

Striatisorolide A, a butenolide metabolite from *Athyrium multidentatum* (Doll.) Ching, as a potential antibacterial agent

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Abstract. The present study aimed to investigate the antibacterial activity of striatisorolide A (SA) against *Escherichia coli* (*E. coli*) and the underlying mechanism. Antibacterial activity was evaluated according to the inhibitory rate and zone of inhibition. The antibacterial mechanism was investigated by analyzing alkaline phosphatase (AKP) activity and ATP leakage, protein expression, cell morphology and intracellular alterations in *E. coli*. The results demonstrated that SA exerted bacteriostatic effects on *E. coli in vitro*. AKP activity and ATP leakage analysis revealed that SA damaged the cell wall and cell membrane of *E. coli*. SDS-PAGE analysis indicated that SA notably altered the level of 10 and 35 kDa proteins. Scanning electron microscopy and transmission electron microscopy analyses revealed marked alterations in the morphology and ultrastructure of *E. coli* following treatment with SA. The mechanism underlying the antimicrobial effects of SA against *E. coli* may be attributed to its actions of disrupting the cell membrane and cell wall and regulation of protein level. The findings of the present study provide novel insight into the antimicrobial activity of SA as a potential natural antibacterial agent.

Introduction

Microbial contamination is a global issue associated with illnesses caused by the ingestion of food contaminated with mycotoxins, which have adverse effects on humans, animals and crops (1). Additionally, multi-drug-resistant pathogens, including strains of *Escherichia coli*, *Staphylococcus aureus*

and *Candida albicans*, have been reported to arise from the increased use of antibiotics in recent years (2). A report on the antimicrobial susceptibility profile revealed that *E. coli* showed high rates of resistance to commonly used antibiotics, such as ampicillin and septrin, and relatively lower resistance rates to amoxicillin/clavulanate, chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin and ceftriaxone (3). Annually, numerous mortalities have been caused by infection with these 'superbugs' worldwide; such infection has become one of the most important problems affecting public health (4,5). Certain synthetic antimicrobial compounds, including nitrites, sulfites and butylated hydroxyanisole, are commonly used in processed foods; however, with safety concerns, considerable interest has arisen in identifying alternative natural antimicrobial agents (6). Plant extracts, including esters, peptides and phenolics, may be sources of natural antimicrobial molecules with potential to eliminate antibiotic-resistant microorganisms. Nisin, a non-toxic natural preservative from lactic acid bacteria strains, is used in the food industry worldwide (7). Numerous mechanisms are involved in the antimicrobial actions of these natural molecules, including damaging the cell membrane and/or cell wall, and inhibiting the synthesis of intracellular substances, including DNA, proteins and enzymes.

Athyrium multidentatum (Doll.) Ching (AMC) belongs to the Athyriaceae family and grows in shady and damp environments, including mountains and forests. It is usually used for treating parasitic infections, anxiety and arthritis in the Changbai Mountains region of China (8); AMC is also a nutritious potherb. The rhizome of AMC is rich in numerous nutritional components, including amino acids, starch and polysaccharides. In our previous study, striatisorolide A (SA) was extracted from the rhizomes of AMC (9). SA is regarded as a butenolide derivative due to its four-carbon heterocyclic ring skeleton of 2 (3H) furanone. This compound is produced by few types of fungi and bacterial strains (10,11). Studies have demonstrated that SA exerted a weak cytotoxic effect on A549 cells, but exhibited notable cytoprotective activity on human umbilical vein endothelial cells (11,12); the antibacterial activity of SA requires further investigation.

The purpose of this study was to investigate the antibacterial activity of SA and the underlying mechanism. On the basis of our preliminary experiments, the present study selected

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E. coli ATCC 8739 as the experimental strain for analysis. The antibacterial potential of SA was determined according to the rate and zone of inhibition. The abundance of alkaline phosphatase (AKP) and ATP was detected to assess the effects of SA on the cell wall and membrane of *E. coli* ATCC 8739. The effects of SA on protein expression, bacterial morphology and intracellular structure of *E. coli* were also determined to investigate the underlying mechanisms.

Materials and methods

Materials. Tryptic soy broth (TSB) was purchased from Hope Bio-Technology Co., Ltd.; 2,3,5-triphenyltetrazolium chloride (TTC), a bicinchoninic acid (BCA) protein assay kit, a pre-stained protein ladder (11-180 kDa), PBS and DMSO ($\geq 99.5\%$) were obtained from Beijing Solarbio Science & Technology Co., Ltd. Luria-Bertani (LB) broth, AKP, ATP and the SDS-PAGE gel quick preparation kit were obtained from Beyotime Institute of Biotechnology. Agar was purchased from neoFroxx GmbH. Ciprofloxacin hydrochloride (CX) was acquired from Jingxin Pharmaceutical Co., Ltd. AMC rhizomes were harvested in the Changbai Mountain area of China in 2017 and stored in a dry and ventilated room.

Preparation of samples. SA was extracted from the rhizomes of AMC as described previously (9). Briefly, the ground AMC rhizomes were immersed in methanol at room temperature for 20 days twice, then filtered. The resultant leach liquor was concentrated under reduced pressure, and successively separated with petroleum ether and ethyl acetate. The ethyl acetate extract was subjected to silica gel-column chromatography and eluted with petroleum ether/ethyl acetate (4:1, v/v) to obtain SA. Finally, the purified SA was dissolved in DMSO and filtered with a 0.22- μm bacteriological filter. CX was used as the positive control. CX was dissolved in normal saline for 10 min via sonication and filtered with a 0.22- μm bacteriological filter. All samples were stored at 4°C for the following assays.

Bacterial cultures. The standard strain of *E. coli* (Migula) Castellani and Chalmers (cat. no. ATCC 8739) was procured from Beijing Zhongyuan Ltd., which was maintained on an LB agar plate supplemented with 1.5% agar, 1% peptone, 0.5% yeast extract and 1% sodium chloride at 4°C, and subcultured once a month. The bacteria were maintained in TSB medium on a rotary shaker at 120 rpm and 35°C for 14 h until the mid-log phase of growth was achieved. The turbidity of the cell suspensions was measured at 600 nm using a microplate reader (Spectramax M5; Molecular Devices, LLC, Sunnyvale, CA, USA) and adjusted to the required concentrations of 1×10^6 or 1×10^8 colony-forming units (CFU)/ml using a McFarland standard prior to analysis in the subsequent experiments.

Determination of antibacterial activity of SA. The antibacterial activity of SA was determined by TTC and Kirby-Bauer disc agar diffusion assays. *E. coli* was incubated in TSB for 20 h at 35°C. The rate of growth inhibition was determined by a modified TTC assay (13). *E. coli* was diluted to 1×10^8 CFU/ml with fresh TSB; 50 μl bacterial suspension was added to each well of a sterile 96-well microplate. Then, 50 μl SA (25-400 μM)

was added. The bacterial suspension incubated without SA served as the normal control and TSB was used as the blank control. Following culturing for 24 h at 35°C, the suspension was mixed with 15 μl TTC reagent (5 mg/ml) and incubated for another 2 h. Subsequently, 50 μl DMSO was added and bacteria were gently agitated for 10 min at 25°C to dissolve the red triphenyl formazan crystals. The optical density (OD) was measured at 532 nm using a microplate reader (Thermo Fisher Scientific, Inc.). The antibacterial activity of SA was expressed as a percentage of inhibition and calculated according to the following formula: Inhibition rate (%) = $(\text{OD}_{\text{sample 532}} - \text{OD}_{\text{blank 532}}) / (\text{OD}_{\text{normal 532}} - \text{OD}_{\text{blank 532}}) \times 100$.

The antibacterial activity of SA was investigated using a Kirby-Bauer disc agar diffusion assay; 200 or 400 μM of SA were applied for analysis. In brief, *E. coli* was diluted to 1×10^6 CFU/ml with fresh TSB and evenly propagated over the surface of an LB agar plate. Sterile filter paper (6 mm) pre-soaked with 200 or 400 μM SA was applied to the plate and incubated for 20 h at 35°C. The sensitivity of *E. coli* to SA was determined according to the diameter of the inhibition zone: A diameter of ≥ 15 mm was considered to indicate high sensitivity to SA; 10-15 mm indicated moderate sensitivity and a diameter of ≤ 10 mm suggested low sensitivity to SA.

Effects of SA on bacterial cell wall. AKP activity was measured according to the manufacturer's protocols to assess the cell wall integrity (Beyotime Institute of Biotechnology). *E. coli* cells (1×10^6 CFU/ml) were plated into a 6-well plate and treated with SA; 50 μl bacterial suspension was collected at various time intervals (0, 3, 6, 9, 12 and 24 h) and centrifuged at 415 g for 3 min under 25°C. The supernatant was mixed with 50 μl para-nitrophenyl phosphate. After 10 min, the reaction was terminated by adding 100 μl stop buffer (Alkaline Phosphatase Assay Kit; Beyotime Institute of Biotechnology). The absorbance was analyzed with a microplate reader (SpectraMax M5; Molecular Devices, LLC). AKP activity was expressed as the OD value at 405 nm.

Effects of SA on the bacterial cell membrane. The integrity of the bacterial membrane was monitored by measuring the levels of ATP, DNA and RNA. *E. coli* was sedimented at 415 x g for 3 min under 25°C and washed two times with distilled water, and then resuspended into 3.6 ml distilled water (1×10^6 CFU/ml) prior to transfer into a 24-well plate. Subsequently, SA (200 or 400 μM) was added and cells were incubated at 35°C. At predetermined sampling times (0, 0.5, 1, 1.5 and 2 h), the bacterial suspension was centrifuged at 415 g and 25°C for 3 min. A total of 50 μl supernatant was mixed with 100 μl ATP detection buffer (ATP Assay Kit; Beyotime Institute of Biotechnology). The luminous intensity was detected within 10 min by a microplate reader (Spectramax M5; Molecular Devices, LLC). The concentrations of DNA and RNA were measured in another experimental system. Briefly, following two washes with PBS, *E. coli* cells (1×10^6 CFU/ml) were plated into a 24-well plate and cultured with SA at 35°C. At various time intervals (0, 2, 4, 6 and 8 h), 100 μl bacterial suspension was collected and centrifuged at 415 g and 25°C for 3 min. The absorbance was determined at 260 nm using a microplate reader (SpectraMax M5; Molecular Devices, LLC).

Effects of SA on protein expression. *E. coli* (1×10^6 CFU/ml) were incubated with 200 or 400 μ M SA in TSB medium for 24 and 32 h, respectively, then centrifuged and washed twice with normal saline. The bacterial suspensions were evenly divided into two portions: One part was mixed with 400 μ l distilled water and the other mixed with RIPA lysis buffer (Solarbio Science & Technology Co., Ltd.). Following determination of the protein content by a BCA assay, the aqueous solution and lysate were separately mixed with 5X SDS and boiled for 5 min at 100°C. Equal amounts of proteins (1 μ g) were separated via SDS-PAGE (10% separating gel/5% stacking gel) at 100 V for 1.5 h. Then, the gels were stained with Coomassie Brilliant Blue overnight at 25°C. Subsequently, the gels were de-stained in water for two days. The effects of SA on *E. coli* protein expression were analyzed according to the staining intensity of the protein bands.

Scanning electron microscopy (SEM). SEM was used to examine the morphology of *E. coli* cells. Following culture in fresh TSB medium with 200 or 400 μ M SA for 20 h at 35°C, the bacterial suspensions were centrifuged at 415 x g for 10 min under 25°C and washed twice with PBS. *E. coli* was fixed in 5% glutaraldehyde for 4 h at 4°C in the dark. Following three washes with PBS, all samples were dehydrated once with 30, 50, 70, 80 and 90% ethanol, twice with 100% ethanol, each for 15 min. Then, the samples were centrifuged at 2,771 x g for 3 min at 25°C. Finally, all samples were sputter-coated with platinum prior to analysis with a scanning electron microscope (magnification, x10,000; SUPRA 55-VP; Carl Zeiss AG).

Transmission electron microscopy (TEM). To prepare samples for TEM, *E. coli* was cultured in TSB medium overnight and treated with 200 or 400 μ M SA for 24 h at 35°C. Untreated and SA-treated bacteria were harvested by centrifugation at 415 x g and 25°C. Colonies of bacteria were fixed with 2.5% glutaraldehyde and 1% OsO₄ fixative solution (pH 7.4) for 4 h at 4°C, and then rinsed with PBS. The samples were dehydrated with increasing concentrations of ethanol (30, 50, 70, 80, 90 and 100%). Following drying for 24 h, the samples were serially sectioned for TEM analysis at a magnification of x60,000. Ultrathin sections (50 nm) were visualized using a transmission electron microscope (1200 EX; JEOL, Ltd. pan). Images were acquired using an AMT 2k CCD camera (Advanced Microscopy Techniques, Corp.) under standard conditions.

Statistical analysis. The antimicrobial experiments were conducted in triplicate. All data were expressed as the mean \pm standard deviation and analyzed by one-way ANOVA followed by Dunnett's test using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to significant to indicate a statistically significant difference.

Results

Antimicrobial activity of SA. The antimicrobial activity of SA against *E. coli* was analyzed in the present study. The highest rate of growth inhibition of SA against *E. coli* was $32.74 \pm 0.058\%$ at 200 μ M and $31.24 \pm 0.065\%$ at 400 μ M (Fig. 1). Dose-dependent responses to 25 and 200 μ M

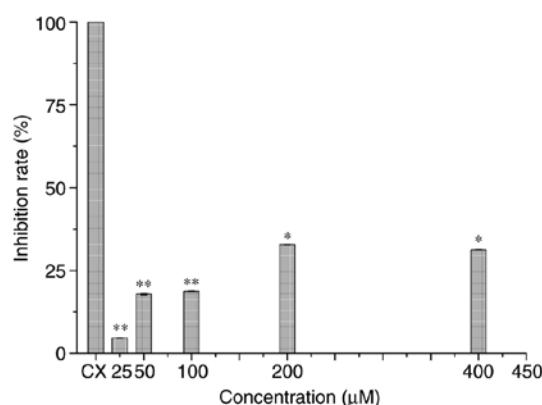


Figure 1. Antimicrobial activity of striatissporolide A against *Escherichia coli*. * $P < 0.05$, ** $P < 0.01$ vs. CX group. CX, ciprofloxacin hydrochloride.



Figure 2. Inhibitory zones growth of *Escherichia coli* treated with SA1 and SA2, and CX. CX, ciprofloxacin hydrochloride; SA, striatissporolide A; SA1, 200 μ M SA; SA2, 400 μ M SA.

SA were observed. CX produced an increased inhibition rate of $100 \pm 0.07\%$ against *E. coli* at 0.5 μ g/ml. Based on these findings, 200 (SA1) and 400 μ M (SA2) SA were used for subsequent analysis.

As presented in Fig. 2, *E. coli* exhibited low sensitivity to SA (200 and 400 μ M) and CX (0.5 μ g/ml) according to the diameter of the inhibition zones (7.03 ± 0.04 , 7.08 ± 0.06 and 7.04 ± 0.03 mm, respectively), which was in accordance with the inhibitory rate of SA. There was no statistically significant difference in the inhibition zone diameters among the groups.

Effects of SA on bacterial cell wall. As presented in Fig. 3, SA notably increased the extracellular levels of AKP. In the normal group (NG), where the cells were treated with TSB medium alone, AKP activity was low. Following treatment with 200 and 400 μ M SA, the absorbance increased from 0.192 to 0.217 ± 0.003 and 0.192 ± 0.001 to 0.286 ± 0.003 at 3 and 24 h, respectively. The activity of AKP exhibited peak values of 0.214 ± 0.008 and 0.299 ± 0.002 at 9 h, which showed significant difference ($P < 0.01$) compared with the normal group. In addition, SA at a concentration of 400 μ M exhibited

a markedly stronger effect on the permeability of the *E. coli* cell wall than SA at 200 μM ($P < 0.05$).

Effects of SA on the bacterial cell membrane. As presented in Fig. 4, the extracellular concentrations of ATP in the SA1 and SA2 groups were 0.83 ± 0.08 and 0.96 ± 0.08 μM at 0.5 h, respectively, but decreased after 1 and 2 h; which showed significant difference at 0.5 ($P < 0.01$), 1 and 2 h ($P < 0.05$) compared with the normal group; the ATP levels in the normal group were the same as that of the blank group, which were zero. These results indicated that SA may rapidly damage the cell membrane of *E. coli* and induce ATP leakage. In another experimental system, it was the DNA and RNA levels in the SA and the normal groups was determined. However, no changes in the DNA and RNA levels were observed between these groups.

Effects of SA on protein levels. Protein bands of 10 and 35 kDa were observed following 10% SDS-PAGE. Treatment with 200 and 400 μM SA for 24 and 32 h notably increased the level of the 35 kDa protein compared with the normal group in aqueous suspensions of bacteria (Fig. 5A). As presented Fig. 5B, SA notably reduced the level of the 10 kDa protein in bacterial lysates. The results demonstrated that SA exhibited marked effects on protein levels in *E. coli*; however, there were no marked differences in the protein expression levels between 24 and 32 h. Thus, SA may interfere in the protein metabolism within 24 h.

SEM analysis. SEM was used to observe the morphological alterations of *E. coli* cells in the presence or absence of antibacterial treatments. Untreated *E. coli* exhibited a smooth and intact surface (Fig. 6A); the rod-shaped morphology of *E. coli* was retained. After 24 h of treatment with SA, the majority of the cells appeared to possess a rough surface, and the size of cells was irregular and shorter compared with the normal group (Fig. 6B and C).

TEM analysis. Similar to the results of SEM, TEM revealed that untreated *E. coli* cells appeared to exhibit typical cellular organization of Gram-negative bacteria (Fig. 7A); however, treatment with SA induced the alteration in the morphology of *E. coli*, including irregularities in the shape of the bacterium (Fig. 7B and C).

Discussion

Butenolides are a class of lactones with a four-carbon heterocyclic ring structure, and possess important antibacterial, antiparasitic and antitumor properties. Evernic acid, an analogue of SA, severely inhibits the growth of *Phytophthora infestans* at 0.5×10^{-5} – 0.5×10^{-3} M (14). Methylenolactocin, an isomer of SA from *Penicillium* sp., has been reported to have prominent antibacterial activity against Gram-positive bacteria (15). Taibi *et al.* (16) synthesized eight butenolide derivatives and revealed that three of these compounds exerted significant antimicrobial activity against *E. coli*. These findings suggest that butenolides have a wide range of antimicrobial targets and may be of potential use as bacterial agents; however, the exact antibacterial mechanism of these

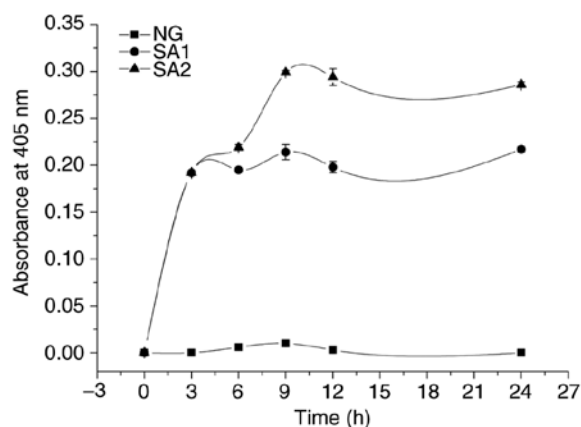


Figure 3. Effects of SA1 and SA2 on alkaline phosphatase activity in *Escherichia coli*. NG, *Escherichia coli* cells treated with TSB medium alone; SA, striatissporolide A; SA1, 200 μM SA; SA2, 400 μM SA.

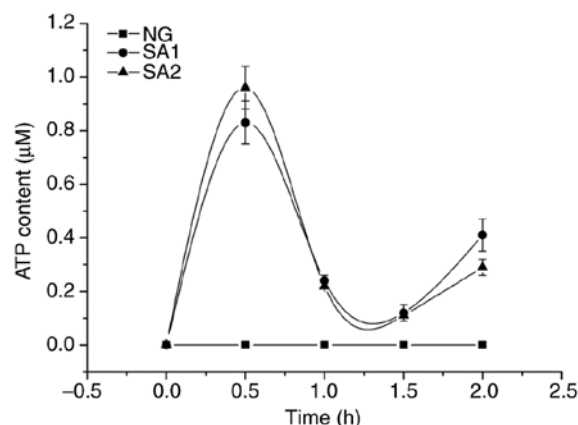


Figure 4. Effects of SA1 and SA2 on extracellular ATP content in *Escherichia coli*. NG, *Escherichia coli* cells treated with TSB medium alone; SA, striatissporolide A; SA1, 200 μM SA; SA2, 400 μM SA.

compounds is still unknown. The results of the present study suggested that SA had fewer antibacterial targets and weak activity compared with these compounds. It may be that structural variations are associated with the antibacterial potential of a compound.

In the current study, CX exhibited a high inhibitory rate at 0.5 $\mu\text{g/ml}$; however, there appeared to be no difference in the inhibition zone diameter produced by CX and SA. We hypothesize that because the content of CX in the filter paper was much lower than that in the well of the 96-well microplate because the filter paper was just soaked in CX solvent (0.5 $\mu\text{g/ml}$) and then placed on the LB agar plate. Furthermore, CX was at a low concentration of 0.5 $\mu\text{g/ml}$ and could not produce a larger inhibition zone in this diffusion state. Therefore, the difference in the inhibition zone diameter between CX and SA was not distinct.

AKP is present between the cell wall and cell membrane of bacteria, and may leak out of damaged cell walls; thus AKP is regarded as a marker of cell wall permeability (17). Diao *et al.* (18) reported that the integrity of the *Bacillus cereus* cell wall could be disrupted by monolauryl-galactosylglycerol, which led to leakage of AKP from the bacteria and into the extracellular environment. The results of the present study

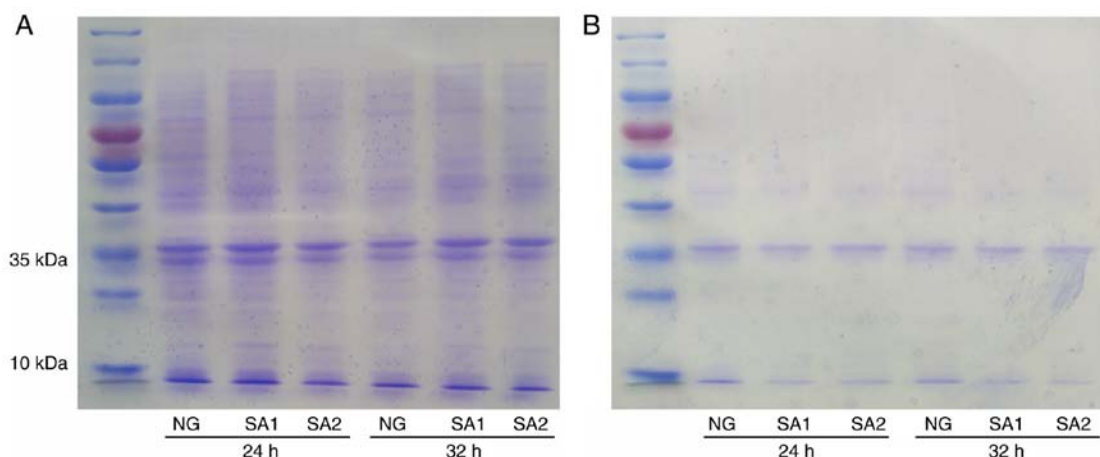


Figure 5. SDS-PAGE-based peptide mapping of *Escherichia coli* treated with SA1 and SA2. (A) Samples from an aqueous suspension of bacteria. (B) Samples from the bacterial lysate. NG, *Escherichia coli* cells treated with TSB medium; SA, striatissporolide A; SA1, 200 μ M SA; SA2, 400 μ M SA.

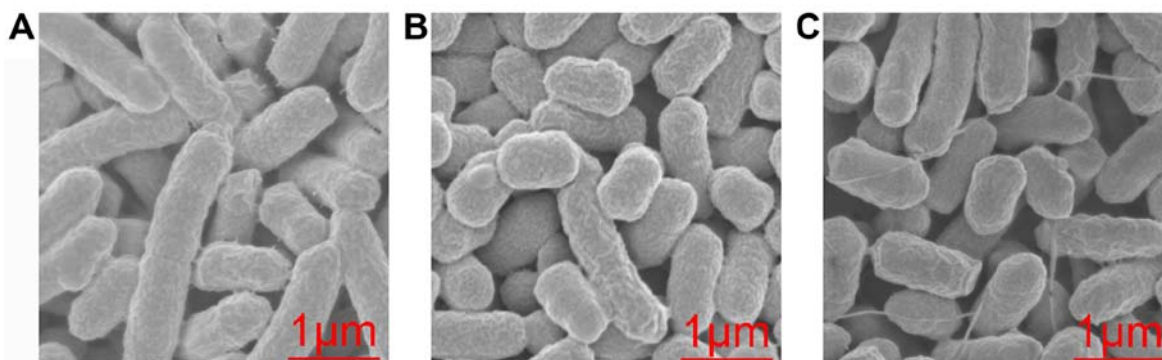


Figure 6. Scanning electron microscopy images of *Escherichia coli* cells treated (A) without SA, and with (B) 200 μ M and (C) 400 μ M SA. SA, striatissporolide A.

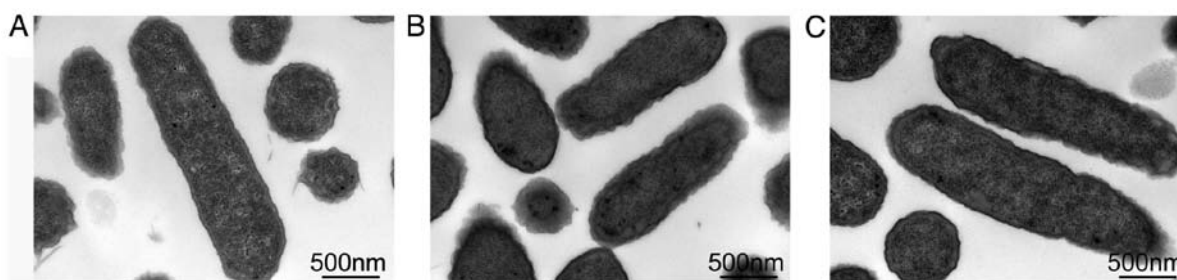


Figure 7. Transmission electron microscopy images of *Escherichia coli* cells treated (A) without SA, and with (B) 200 μ M and (C) 400 μ M SA. SA, striatissporolide A.

demonstrated that SA damaged the cell wall of *E. coli* and led to the leakage of AKP.

The leakage of cytoplasmic material has been associated with severe or irreversible damage to the cytoplasmic membrane; the levels of specific cell leakage markers, including ATP, nucleic acids and proteins, may be used to determine the integrity of the membrane (19). ATP is a complex biomolecule produced in all living organisms. It provides energy to drive all processes in living cells, including muscle contraction, the propagation of nerve impulses and chemical synthesis (20). ATP is an important index for interpreting the mechanisms of action of antimicrobial agents. The results from the present

study suggested that the extracellular concentrations of ATP in the SA1 and SA2 groups increased from 0 to 0.5 h, then decreased from 0.5 to 1.5 h. As of the abnormal reductions in the levels of ATP, the present study proposed that damaged bacteria had undergone a process of self-repair, which inhibited the leakage of ATP from the damaged cell membrane induced by SA. In the other parallel experimental system, no notable leakage of DNA, RNA or protein was reported in the extracellular environment. Thus, it was proposed that SA-induced cell membrane damage may not cause leakage of the aforementioned molecules. It was previously reported that damage to the cell membrane of *Enterobacter cloacae*

induced by 0.1% 3-phenyllactic acid resulted in the leakage of low-molecular weight substances, such as ATP; however, the leakage of high-molecular weight substances, including nucleic acids and proteins were not detected (21). These findings were consistent with the observations of the present study.

Inhibition of bacterial protein synthesis is an important mechanism underlying the antibacterial properties of certain compounds. Wolf *et al* (22) reported that inhibition of protein synthesis by kirromycin occurred by blocking the release of elongation factor Tu from the ribosome following the enzymatic binding of aminoacyl-transferRNA and subsequent GTP hydrolysis. These findings suggest that SA may affect protein synthesis in *E. coli*; however, the underlying mechanism requires further investigation.

Damage to the cell wall and cell membrane can affect the permeability of these regions and lead to cell death (23). SEM indicated that SA-induced morphological alterations in *E. coli* may be responsible for the leakage of AKP and ATP, which serve pivotal roles in cell growth, proliferation and survival. Henning *et al* (24) reported that the antimicrobial effects of the nisin polypeptide occurred via interactions with the phospholipid components of the cytoplasmic membrane, which led to suppressed membrane function. The target site of SA on the cell wall and cell membrane of bacteria remains unknown; however, SA may penetrate cells and interact with intracellular substances due to its small molecular weight of 224 Da and lipophilic structure (12). To further investigate the antibacterial mechanism, TEM analysis was conducted to observe ultrastructural alterations of the intracellular substances in *E. coli*.

ATP is an important precursor for DNA and RNA. It is required in RNA synthesis, and the replication and transcription of DNA. Therefore, the leakage of ATP may result in reduced levels of nucleic acids and ribosomes in *E. coli*, which may be involved in the antibacterial mechanism of SA. Antimicrobial agents can directly inhibit the synthesis and function of the intracellular biopolymers, such as proteins, DNA or ribosomes (25). Miao *et al* (26) reported that an antimicrobial peptide present in Tibetan kefir may inhibit the synthesis of *E. coli* DNA via direct interactions. These findings indicated that certain antimicrobial molecules may have numerous targets involved in eliminating bacteria. Furthermore, as SA has a low molecular weight and can penetrate bacteria, it may bind to bacterial DNA. A gel shift assay and AFM should be conducted in future to investigate the association between SA, DNA and protein.

The findings of the present study demonstrated the antibacterial activity of SA against *E. coli*. However, the highest reduction rate of the viable *E. coli* was only 32.74±0.058% by SA. Therefore, structural modifications of SA will be generated to explore the structure-activity relationships and improve its antibacterial activity in future experiments. The mechanisms of action of SA against *E. coli*, including how SA destroys the cell membrane and cell wall, regulates protein levels and abates nucleic acid levels in *E. coli* require further investigation.

In conclusion, the current study explored the antibacterial activity and the mechanism of SA for the first time. Although SA exhibited modest activity against *E. coli*, it did alter the protein level and damage the cell membrane and cell wall markedly, which provides an experimental basis for the antibacterial mechanism of butenolides. The present study

proposed that SA may possess notable features as an effective and natural antibacterial agent.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JWS designed this study and wrote the paper. DML, LJ and GXX performed the research. WFZ and JRJ checked and analyzed the data. JBT contributed to the acquisition of data, and critically revised the manuscript for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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