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In-Vitro and In-Vivo Antibacterial Effects of *Saxifraga umbellulata* var. *Pectinata* on *Escherichia coli* Isolated from Yaks

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Key words
Anti-microbial, Diarrhea, *Escherichia coli*, MIC, MBC, *Saxifraga umbellulata* var. *Pectinata*, Yak

ABSTRACT

Yaks are economically important animals in plateau regions. Bacterial diarrhea especially caused by drug-resistant pathogens like *Escherichia coli* has been a persistent issue among them. In this study, we explored the antibacterial effects of *Saxifraga umbellulata* var. *Pectinata* (SUP) on *E. coli* isolated from yaks. Results showed that the MIC and MBC of SUP against *E. coli* was 8 mg/mL and 16 mg/mL, respectively. The growth inhibition curve demonstrated that SUP significantly inhibited the growth of *E. coli* in a dose-dependent manner. Animal studies showed that SUP increased the survival rate of animals by alleviating intestine damage. In infected animals, villus height (P<0.0001) and the ratio of villus height/crypt depth (P<0.001) were lower, while crypt depth was significantly higher (P<0.0001). However, animals treated with SUP showed significant increase in villus height (P<0.0001), the ratio of villus height/crypt depth (P<0.0001) and decrease in crypt depth (P<0.001). Organ bacterial loads demonstrated that animals fed with SUP had markedly lower bacteria loads in heart (P<0.5), liver (P<0.01), spleen (P<0.5), lung (P<0.001), duodenum (P<0.05), jejunum (P<0.01), ileum (P<0.01), cecum (P<0.01), colon (P<0.01) and rectum (P<0.05). Crystal violet staining revealed that SUP inhibited the biological film formation of *E. coli* (P<0.0001). Membrane permeability analysis of *E. coli* indicated that SUP markedly increased bacterial leakage (P<0.0001). This study demonstrates that *Saxifraga umbellulata* var. *Pectinata* can inhibit *E. coli* both *in vitro* and *in vivo* by affecting the bacterial biofilm and membrane permeability, which may provide insights for the development of novel anti-*E. coli* drugs or preventive measures against diarrhea in plateau yaks.

INTRODUCTION

Yaks are economically important and essential bovine ruminant on the Qinghai-Xizang plateau (Li *et al*., 2023; Chen *et al*., 2022). These animals provide milk, meat, fur or related products and as well as serving as means of transport for native people (Lu *et al*., 2023; Wang *et al*., 2023). However, diarrhea poses a frequent problem in these animals, leading to severe losses and affecting ruminant health (Li *et al*., 2022). Diarrhea in cattle, especially calf diarrhea is a world-wide issue in the cattle industry (Choi *et al*., 2021; Rasheed *et al*., 2022; Anwar *et al*., 2022). Previous studies have confirmed that pathogens such as viruses (bovine viral diarrhea virus, torovirus), bacteria (*Escherichia coli*, *Salmonella* spp.), and parasites (*Cryptosporidium parvum*, *Giardia duodenalis*) are factors that lead to diarrhea in animals (Chang *et al*., 2021; Shi *et al*., 2020; Gelalcha *et al*., 2022; Arsenault *et al*., 2022; Ali *et al*., 2024; Taghipour *et al*., 2022). Among them, *E. coli* is a commonly detected bacterial pathogen that bring severe challenges to public and livestock health (Frankel and Ron, 2018; Li *et al*., 2023). Moreover, an increasing number of multi-resistant bacteria have been isolated from food animals, further complicating the issue

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due to limited available antibacterial agents (Roth *et al*., 2019; Ullah *et al*., 2023).

Traditional Chinese medicines are valuable and effective means for curing diseases and enhancing health (Chi *et al*., 2021). Among them, many herbs have antibacterial effects, such as *Andrographis paniculata*, *Sanguisorba officinalis* L. and garlic (Dai *et al*., 2019; Zhou *et al*., 2021; Tesfaye, 2021). The long history of Xizang medicine has integrated medical systems developed from traditional Chinese medicine and other medicines like Arabian medicine, which have greatly contributed to the health of plateau herdsmen (Huang *et al*., 2023).

The Xizang medicine of *Saxifraga umbellulata var. Pectinata* (SUP) is a representative plateau perennial herb, which belongs to Saxifragaceae family. In regions with altitude over 3 km, this herb is a recognized traditional Xizang medicine named Songdi used for treating liver and gallbladder diseases as well as digestive diseases (Huang *et al*., 2023). Previous reports have indicated that SUP extraction has antibacterial activity, hepatoprotective effect (Huang *et al*., 2023). However, not much information is available about the antibacterial effect of SUP on *E. coli* isolated from yaks. Therefore, we conducted this study to investigate the *in vitro* and *in vivo* antibacterial effect of SUP on *E. coli* isolated from yaks.

MATERIALS AND METHODS

Bacterial isolate

Multi-drug resistant *E. coli* was previously isolated from diarrhea yaks and stored in the clinical veterinary laboratory of Nanjing Agricultural University. This bacterium was resistant to penicillin, ampicillin, erythromycin, tetracycline, streptomycin and gentamicin.

Preparation of the SUP extracts solution

SUP (200 g) was obtained from Tibetan medicine factory (Lhasa, China). Ethyl acetate extraction of SUP was performed according to previously described protocols (Hou *et al*., 2022; Liu *et al*., 2022). Initially the herbs were crushed and soaked with 2 L of ethanol (75%) overnight. The following day, herbs were heated to 85 ℃ and subjected to reflux extraction for 2h, followed by filtration. The SUP herbs were extracted and filtered three times and all the products were mixed together. At last, the mixed SUP products were dried and reconstituted in sterile water at 1g/mL for further use.

In vitro *antibacterial effect of SUP on* E. coli

The anti-*E. coli* activity of SUP was examined via commonly utilized methods of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) detection (Han and Guo, 2012; Parvekar *et al*., 2020). In a 96-well plate, 100 µL of SUP at various concentrations (0, 512 mg/mL, 256 mg/mL, 128 mg/mL, 64 mg/mL, 32 mg/mL, 16 mg/mL, 8 mg/mL, 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL) was added to an equal volume of bacteria solution (10^6 CFU/mL). Subsequently, 50 µL of LB medium (Hangzhou Binhe Microorganism Reagent Co., Ltd, China) was added to each well and the plate was incubated at 37 ℃ for 18 h. The MIC endpoint was determined when no visible growth of *E. coli* was observed in the well. To determine the MBC, 100 µL of medium from wells before and after MIC well, and MIC well plated onto LB agar plates and incubated at 37 ℃ overnight. The MBC endpoint was reached when the plates had fewer than 0.25×10^5 CFU (0.1%) colonies.

Next, we evaluated the growth inhibition curve of *E. coli* in response to different doses of SUP. In sterile tubes containing 4.90 mL LB medium, 50 µL of bacteria solution (10^6 CFU/mL) was added along with 50 µL of SUP at concentration of 0, MIC, 2MIC and 4MIC mg/mL. Each concentration had 18 independent repeat tubes. The tubes were then incubated at 37 ℃ in shaker and at time point of 0, 2h, 4h, 6h, 8h, 10h and 12h, three tubes of each concentration were sampled to check OD_{600} value.

The effect of SUP on the biological film and membrane permeability of E. coli

The effect of SUP on the biofilm was assessed using crystal violet staining (Bai *et al*., 2022). Ina 96 well plate, 100 µL of *E. coli* (OD_{600}=0.05) was mixed with 100 µL of SUP (4MIC) and incubated at 37 ℃ for 6 h and 12 h. Then the OD_{620} value was measured, and the plate was washed by PBS three times and fixed with 200 µL methanol for 15 min. Finally, the plate was washed with PBS for three times and stained with 0.1% crystal violet for 30 min. Finally, the plate was washed with PBS and 200 ul of 30% glacial acetic acid added for measuring absorbance at 540 nm. Three independent repeats were performed for all wells, and equal volume of sterile water was added to control wells. The ratio value of OD_{540}/OD_{620} was calculated to determine the effect of SUP on the biofilm.

To evaluate the effect of SUP on the membrane permeability of *E. coli*, 100 uL bacteria solution (10^6 CFU/mL) was mixed with SUP (4MIC) in a 96-well plate, and then incubated at 37 ℃. The OD_{260} and OD_{280} values of supernatant were examined at 0, 2, 4 and 8 h. Three independent repeats were set for all wells, and an equal volume of sterile water was added in control wells.

Animal experiments

A total of 39 male Kunming mice (five weeks, average weight of 23.5±1.3 g) were obtained from Qinglongshan

animal breeding (Nanjing, China). The mice were reared and housed in the animal facility having free access to feed and water. All of the mice were given three days for acclimatization and then grouped into control (C), infection (I) and treatment (T) groups. Mice in group I and T were intra-peritoneally infected with a bacteria solution (10^7 CFU/mL), while group T received treatment with SUP (200 mg/Kg) for three days. Group C and I were treated with an equal volume of sterile water. On the second day, three mice from each group were euthanized to collect organ and intestines samples. Daily weights and mortality of mice were recorded. Those collected tissues were ground with sterile PBS, and then used for bacteria culture on LB agar plate. Colony forming units (CFUs) were counted to analyze tissue bacterial loads. Additionally, the jejunum was employed for pathologic analysis.

Pathologic analysis

The jejunum from Kunming animals was fixed in paraformaldehyde (4%) and then subjected to H & E staining in Pinuofei Biological Technology (Wuhan, China). An Olympus CX23 microscope (Olympus Co., Japan) was used for pathologic analysis. Statistical analysis of villus height and crypt depth of mice in C, I and T were performed.

Statistical analysis

Non-parametric tests were conducted using IBM SPSS (27.0) software. Data are presented as means ± SD and statistical significance was determined at $P<0.05$.

RESULTS

In vitro *antibacterial effect of SUP on* E. coli

The MIC and MBC of SUP anti-against *E. coli* were 8 mg/mL and 16 mg/mL, respectively. The growth inhibition curve clearly demonstrated that SUP significantly inhibited the growth of *E. coli*, indicating a dose-dependent bactericidal effect (Fig. 1).

Crystal violet staining showed that SUP significantly inhibited the formation of *E. coli* biofilm at 6 h ($P<0.0001$) and 12 h ($P<0.0001$) (Fig. 2A). Membrane permeability analysis of *E. coli* revealed that SUP markedly increased bacterial leakage at 4 h ($P<0.0001$) and 8 h ($P<0.0001$) (Fig. 2B).

In vivo *antibacterial effect of SUP on* E. coli

Animal study revealed that *E. coli* infection led to mice mortality within 2-24h, while treatment with SUP saved the animals lives (Fig. 3A). Weight analysis showed that *E. coli* caused weight loss in mice, while animals

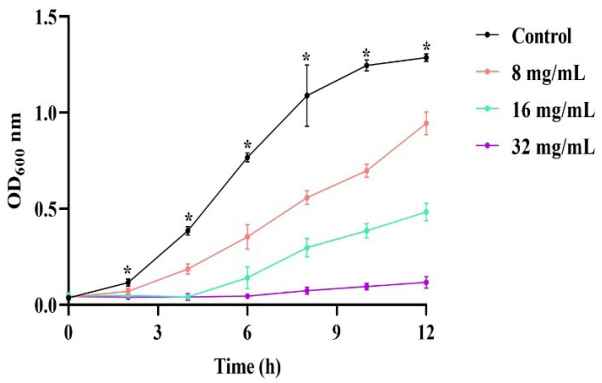

Fig. 1. The inhibition effect of SUP on the growth curve of *E. coli.* Data are described as the mean ± SEM (n = 3).

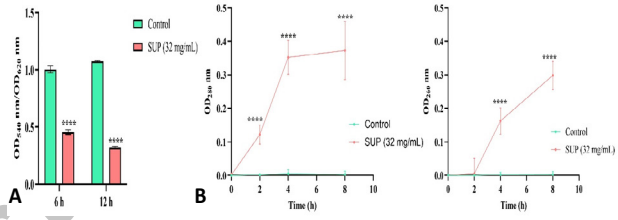

Fig. 2. SUP inhibited the biological film formation (a) and increased membrane permeability (b) of *E. coli*. Significance is presented as ****P < 0.0001; data are presented as the mean ± SEM (n = 3).

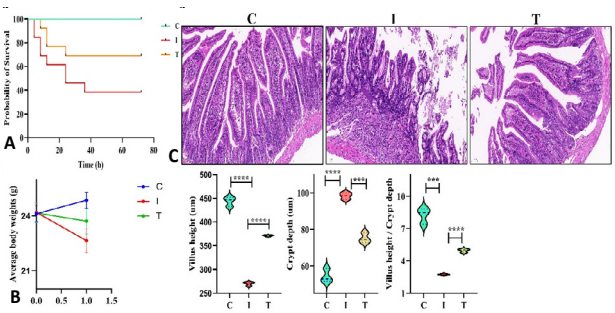

Fig. 3. The *in vivo* inhibitory effect of SUP on *E. coli.* (A) Survivorship curve, (B) average body weights, (C) intestine pathologic analysis. Scale bar 50 μm. Significance is presented as ***P < 0.001 and ****P < 0.0001; data are presented as the mean ± SEM.

treated with SUP showed slightly higher body weights (Fig. 3B). Pathologic analysis indicated that *E. coli* obviously disrupted the integrity of intestinal villi in mice, whereas SUP alleviated intestinal damage in animals (Fig. 3B). Villus height ($P<0.0001$) and the ratio of villus height/crypt depth ($P<0.001$) in group I were markedly lower than in group C, while crypt depth in group I was

significantly higher (P<0.0001). However, animals treated with SUP presented noticeably increased villus height (P<0.0001), ratio of villus height/crypt depth (P<0.0001), and decreased crypt depth (P<0.001) (Fig. 3B).

Organ bacterial loads showed that *E. coli* loads in the heart (P<0.5), liver (P<0.001), spleen (P<0.001), lung (P<0.001), kidney (P<0.001), duodenum (P<0.001), jejunum (P<0.001), ileum (P<0.001), cecum (P<0.01), colon (P<0.01) and rectum (P<0.001) in group I were significantly increased. Interestingly, animals fed with SUP exhibited markedly lower bacteria loads in heart (P<0.5), liver (P<0.01), spleen (P<0.5), lung (P<0.001), duodenum (P<0.05), jejunum (P<0.01), ileum (P<0.01), cecum (P<0.01), colon (P<0.01) and rectum (P<0.05) (Fig. 4).

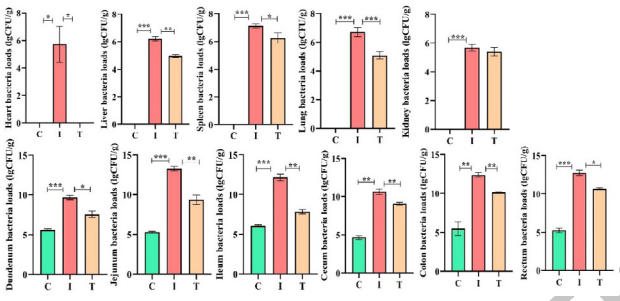

Fig. 4. Organ bacterial loads analysis of mice. Significance is presented as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$; data are presented as the mean ± SEM (n = 3).

DISCUSSION

Cattle are important food-producing ruminants, providing nutritious products for citizens. Therefore, cattle disease not only harm animal health, but potentially threaten the protein food supply. Especially on the cold plateaus, yaks are crucial food resources (Li and Liu, 2022). *E. coli* is a common opportunistic pathogen causing diarrhea, resulting in significant economic losses to farming industry due to medical costs and animal deaths (Zhang *et al*., 2022). With antibiotic abuse in veterinary and medical practices, drug-resistant *E. coli* has been detected in the environment, animals and people (Hu and Cheng, 2016). There is an urgent need to screen novel antibacterial drugs with fewer side effects.

Medicinal herbs have been popularly used for thousands of years due to their antimicrobial properties, offering promising alternatives to conventional antibacterial drugs (Alanazi *et al*., 2023). Previous research has found herbs such as *Chrysanthemum*, *Lagotis brachystachya* and *Oak bark* for their anti-*E. coli* effects (Kim *et al*., 2013; Hou *et al*., 2022; Šukele *et al*., 2022). Consistent with these findings, our study confirmed that SUP could inhibit the growth of multi-drug resistant *E. coli* from yaks both *in vitro* and *in vivo* (Figs. 1, 3). *In vitro* studies showed that SUP could inhibit *E. coli* at 8 mg/mL, with MBC was 16 mg/mL. The growth inhibition curve indicated that SUP (32 mg/mL) could nearly completely inhibited bacteria growth (Fig. 1). Similar to a previous a study reported gut injuries caused by *E. coli* (Ismael *et al*., 2023), the strain of *E. coli* isolated from yaks proved lethal to mice causing severe intestine damage. However, our *in vivo* results showed that SUP decreased mice mortality by mitigating intestine damages and reducing bacteria loads (Figs. 3, 4).

Furthermore, we investigated the anti-*E. coli* mechanism of SUP by examining the biofilm and membrane permeability of *E. coli*. Biofilms consist of numerous bacterial cells aggregated together with extracellular matrix, which can shield bacteria from antibacterial agents and confer resistance to drugs (Lu *et al*., 2021), as well as protect from the host immune system (Roy *et al*., 2018). Following treatment for 6 h and 12 h, SUP significantly inhibited the biofilm formation (Fig. 2A), suggesting that SUP may hinder *E. coli* survival by impeding the biofilm formation. Our results are consistent with previous studies that have shown various herbs can inhibit the bacterial biofilm formation (Hou *et al*., 2022; Lu *et al*., 2019). Membrane integrity and permeability are crucial for bacteria growth (Yang *et al*., 2021), as a compromised membrane can result in the leakage of cell contents (Xu *et al*., 2017). In this study, proteins and nucleic acids were detected in *E. coli* treated with SUP (Fig. 2B), indicating that SUP could increase the permeability of *E. coli* membrane.

CONCLUSION

In this study, we demonstrated that *Saxifraga umbellulata* var. *Pectinata* could inhibit *E. coli in vitro* and *in vivo* by affecting the biological film and membrane permeability of bacteria. These findings provide insights that could potentially lead to develop novel anti-*E. coli* drugs or prevent measures for diarrhea in plateau yaks.

DECLARATIONS

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Ethical statement

All the experiment procedures were conducted in accordance with the guidelines and approval of the Ethics Committee of Nanjing Agricultural University (NJAU. No20240226021).

Statement of conflict of interest

The authors have declared no conflict of interest.

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