Zanthoxylum fruit extract from Japanese pepper promotes autophagic cell death in cancer cells

SUPPLEMENTARY FIGURES

Supplementary Figure S1: Effects of ZFE on morphology and proliferation of human colon cancer cells. a. Effect of ZFE on the morphology of the indicated cells. Cells were incubated with 200 μg/ml of ZFE or 0.2% v/v DMSO (control) for 24 h. Scale bars, 50 μm. b. Cells were incubated with 200 μg/ml of ZFE or 0.2% v/v DMSO (control) for the indicated time and cell viability was measured using a cell proliferation assay kit. Data are the mean ± SD of three independent experiments. *p < 0.05 vs. control at 48 h. ††p < 0.01, vs. control at 72 h (Student’s t-test). Ctrl; control.
Supplementary Figure S2: Knockdown of ATG5 protein inhibits the anticancer effect of ZFE in DLD-1 cells. 

a. DLD-1 cells were transfected with scrambled siRNA or ATG5 siRNA #2 (10 nM final concentration) or subjected to transfection in the absence of siRNA (Mock). Twenty-four hours after transfection, RNAs were extracted, and quantitative RT-PCR was performed to measure knockdown efficiency. Fold-changes in ATG5 mRNA levels were calculated by the ΔΔCt method using GAPDH as a reference gene. Error bars represent S.D. of mean values (n=3). **p < 0.01 vs. control (Dunnett’s test).

b. DLD-1 cells were transfected with scrambled siRNA or ATG5 siRNA #2 (10 nM final concentration) or subjected to transfection in the absence of siRNA (Mock). Twenty-four hours after transfection, cell lysates were subjected to Western blotting with the indicated antibodies. Similar results were obtained in three independent experiments.

c. Effect of ZFE on the morphology of ATG5 knockdown DLD-1 cells. Transfected DLD-1 cells were incubated with 200 μg/ml of ZFE for 24 h. Scale bars, 50 μm.

d. DLD-1 cells were transfected with scrambled siRNA or ATG5 siRNA #2 (10 nM final concentration) or subjected to transfection in the absence of siRNA (Mock). Twenty-four hours after transfection, the cells were harvested and treated with 200 μg/ml of ZFE or 0.2% v/v DMSO (control) for the indicated time. Cell viability was measured using a cell proliferation assay kit. Error bars represent S.D. of mean values (n=3). **p < 0.01 at 72 h (Student’s t-test). Ctrl; control.
Supplementary Figure S3: Effects of ZFE on phosphorylation of JNK in intestinal cells. 

a. Cells were treated with 200 μg/ml of ZFE for the indicated time. The cell lysates were prepared and subjected to Western blotting with the indicated antibodies. Similar results were obtained in three independent experiments.

b. IEC-6 cells were treated with 200 μg/ml of ZFE for the indicated time. The cell lysates were prepared and subjected to Western blotting with the indicated antibodies. Similar results were obtained in three independent experiments.
Supplementary Figure S4: Sanshools have no anticancer activity in DLD-1 cells. 

**a.** Effect of sanshools (mixture of HAS and HBS at the same concentration) on the morphology of DLD-1 cells. Cells were incubated with 38 μM of sanshools or 0.2% v/v DMSO (control) for 24 h. Scale bars, 50 μm. 

**b.** DLD-1 cells were treated with 38 μM of sanshools or 0.2% v/v DMSO (control) for the indicated time. The cell lysates were prepared and subjected to Western blotting with the indicated antibodies. Similar results were obtained in three independent experiments. 

**c.** DLD-1 cells were incubated with 38 μM of sanshools or 0.2% v/v DMSO (control) for the indicated time and cell viability was measured using a cell proliferation assay kit. Data are the mean ± SD of three independent experiments. Ctrl; control.