which are capable of extending survival and preventing or delaying age-associated diseases (24). *Drosophila* fed with the standard medium with 1% extract of *C. sativus* L.

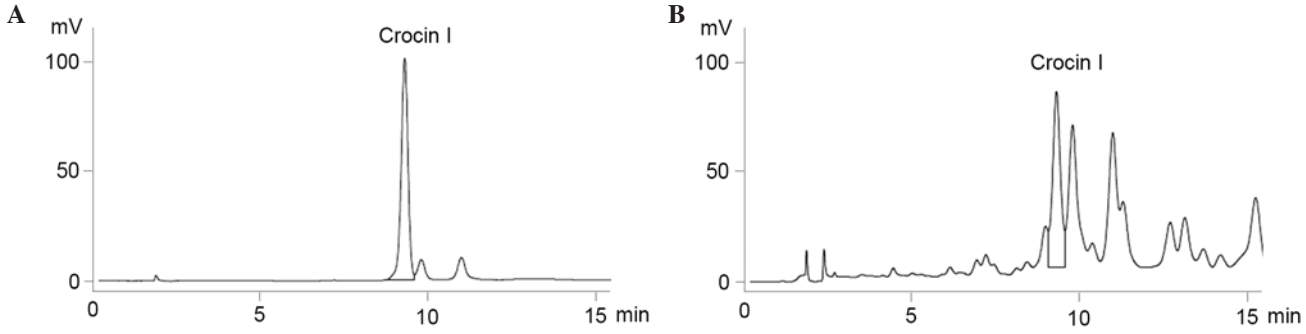

Figure 1. Identification of Crocin I in *C. sativus* L. aqueous extracts by high-pressure liquid chromatography chromatographs. (A) Reference; (B) aqueous extracts.

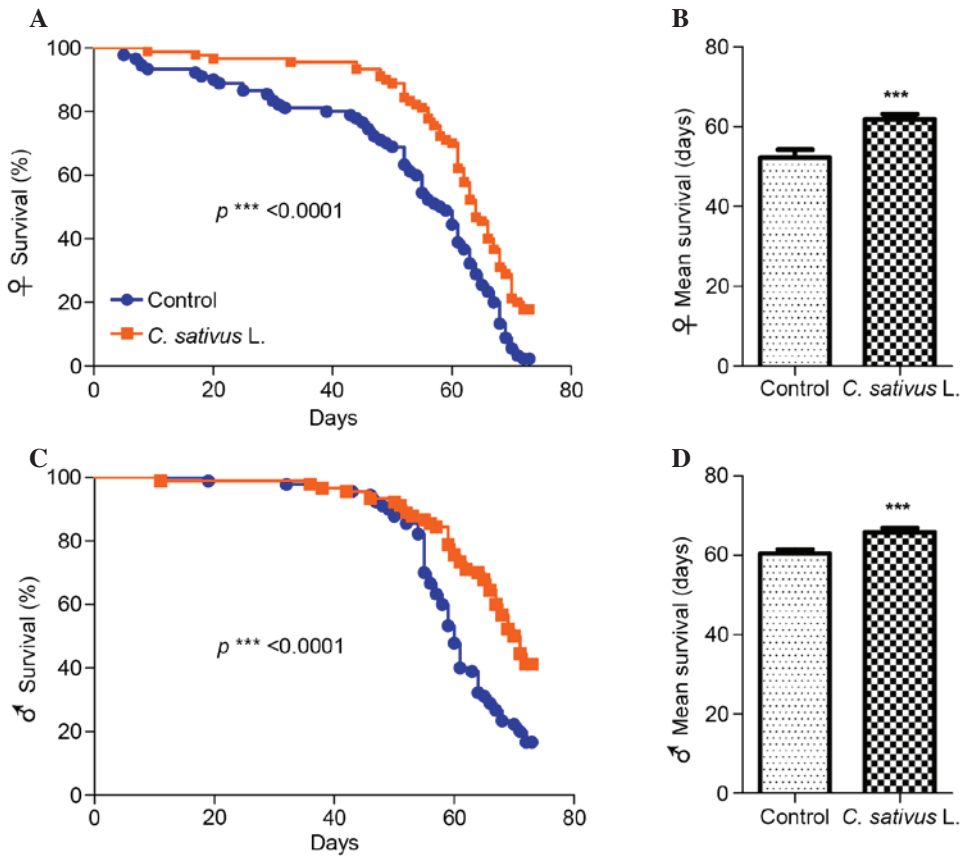

Figure 2. *C. sativus* L. may extend the *Drosophila* lifespan. (A) Maximum and (B) mean lifespan of females fed with the standard diet or a diet supplemented with 1% *C. sativus* L. extract. (C) Maximum and (D) mean lifespan of males fed with the standard diet or a diet supplemented with 1% *C. sativus* L. extract The results are shown from three independent experiments, with a total of 90 flies used in each lifespan assay. ***P<0.0001, Control vs. *C. sativus* L.

were used as the experimental group (Fig. 2). It was determined that significant increases in the maximum lifespan of female and male flies occurred in the group fed with the *C. sativus* L. extract compared with the control group (P<0.0001; Fig. 2A and C). In addition, the mean lifespan increased by 18.3% in females and 8.8% in males (Fig. 2B and D). These findings suggest that *C. sativus* L. extract may promote longevity and increase the mean lifespan, and that the magnitude of this increase may be associated with gender.

C. sativus L. extract increases the survival rate following the ingestion of toxic compounds. The intestinal epithelium is susceptible to pathogen damage, oxidative stress and toxic compounds. In order to determine the protective effect of *C. sativus* L. extract, adult flies from both culture conditions (the control and experimental groups) were orally treated with the inflammatory reagent, SDS or DSS. These chemicals may interfere with the normal function of the intestinal barrier and stimulate local and systemic inflammation. This may result in tissue damage and melanotic phenotypes in the adult *Drosophila* intestine (8,10). As presented in Fig. 3A and B, survival rates were significantly increased in the *C. sativus* L. feeding groups compared with the control group, following exposure to SDS or DSS for 6-7 days (P<0.001). The survival rate in the treatment groups fed with *C. sativus* L. extract were 35.5 and 67.7% for SDS and DSS, respectively, which

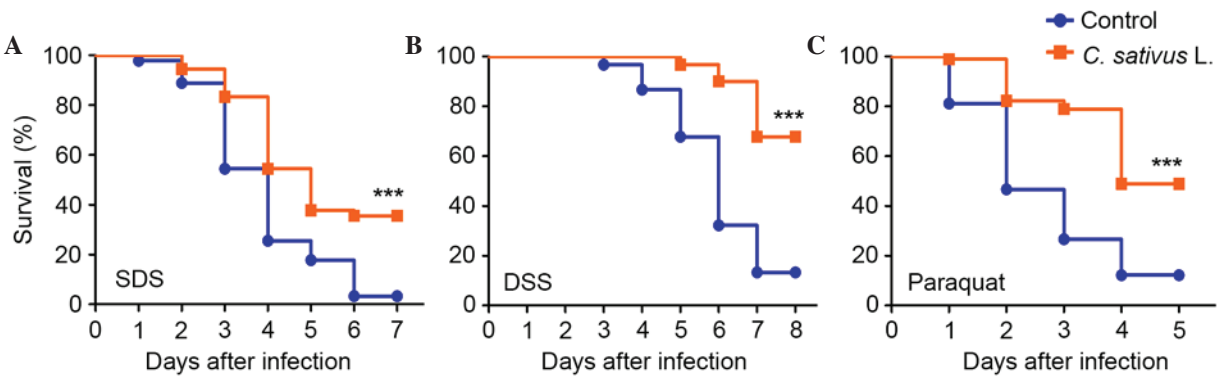

Figure 3. Survival rates of the control and *C. sativus* L. feeding groups following treatment with SDS, DSS or Paraquat. Adult flies in the control and *C. sativus* L. feeding group were treated with 5% sucrose containing (A) 0.6% SDS, (B) 4% DSS or (C) 6 mM paraquat at 25˚C for 4-7 days. The survival curves were derived from three independent experiments. The control contained standard medium, whereas the *C. sativus* L. group had standard medium containing 1% extract of *C. sativus* L. ***P<0.001, Control vs. *C. sativus* L. SDS, sodium dodecyl sulfate; DSS, dextran sulfate sodium.

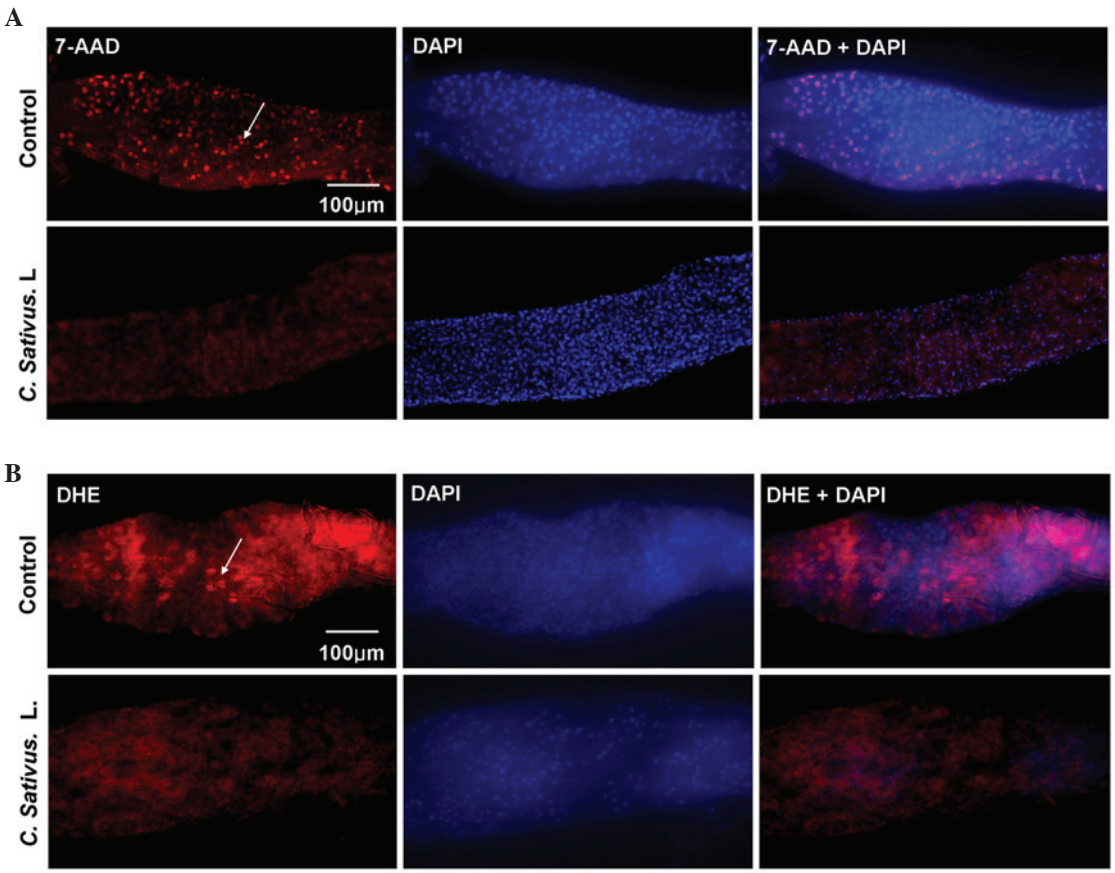

Figure 4. *C. sativus* L. extracts protect against SDS-induced epithelial cell damage. (A) The 7-AAD staining of mid guts of female flies following ingestion of SDS (0.6%, w/v) for 96 h. (B) DHE staining of mid guts of female flies following ingestion of SDS (1%, w/v) for 48 h. The control contained standard cornmeal-yeast medium, whereas the *C. sativus* L. group had a standard medium containing 1% *C. sativus* L. extracts (w/v). The results represent at least three independent experiments. 7-AAD, dead cells (red); DHE, reactive oxygen species levels (red); DAPI, nucleus (blue). 7-AAD, 7-amino-actinomycin D; DHE, dihydroethidium; DAPI, 4',6-diamidino-2-phenylindole.

were significantly higher compared with the survival rates of the control group (3.3 and 13.2%, respectively). Following ingestion of an ROS-producing agent, paraquat, for 4 days, the *C. sativus* L. feeding groups exhibited significantly higher survival rates (48.9%) compared with the control group (P<0.001; Fig. 3C). This survival rate was >36.7% compared with the control group (12.2%; Fig. 3C). These findings indicated that *C. sativus* L. extract may increase the *Drosophila* survival rate following oral infection with SDS, DSS and paraquat. Therefore, *C. sativus L.* may contribute to the resistance of infection due to pro-inflammatory reagents.

C. sativus L. extract protects the adult intestine against SDS-induced epithelial cell damage. The ingestion of toxic

compounds, such as SDS, has been identified to induce epithelial cell damage (8). To determine whether the reduced survival rate of adult flies resulted from increased cell death in response to SDS treatment, adult flies in the control and *C. sativus* L. feeding groups were treated with 0.6% SDS for 96 h. Subsequently cell viability of intestinal epithelial cells was detected using 7-AAD staining, which is a type of nucleic acid dye that is capable of passing through the plasma membrane and combining with the DNA of the dead cells. An increase in the number of dead cells was observed in the control group, whereas only a few dead cells were detected in the experimental group (Fig. 4A).

Various stresses may lead to the production of excessive ROS, which may then cause oxidative damage, and ultimately cell death. This depends on the maintenance of an equilibrium between ROS production and scavenging. Bandegi *et al* (25) have proposed that the *C. sativus* L. extract and its active constituent, Crocin I, may prevent chronic stress-induced oxidative stress damage to the brain, liver and kidneys in rats (25). The present study determined whether treatment with *C. sativus* L. eliminated the excessive ROS levels in the *Drosophila* intestine. DHE, a redox sensitive dye that intercalates into cellular DNA when oxidized (26), was used to quantify ROS levels. Adult flies were exposed to SDS for 48 h. DHE fluorescence was reduced in the posterior midgut of the *C. sativus* L.-fed group compared with the control (Fig. 4B). These findings demonstrated that *C. sativus* L. extract may maintain the host redox homeostasis following SDS exposure, therefore protecting against damage of intestinal epithelial cells.

C. sativus L. extract maintains Drosophila intestinal morphology following SDS ingestion. A previous study revealed that SDS may induce melanotic tumors and morphological changes in the *Drosophila* intestine (21). Following treatment with 0.6% SDS for 4 days, the intestinal length of the control group was observably shorter than the *C. sativus* L.-fed group. Additionally, melanotic tumors were observed in the posterior midgut of the control group. No melanotic masses were observed in the *C. sativus* L.-fed group (Fig. 5). These findings indicated that increased epithelial cell death may induce morphological changes in the adult *Drosophila* intestine.

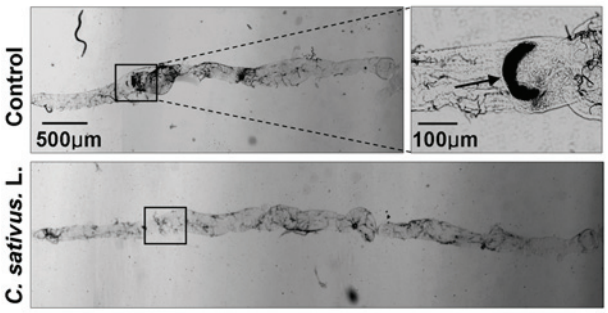

Figure 5. *C. sativus* L. extract protects the morphological change of the intestine against SDS-induced damage. Nomarski images of the adult gut following ingestion of 0.6% SDS (w/v, containing 5% sucrose) for 96 h. The arrow indicates the melanotic mass in the magnified image within the square box for the control group. All experiments were independently performed three times.

Discussion

The intestinal epithelium in the majority of animals undergoes rapid regeneration under homeostatic conditions and in response to tissue damage (3). The generation of ROS and local production of AMPs are two complementary effector mechanisms of controlling pathogen infection in the *Drosophila* intestine (4). In addition, nutritional status and tissue damage may influence stem cell turnover rates. The loss of intestinal homeostasis is critical for inflammatory diseases of the intestine, and may influence overall health and lifespan (27).

C. sativus L., also termed saffron, is a small perennial plant from the Iridaceae family, which is widely cultivated, and its stigma is used medicinally. *Drosophila* flies frequently inhabit decaying and fermenting matter; therefore, they are exposed to numerous bacteria, fungi and viruses, which they must defend themselves against. The simplicity of the structure and the multipotency of the *Drosophila* posterior midgut make it a suitable model for investigating adult epithelial cell homeostasis and regeneration. The present study investigated the protective effect of *C. sativus* L in intestinal immunity using *Drosophila* as a model system to analyze lifespan and intestinal homeostasis following the ingestion of toxic compounds. Significant increases in the survival rate of *Drosophila* following SDS, DSS and paraquat treatment were observed in the *C. sativus* L. feeding group compared with the control group. This may be due to the elimination of excess ROS by treatment with *C. sativus* L., therefore decreasing epithelial cell death and improving intestinal morphology. In order for intestinal homeostasis to be maintained, the rate of stem cell division is increased in response to intestinal damage (8). However, 1% *C. sativus* L. extract did not significantly change the proliferation of stem cells (data not shown). Therefore, the mechanism by which *C. sativus L.* protects the intestine may be due to a decrease in epithelial cell death.

Aerobic metabolism, with the concomitant generation of ROS, remains the most widely accepted cause of aging. The present study determined higher survival rates in the *C. sativus* L. feeding group following treatment with ROS-producing agents, such as paraquat. Furthermore, the maximum and mean lifespan was significantly increased in female and male adult flies (Fig. 2). These findings indicated that *C. sativus* L. extracts may eliminate ROS in the *Drosophila* intestine, thus protecting adult flies. A previous study determined that *C. sativus* L. and its active constituent, Corcin I, may exert a protective effect against chronic stress-induced oxidative damage of the brain, liver and kidneys in rats (23). Furthermore, safranal (an organic compound isolated from saffron) may be effective in protecting the susceptible brains of aged rats from oxidative damage by increasing antioxidant defenses (28). Further investigation is required to determine the active components that exert protective effects on the *Drosophila* intestine.

In conclusion, the findings of the present study revealed the preventive effect of *C. sativus* L. on *Drosophila* intestinal damage, and its ability to increase lifespan. A possible mechanism to account for these findings may be that the active constituent exerts anti-inflammatory and anti-oxidant effects.

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