Antimicrobial ability of indoxyl, an intermediate product in the formation of indigo and indirubin, against Staphylococcus aureus

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Indoxyl is an intermediate product in the production of indigo and indirubin. There are no studies on the inhibitory effect of indoxyl on microorganism. In this study, it was found that temperature-stress induced the synthesis of intracellular β-glucosidase, which had a great influence on the ratio of indican, indoxyl, indigo and indirubin in Strobilanthes cusia. At 100 , only indican was detected, whereas a large amount of indoxyl was found at 50–70 . HPLC analysis indicated that the use of indoxyl reduced glucose consumption and metabolite production by Staphylococcus aureus. The cells treated with indoxyl had irregular shapes, and fragments of cells were found in SEM images. The images of CLSM suggest that cell morphological changes would be caused by the inhibition of indoxyl on EMs formation. Moreover, indoxyl decreased intracellular ATP content and increased the NAD+/NADH ratio, which promoted the generation of H2O2, damaging cells.

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Highlights

- Temperature showed a large influence on the components of the bioactives of *Strobilanthes cusia* .
- β-Glucosidase activity was the key point for the change of components of bioactives.
- The bioactives consisted mainly of indican, indoxyl, indigo and indirubin.
- Indoxyl exhibited the highest antimicrobial ability among the components.
- Indoxyl decreased the formation of extracellular matrices and altered the intracellular redox status of *Staphylococcus aureus* .

Abstract

Indoxyl is an intermediate product in the production of indigo and indirubin. There are no studies on the inhibitory effect of indoxyl on microorganism. In this study, it was found that temperature-stress induced the synthesis of intracellular β-glucosidase, which had a great influence on the ratio of indican, indoxyl, indigo and indirubin in*Strobilanthes cusia* . At 100 , only indican was detected, whereas a large amount of indoxyl was found at 50–70 . HPLC analysis indicated that the use of indoxyl reduced glucose consumption and metabolite production by *Staphylococcus aureus* . The cells treated with indoxyl had irregular shapes, and fragments of cells were found in SEM images. The images of CLSM suggest that cell morphological changes

would be caused by the inhibition of indoxyl on EMs formation. Moreover, indoxyl decreased intracellular ATP content and increased the NAD^+/NADH ratio, which promoted the generation of H_2O_2, damaging cells.

Keywords

Antimicrobial activity; *Staphylococcus aureus* ;*Strobilanthes cusia* ; Indigo; Indoxyl.

Introduction

Staphylococcus aureus is a Gram-positive bacterium, which often exists on human skin and the surface of medical equipment. The pathogen can also be found in the nose of healthy individuals (Grundmann et al., 2006). In the last several decades, the excessive use of antibiotics has led to multidrug resistance of the strain, which causes serious challenges to clinical medicine and disease treatment (Gatadi et al., 2019; Liu et al., 2021; Singh et al., 2021).

Extracellular matrices (EMs) are complex growth structures formed by many cells gathered together (Roy et al., 2018). This mechanism can make microorganisms adsorb more firmly on the surface of objects (Satpathy et al., 2016). The inhibitory effect of plant-derived bioactives on*S. aureus* has been widely studied (Cui et al., 2019; Shi et al., 2020). Cui et al. found that phenolic acids derived from rice straw can effectively inhibit the formation of EMs of *S. aureus*[8]. The combination of phenolic acid and antibiotics can make it easier for antibiotics to enter the cell, which can destroy the redox and generate peroxides in *S. aureus* cells, thus, achieving the lethal effect of the pathogen (Wei et al., 2022).

Strobilanthes cusia (Nees) Bremek is one of the important raw materials of traditional Chinese medicine widely distributed in Asia, commonly called "Malan," which mostly grows in Southwest China (Liau et al., 2007). *S. cusia* plays an important role in maintaining people's health (Zhang et al., 2021). As a traditional Chinese medicine,*S. cusia* contains a large number of alkaloids, such as indigo and indirubin (Li et al., 2021), which have anti-inflammatory, antivirus and other effects (Li & Peng, 2013; Zhou et al., 2017). Indigo showed a significant antibacterial effect in an in vitro antibacterial test. Indirubin could activate the CYP3A4 gene in the human body and plays an important role in detoxification (Kumagai et al., 2016). However, the solubility of indirubin and indigo in water is small, which limits their clinical application (Wang et al., 2021).

Indican (indoxyl-β-d-glucosidase) is the major alkaloid in*S. cusia* (Yu et al., 2021). It is the precursor of indigo and indirubin. Indoxyl is an intermediate product in the formation of indigo and indirubin. When plants are subjected to environmental stress, indican in vacuoles could be hydrolyzed by β-glucosidase to generate indoxyl, which could protect plants from environmental disturbance. During this process, parts of indoxyl can be oxidized to form indigo and indirubin (Zhang et al., 2020). Because indoxyl is very unstable, previous studies were mainly focused on the final products, indigo and indirubin, whereas there have been few investigations on the influence of indican and indoxyl on microbial growth.

In this study, the extraction of bioactives from the leaves of *S. cusia* was carried out to investigate the influence of temperature on the component bioactives. Then, the effects of different bioactive substances, especially indoxyl, on the growth and metabolism of *S. aureus* were evaluated using microbial growth, metabolites, intracellular redox and observing the morphological changes of cells. The results of this study can provide new ideas for improving the medicinal properties of Chinese herbal medicines such as Banlangen and put forward new directions for improving the extraction methods and conditions of their biologically active substances.

Material and Methods

2.1 Preparation of indican solution from leaves of S. cusia

The fresh leaves of *S. cusia* were harvested from Guizhou. The extraction conditions of indican were 100 water bath heat treatment. The fresh leaves were treated with deionized water, and 10 g of it was treated with 100 mL deionized water at 100 in a water bath for 10 min. The obtained liquid was then immediately

cooled rapidly in a 4 water bath for 10 min to stop the extraction reaction that was still going on. The cooled solid–liquid mixture was filtered with gauze and the leaves and indican solution were separated.

2.2 Enzyme activity assay

2.2.1 Extraction of crude enzyme solution

A total of 10 mL of deionized water was preheated at different temperatures for 10 min, and 1 g of the fresh leaves was accurately weighed and added to the preheated water. After 10 min of heat treatment, the leaves were immediately removed and placed in a 4 ice bath. After the ice bath, 30 mL 0.5 M acetic acid buffer was added to the leaves, which were fully ground and centrifuged at 7370 × g at 4 for 20 min. After centrifugation, the supernatant was taken and placed on ice for storage.

2.2.2 Determination of enzyme activity

A total of 10 mmol/L *p* -nitrophenyl–β-d-glucopyranoside was used as the substrate to analyze the activity of β-d-glucosidase. We then mixed 50 μL of suitably diluted enzyme solution and 100 μL substrate with 200 μL 0.05 mol/L acetic acid buffer (pH 5.0), then added 650 μL deionized water to preheat at 45 for 5 min. Then, 500 μL 1 mol/L sodium carbonate was added to the mixture to stop the reaction at 45 for 10 min. The absorbance value was measured at 420 nm. The amount of enzyme required to produce 1 μmol/L*p* -nitrophenol per unit time per unit volume defines the β-d-glucosidase activity. The standard curve was plotted by dissolving different concentrations of *p* -nitrophenol. The calculation formula of enzyme activity is as follows:

$$\text{Enzyme activity assay} = \frac{\text{A} \times \text{N} \times \text{Coefficient}}{\text{V} \times \text{t}},$$

A : Absorbance, N : Diluted multiples, V : Sample volume, t : Time, *Coefficient* : 5.7615.

2.3 Conversion of indican to indoxyl

The indican solution was added to four times the volume of anhydrous ethanol and stood overnight at room temperature for separation of protein, starch and other macromolecules that are insoluble in ethanol. The suspension was centrifuged at 11,510 × g for 5 min. The ethanol was removed and the indican solution was concentrated using a rotary evaporator. Cellulase was separated from *Acremonium cellulolyticus* which has the activity of β-d-glucosidase was added to concentrate for converse indican to indoxyl at 45 for 30 min.

2.4 High-performance liquid chromatography

2.4.1 Determination of indican and indoxyl

The qualitative quantification of indican and indoxyl was performed using high-performance liquid chromatography (HPLC) (UV detector at 280 nm, EX1600, Exformma, USA). An Eclipse XDB C18 column (250 mm × 4.6 mm, Agilent, USA) was used for separation at a flow rate of 1.0 mL/min and the temperature of the column oven was 30 . Mobile phase A (deionized water–acetic acid 100:0.5) and mobile phase B (acetonitrile). The elution ratio was A-80%: B-20% for 15 min. The UV detection wavelength was 240 nm.

2.4.2 Determination of indigo and indirubin

Indigo and indirubin were quantified using HPLC (UV detector at 280 nm, EX1600, Exformma). An Eclipse XDB C18 column (250 mm x 4.6 mm, Agilent) was used for separation at a flow rate of 1.0 mL/min and the temperature of the column oven was 35 . Mobile phase A (deionized water–acetic acid 100:0.5) and mobile phase B (acetonitrile). The elution ratio was A-60%: B-40% for 10 min. The UV detection wavelength was 280 nm.

2.5 Culture conditions

2.5.1 Medium, strain and culture conditions

This study used *S. aureus* (ATCC 6538) which was stored at –80 . The strain was precultured in 20 mL of 25 g/L Luria–Bertani (LB) medium at 37 for 12 h. The initial inoculation concentration was set at OD_{600}

= 0.05. The suspension culture medium (5 mL) was a mixture of LB (25 g/L) and glucose (5 g/L). Different concentrations of indoxyl were added. Test tubes were incubated (200 rpm) at 37 for 10 h.

2.5.2 Determination of components in medium

Glucose consumption and bacterial metabolite production were analyzed using HPLC (LC-20AD, Shimadzu, Kyoto, Japan) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The mobile phase was 5 mM H_2SO_4 and the flow rate was 0.6 mL/min at 65 .

2.6 Confocal laser scanning microscopy (CLSM)

S. aureus was cultured on glass slides in 6-well plates. Then, it was stained with four fluorescent dyes, namely, Calcofluor White (Sigma), Congo Red (40 μg mL^{-1}, Sigma-Aldrich), SYTO 9 (Thermo Fisher) and propidium iodide (PI; Thermo Fisher). Samples were stained for 30 min and washed with sterile PBS buffer three times. Finally, 0.5 M carbonate buffer solution mixed with an equal volume of glycerol was used to fix cells. The cells were observed with an Olympus FV3000 confocal laser scanning microscope.

2.7 Activity and metabolic assays

2.7.1 ATP assays and ADP/ATP ratio

The adenosine 5'-triphosphate (ATP) level was detected using an ATP Assay Kit (Beyotime, China). The ADP/ATP ratio was measured using an ADP/ATP Assay Kit (Sigma-Aldrich). An aliquot of each sample was transferred to a white 96-well plate for detection.

2.7.2 Metabolic assay

The content of intracellular hydrogen peroxide was measured with a Hydrogen Peroxide Assay Kit (Beyotime, China). The NAD^+/NADH and $NADP^+$/NADPH were measured with a NAD^+/NADH Quantification Kit and $NADP^+$/NADPH Quantification Kit (Sigma-Aldrich), respectively. All kits were used according to the manufacturer's instructions.

2.7.3 Scanning electron microscopy

Cells of *S. aureus* were prepared as described. Take several cells cultured for 10 h (OD_{600} = 0.5), remove the culture medium after centrifugation, wash twice with PBS, and discard the supernatant. After precooling, slowly add 2.5% glutaraldehyde fixed solution along the test tube wall and store it at 4 overnight. The cells were observed using scanning electron microscopy (SEM) (SU8010, Hitachi, Tokyo, Japan) as previously described (Kumari et al., 2019).

Results and Discussion

3.1 Components of bioactive substances

3.1.1 Influence of temperature on the components of leaves

As a perennial herb, *S. cusia* has broad application prospects in antibacterial medication, especially in antiviral and tumor treatments (Xu et al., 2021). Indole alkaloids are mainly synthesized by the indole pathway in *S. cusia* (Yu et al., 2019). Indigo and indirubin are the main active substances in the leaves of *S. cusia* (Yang et al., 2020). According to the traditional extraction methods, the temperature has a great impact on the extraction efficiency of bioactive substances from different biomass. In this study, the fresh leaves of*S. cusia* were treated with hot water at different temperatures, and the effect of temperature on the components of the leaves was investigated by determining indigo, indirubin and their precursors.

As shown in Fig. 1a, even though the increase in temperature could improve the extraction rate of indigo below 80 , the indigo content extracted at higher temperatures was significantly less than that at lower temperatures along with time. At 100 , no indigo was detected in the solution. The extraction results of indirubin were significantly different from those of indigo (Fig. 1b). At 80 , indirubin was detected after 15 min; meanwhile, the content of indirubin increased with the increase in temperature. However, indirubin

was not detected at 100 . This result indicates that the extraction temperature had a great influence on what was extracted. In general, an increase in temperature aids in the extraction of substances. This tendency of indigo indicates that temperature not only affected the extraction efficiency of active substances but also affected the formation or decomposition of indigo. Because indigo and indirubin still maintained good thermal stability after short-time heat treatment (Sun et al., 2021), it could be considered that the change in temperature would affect the formation of indigo and indirubin.

It was found that the content of indigo in the solution was the highest at about 1.91 mg/g when *S. cusia* was treated at 60 for 30 min (Fig. 1a). The contents of indirubin in the solution were the highest at about 0.12 mg/g after it was treated at 80 for 30 min (Fig. 1b). Indigo and indirubin were not detected in the 100 treatment group. Indigo and indirubin do not exist in the leaves of *S. cusia* , so these two bioactive substances must be produced in the extraction process.

Indican is a precursor of indigo and indirubin. At 80 and 100 , indican was detected, whereas there was no indican present in the solution at 60 and 40 (Fig. 1c). On the other hand, the intermediate in the conversion of indican to indigo and indirubin, indoxyl, was found under such conditions (Fig. 1d). The contents of indican and indoxyl in the solution were inversely proportional (Fig. 1c, d). In the general understanding, the extraction conditions affect the yield of the extracts with little effect on their components. In this study, the extraction conditions could not only change the yield of bioactives, but also the components and proportions of the bioactives, indicating that some reactions that affected the components of the biologically active substances took place during the extraction process. We assumed that revealing the reaction mechanism would help us to provide a theoretical and methodological basis for the evaluation and application of the bioactives in the future.

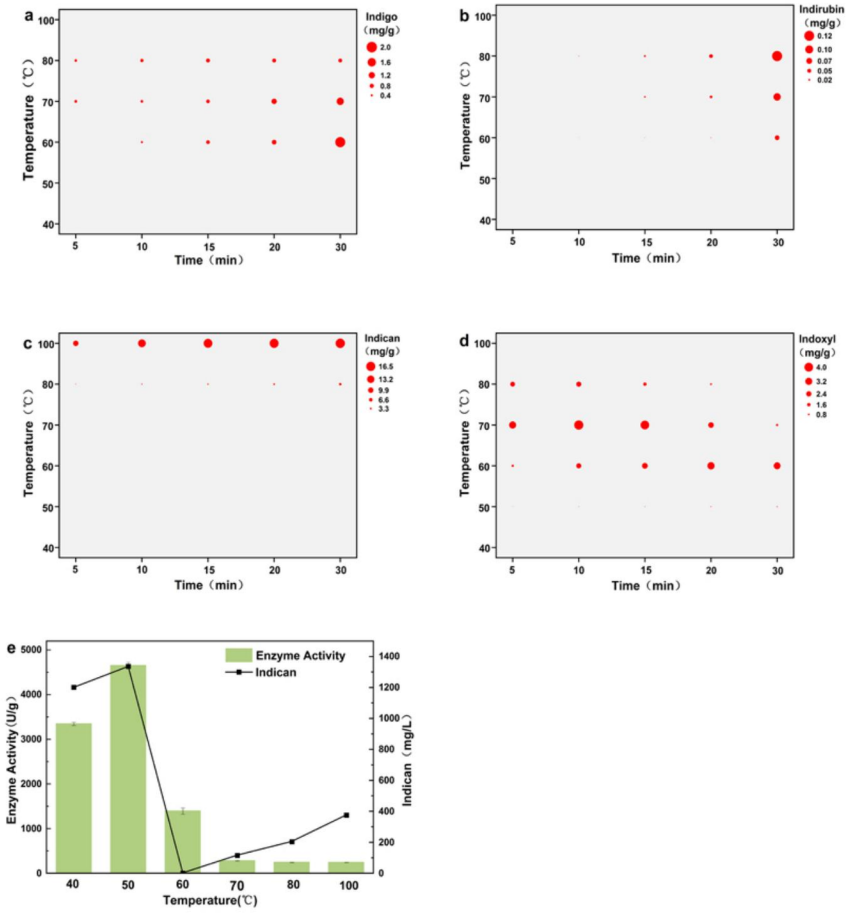


Figure 1. (a–d) Effects of temperature on the components of the bioactives of *S. cusia* . The content of (a) indigo, (b) indirubin, (c) indican (d) indoxyl. (e) The enzyme activity and the residual indican content in leaves.

3.1.2 Influence of temperature on enzyme activity

The changes in the components of the bioactives during the extraction process were all related to the content of indican. We hypothesized that temperature stress during extraction induced cells to synthesize β-glucosidase, which catalyzed the hydrolysis of indican released from vacuoles, thereby changing the components of the bioactives.

β-Glucosidase belongs to cellulase and exists in many plants and microorganisms. To study the influence of temperature on enzyme activity in leaves, the leaves were treated with water of different temperature at a solid–liquid ratio of 1:10 for 10 min. Then, the leaves were thoroughly ground and centrifuged to obtain the crude enzyme solutions for the determination of β-glucosidase activity. The experimental results show that there was a great difference in the enzyme activity of the crude enzyme solutions (Fig. 1e). The crude enzyme solution prepared at 40 had an enzyme activity of 3348.87 ± 36.12 U/g. Compared with the crude enzyme solutions obtained at the other temperatures, the enzyme activity in leaves was up to 4658.69 ± 64.13 U/g at 50 and gradually decreased with the increase in temperature. The β-glucosidase in leaves was basically inactivated at 70 , at which temperature the enzyme activity was only 283.98 ± 9.85 U/g. A further increase in temperature only slightly affected enzyme activity.

Taken together, it was considered that temperature-stress induced the synthesis of intracellular β-glucosidase, which could hydrolyze indican to indoxyl and glucose. At lower temperatures, more indoxyl condensed to form indigo; at relatively high temperatures, indoxyl was unstable and tended to form isatin, which eventually formed indirubin. At 100 , because of the low enzyme activity, indican cannot be hydrolyzed, so there was only indican without indigo and indirubin.

3.1.3 Influence of temperature on indican content in leaves

To further study the effect of β-glucosidase on indican, the leaves treated at different temperatures for 10 min were covered with boiled water and the released indican from the leaves was determined using HPLC. As shown in Fig. 1e, when the leaves were treated at lower temperatures (40 and 50), larger amounts of indican were detected even though the enzyme activity was higher. This could be because β-glucosidase cannot efficiently react with indican in vacuoles to produce indoxyl under such conditions so that the residual indican was high, reaching 1201.02 mg/L and 1336.31 mg/L, respectively (Fig. 1e). With an increase in temperature, the leaves were seriously damaged, and the indican released from vacuoles would be hydrolyzed by β-glucosidase even with low activity. When the leaves were treated at 100 for 10 min, the enzyme activity was too low to hydrolyze indican released from the vacuoles. Therefore, the largest amount of indican (376.16 g/L) was obtained from leaves.

According to the experimental results and previous studies, it could be considered that little indigo and indirubin were present in leaves before extraction. When suffering from the stresses from the outside, the plant starts an emergency mechanism to avoid the stress of the environment, i.e., indican is released from plant vacuoles and hydrolyzed by enzymes to form indoxyl and glucose. Previous studies have shown that indican is stored in plant vacuoles and released by chloroplasts. During natural fermentation, β-glucosidase hydrolyzes indican to form indole groups and glucose (Inoue et al., 2018). Hence, the morphology of some vacuoles would be destroyed under the temperature stress, resulting in the release of indican, to form indoxyl under the action of β-glucosidase.

3.2 Antimicrobial effects of the bioactives in B. cusia

The contents of indole alkaloids, mainly including indican, indoxyl, indigo and indirubin, were very different after the pretreatment of the fresh leaves of *B. cusia* at different temperatures (Fig. 1). To investigate the antibacterial activities of the different bioactives, the consumption of carbon sources and secondary metabolites during*S. aureus* culture with different inhibitors was measured. As shown in Fig. 2, the temperature had a strong effect on the antibacterial activity of the extract. When the bioactives extracted under the condition of 100 were used, the consumption of glucose and the production of acetic acid did not differ from those of the control group, indicating that there were no inhibitors in the bioactives extracted under the conditions of 100 . As shown in Fig. 1, there was only indican in the extract under the condition of 100 without indigo, indirubin and indoxyl. This result indicates that indican had no inhibitory effect on the growth and metabolism of microorganisms. When the bioactives extracted at 70 were used, the consumption of glucose and the generation of acetic acid were the slowest, indicating that the bioactives extracted at 70 had the strongest inhibitory effect on the growth and metabolism of microorganisms. As shown in Fig. 1, the bioactives extracted at 70 contained indigo, indirubin and indoxyl and its indoxyl content was significantly higher than other bioactives. Therefore, it could be considered that indoxyl should have a strong inhibitory effect on the growth and metabolism of microorganisms.

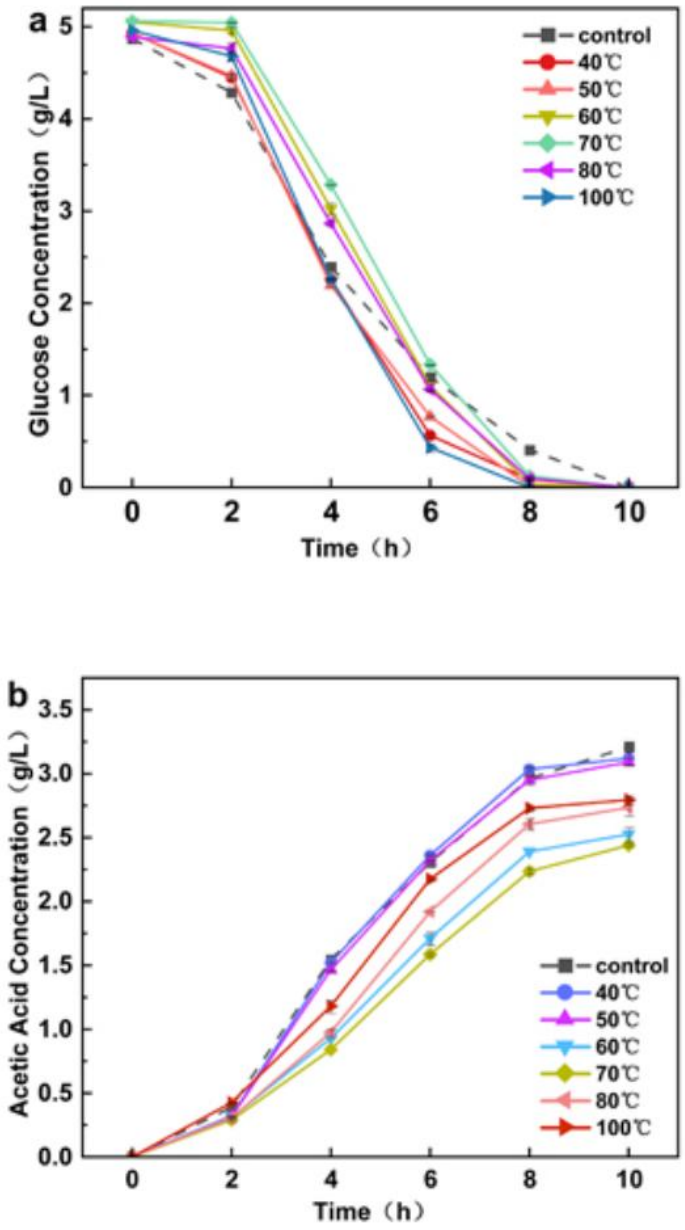


Figure 2. Inhibitory effects of the bioactives on *S. aureus* growth. Influence on (a) glucose consumption, (b) acetic acid production.

3.3 Antimicrobial effect of indoxyl

Indoxyl played a key role in the changes of indole alkaloids (Fig. 1), and during the dynamic change of the indole alkaloids, the content of indoxyl exhibited the highest inhibitory effect on the growth of*S. aureus* (Fig. 2). To investigate the inhibitory effect of indoxyl, indoxyl solution was first prepared by enzymatic treatment of the indican solution obtained at 100 in a nitrogen environment. Because indican extract contains a large

number of impurities such as protein, organic acid and chlorophyll (Feng et al., 2016), ethanol precipitation was used to remove the impurities from the indican solution in this study, followed by rotary evaporation to remove ethanol. The indoxyl concentration in the resulting solution was 1.71 g/L.

Indoxyl of different concentrations was added to the medium for *S. aureus* culture. As shown in Fig. 3a, OD_{600} decreased with the increase in indoxyl concentration. When the indoxyl content was low, the growth of *S. aureus* in the experimental group was similar to that in the control group, and glucose could be completely consumed within 10 h (Fig. 3b). With the increase in indoxyl concentration, the trend of glucose consumption gradually weakened. In particular, when the inhibitory concentration was 0.6 g/L, the value of OD_{600} in the solution remains basically unchanged, little glucose was consumed, and the production of acetic acid was suppressed (Fig. 3c). To further confirm the inhibitory effect of indoxyl on *S. aureus* , cells were cultured for 10 h and then coated with a plate to calculate the number of living cells. The results showed that the cell activity of *S. aureus* would be affected by indoxyl. The number of living cells per unit volume was inversely proportional to the concentration of indoxyl (Fig. 3d).

3.4 Comparison of the inhibitory effects of indigo, indirubin and indoxyl

Previous studies showed that indigo and indirubin exhibited antimicrobial effects (Chiang et al., 2013). On the other hand, there have been no studies on the inhibitory effect of indoxyl. Because indoxyl is unstable in the presence of oxygen, it is possible that parts of indoxyl may be oxidized to indigo and indirubin during microbial growth. To determine if the indigo and indirubin produced during the oxidation of indoxyl had an inhibitory effect on the growth of *S. aureus* , the bacteria were cultured in the media containing the same concentrations of indigo and indirubin with indoxyl.

As shown in Fig. 3e and 3f, indigo and indirubin had little effect on glucose consumption and acetate production. On the other hand, the inhibition by indoxyl of glucose consumption and acetic acid formation was significantly higher than those of indigo and indirubin. This could be because indigo and indirubin had poor solubility in aqueous solution so that they did not have a strong antibacterial effect, whereas it was easier for indoxyl as a soluble small molecular substance to enter into cells, affecting their growth.

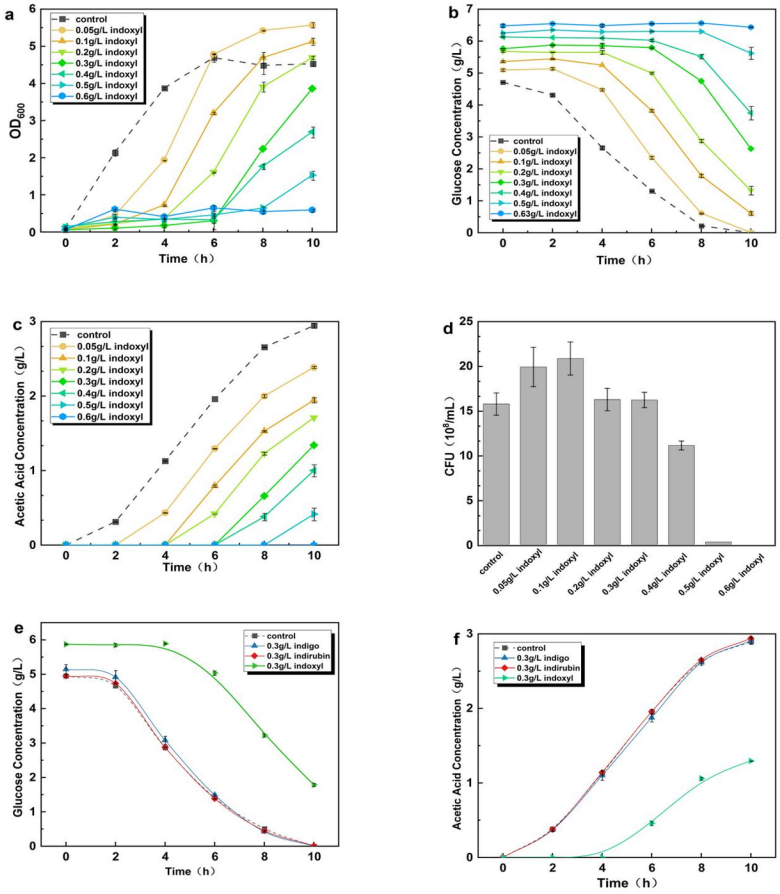


Figure 3. Inhibitory effect of indoxyl on *S. aureus*growth. Influence on (a) OD_{600}, (b) glucose consumption, (c) acetic acid production, (d) Colony–Forming Unit (CFU) at 10 h. (e, f) A comparison among the antimicrobial effects of indigo, indirubin and indoxyl at the same concentration. Influence of indigo, indirubin and indoxyl on (e) glucose consumption, (f) acetic acid production.

3.5 Influence of indoxyl on the morphology ***ofS. aureus***

SEM was used to confirm the influence of indoxyl on cell morphology. As shown in Fig. 4, the untreated cells were complete spheres with a smooth surface. After treatment with indoxyl, the cells had wrinkles with irregular shapes (Fig. 4c, d). Moreover, some fragments of cells could be found in the images, indicating that the indoxyl destroyed the integrity of the cells.

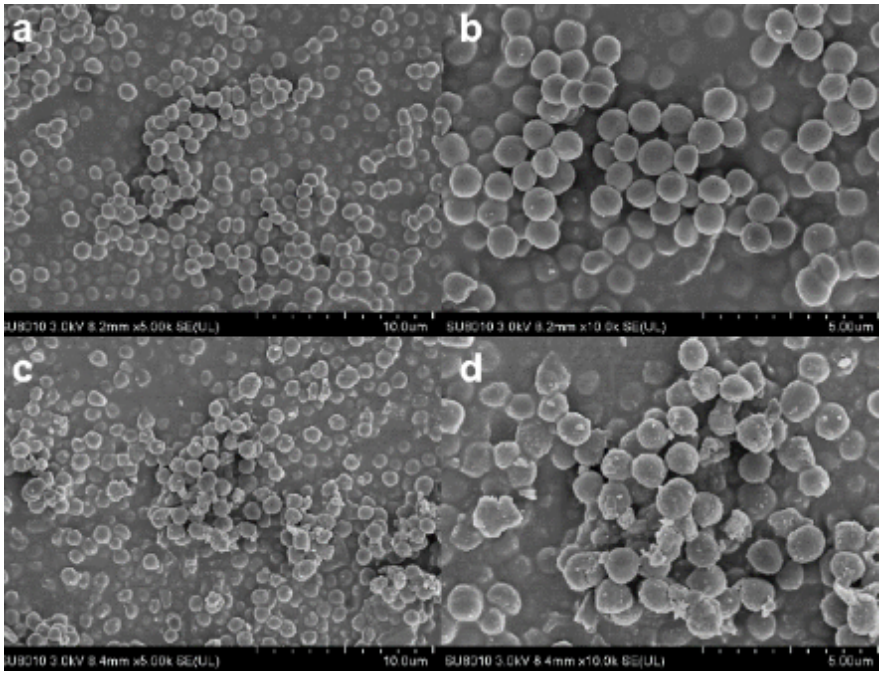


Figure 4. Observation of cell morphology using SEM. (a, b) Control culture. (a) ×5.0k, (b) ×10.0k. (c, d) IC_{50}culture. (a) ×5.0k, (b) ×10.0k. Culture was carried out at 37 and 200 rpm for 12 h in test tubes.

Furthermore, the cells cultured on glass slides were observed using CLSM. The cells were stained with Calcofluor White (blue fluorescence), SYTO 9 (green fluorescence) and PI (red fluorescence) and were examined using CLSM. Calcofluor White has high specificity to specifically bind to β-1,3 and β-1,4 polysaccharides in cellulose and chitin (Zhao et al., 2020), which are the major components of EMs (Cui et al., 2019). SYTO 9 could stain the cells with intact cell membranes and PI could stain the cells with damaged cell membranes (Takenaka et al., 2001). All samples were stained with the three dyes. As shown in Fig. 5, when indoxyl was absent, blue fluorescence was concentrated and strong. This phenomenon indicates that large amounts of EMs were formed. In the presence of indoxyl, blue fluorescence decreased compared with the control group, and the higher concentration of indoxyl resulted in a weaker blue fluorescence, indicating that the presence of indoxyl can inhibit the formation of EMs. Moreover, indoxyl decreased green fluorescence and increased red fluorescence, indicating the destruction of the cell membrane by indoxyl. When indoxyl was 0.6 g/L, no emission was detected, indicating that *S. aureus* could not grow under such a condition.

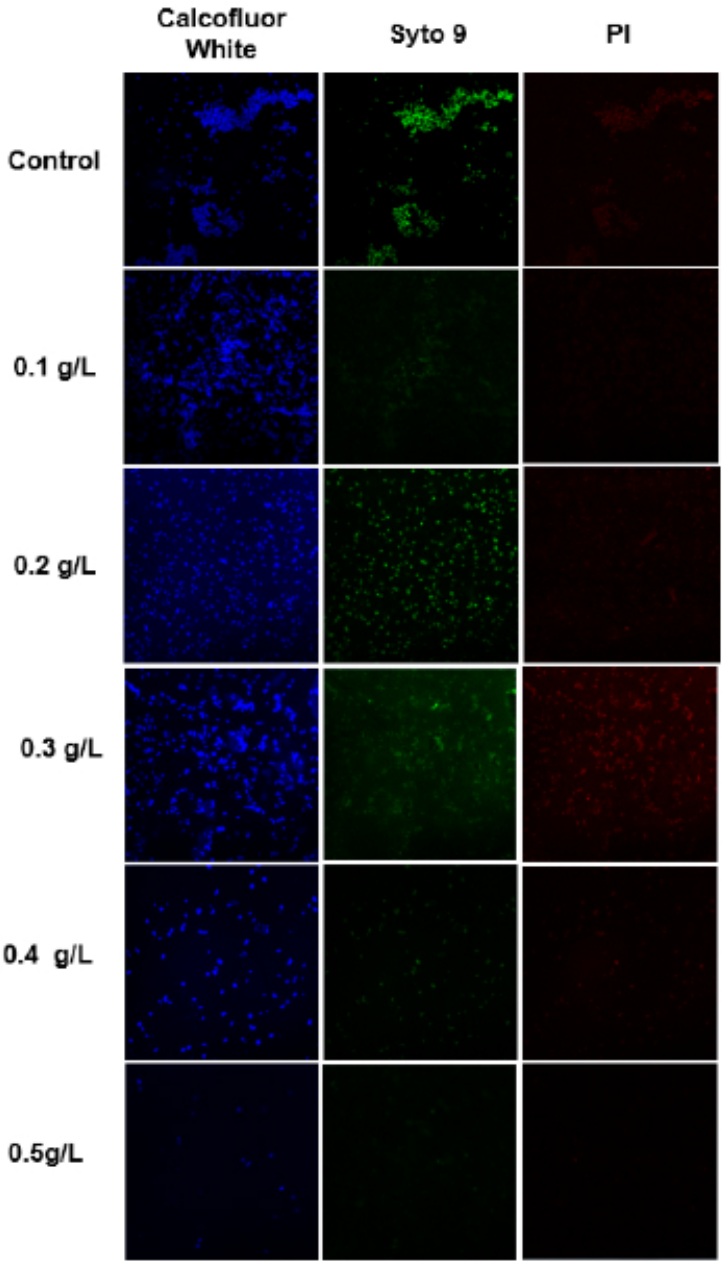


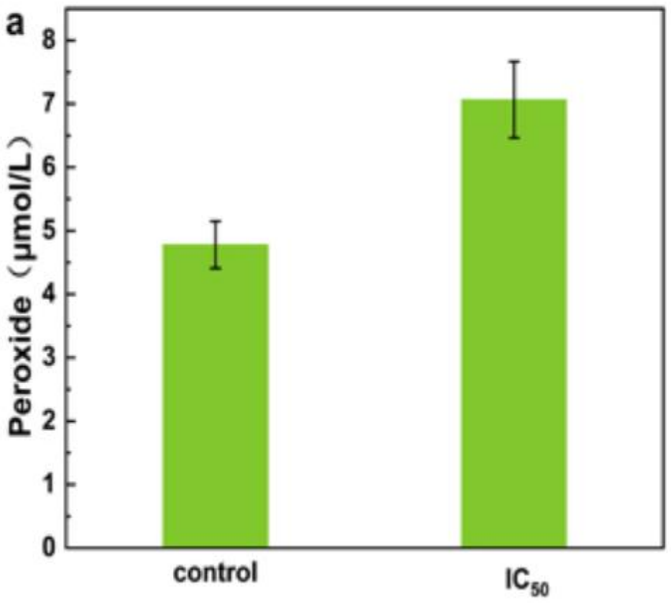
Figure 5. Observation of EM formation using CLSM images. SYTO 9 (green area) stained the cells with intact cell membranes, PI (red area) stained the cells with damaged cell membranes, and Calcofluor White (blue area) stained polysaccharides.

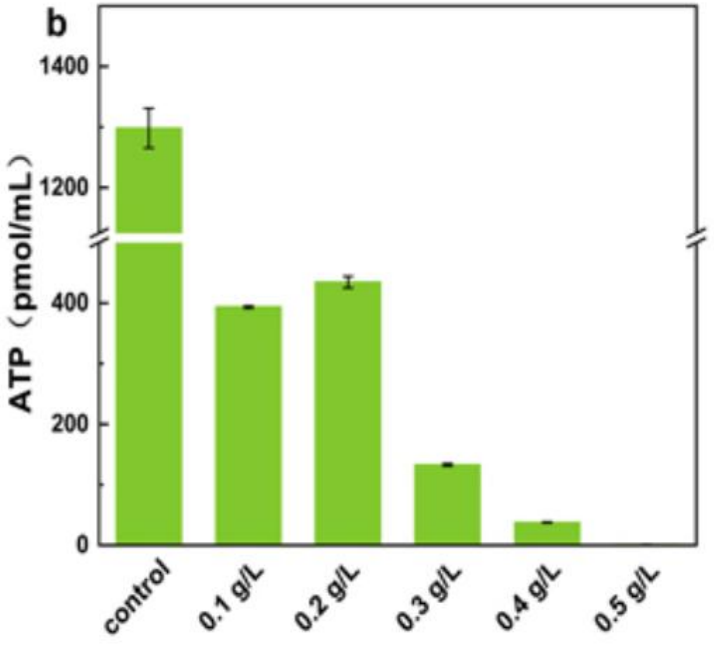
The CLSM and SEM images suggest that cell morphological changes would be caused by the inhibition of EM formation by indoxyl. Decreased EMs can promote cytoplasmic loss, and transmission electron microscopy can observe the separation of the cell membrane from the cell wall due to the loss of cytoplasm (Wei et al., 2021). Atomic force microscopy can observe cell shrinkage caused by external stress, resulting in cell surface folds (Wang et al., 2020). In this study, the damage to the cell membrane observed using CLSM may also

lead to the loss of cytoplasm, and then the cells undergo morphological changes.

3.6 Influence of indoxyl on intracellular redox status

To explore the influence of indoxyl on the intracellular redox status of*S. aureus* , the activity level of cells was studied and analyzed during cell growth. As shown in Fig. 6a, indoxyl increased the level of intracellular peroxide, indicating that it affected the intracellular redox status. Moreover, the production of peroxides also has a very large side effect on cell activity (Cui et al., 2019). The results were also consistent with this conclusion (Fig. 3). As the main products in the tricarboxylic acid (TCA) cycle, ATP and NADH can be used to determine the growth of cells. The gradual decrease of intracellular ATP content and increase of NAD^+/NADH ratio confirmed the inhibitory effect of indoxyl on the TCA cycle (Fig. 6a, c). Therefore, it could be considered that indoxyl destroyed the TCA cycle and reduced the generation of ATP and reducing power. The change in redox status promoted the generation of H_2O_2, which may damage cells through the oxidation of lipids, proteins and DNA.





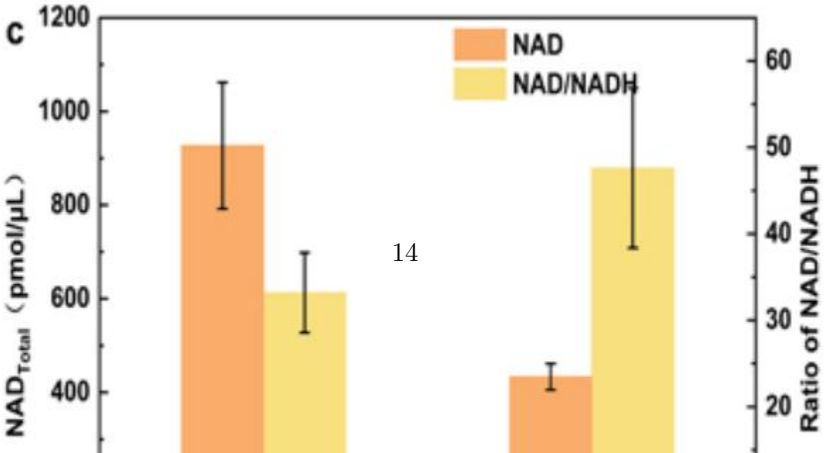


Figure 6. Effect of indoxyl on intracellular redox status of*S. aureus* . (a) Peroxide content in cells collected at 10 h, (b) changes in intracellular ATP, (c) NADH and the ratios to NAD^+ in cells collected at 10 h.

Conclusion

In this study, it was found that the extraction temperature had a huge effect on the bioactive components of *S. cusia* . Indoxyl formed in this extraction process inhibited cell growth and metabolism by disrupting EM formation and altering intracellular redox, and its inhibitory effect was much higher than the traditionally recognized bioactives in banlangen, such as indigo and indirubin. Moreover, the precursor of indoxyl, indican, did not have any antimicrobial activity. Simulating the hydrolysis of indican by β-glucosidase in plants, a novel antibacterial mode with indican could be formed, improving the medicinal properties of antimicrobial agents.

Abbreviations

S. cusia , *Strobilanthes cusia* ; EMs, extracellular matrices; *S. aureus Staphylococcus aureus* ; HPLC, high-performance liquid chromatography; Indican, indoxyl-β-d-glucosidase

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Reference

Chiang, Y.R., Li, A., Leu, Y.L., Fang, J.Y., Lin, Y.K. 2013. An in vitro study of the antimicrobial effects of indigo naturalis prepared from Strobilanthes formosanus Moore. *Molecules* , **18** (11), 14381-96.

Cui, S., Ma, X., Wang, X., Zhang, T.-A., Hu, J., Tsang, Y.F., Gao, M.-T. 2019. Phenolic acids derived from rice straw generate peroxides which reduce the viability of Staphylococcus aureus cells in biofilm.*Industrial Crops and Products* , **140** , 111561.

Feng, L., Yin, J., Nie, S., Wan, Y., Xie, M. 2016. Fractionation, physicochemical property and immunological activity of polysaccharides from Cassia obtusifolia. *Int J Biol Macromol* , **91** , 946-53.

Gatadi, S., Madhavi, Y.V., Chopra, S., Nanduri, S. 2019. Promising antibacterial agents against multidrug resistant Staphylococcus aureus.*Bioorg Chem* , **92** , 103252.

Grundmann, H., Aires-de-Sousa, M., Boyce, J., Tiemersma, E. 2006. Emergence and resurgence of meticillin-resistant Staphylococcus aureus as a public-health threat. *The Lancet* , **368** (9538), 874-885.

Inoue, S., Morita, R., Kuwata, K., Kunieda, T., Ueda, H., Hara-Nishimura, I., Minami, Y. 2018. Tissue-specific and intracellular localization of indican synthase from Polygonum tinctorium. *Plant Physiol Biochem* , **132** , 138-144.

Kumagai, T., Aratsu, Y., Sugawara, R., Sasaki, T., Miyairi, S., Nagata, K. 2016. Indirubin, a component of Ban-Lan-Gen, activates CYP3A4 gene transcription through the human pregnane X receptor. *Drug Metab Pharmacokinet* , **31** (2), 139-45.

Kumari, M., Giri, V.P., Pandey, S., Kumar, M., Katiyar, R., Nautiyal, C.S., Mishra, A. 2019. An insight into the mechanism of antifungal activity of biogenic nanoparticles than their chemical counterparts.*Pestic Biochem Physiol* , **157** , 45-52.

Li, R.L., Zhang, Q., Liu, J., He, L.Y., Huang, Q.W., Peng, W., Wu, C.J. 2021. Processing methods and mechanisms for alkaloid-rich Chinese herbal medicines: A review. *J Integr Med* , **19** (2), 89-103.

Li, T., Peng, T. 2013. Traditional Chinese herbal medicine as a source of molecules with antiviral activity. *Antiviral Res* ,**97** (1), 1-9.

Liau, B.C., Jong, T.T., Lee, M.R., Chen, S.S. 2007. LC-APCI-MS method for detection and analysis of tryptanthrin, indigo, and indirubin in daqingye and banlangen. *J Pharm Biomed Anal* , **43** (1), 346-51.

Liu, W.T., Chen, E.Z., Yang, L., Peng, C., Wang, Q., Xu, Z., Chen, D.Q. 2021. Emerging resistance mechanisms for 4 types of common anti-MRSA antibiotics in Staphylococcus aureus: A comprehensive review.*Microb Pathog* , **156** , 104915.

Roy, R., Tiwari, M., Donelli, G., Tiwari, V. 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. *Virulence* , **9** (1), 522-554.

Satpathy, S., Sen, S.K., Pattanaik, S., Raut, S. 2016. Review on bacterial biofilm: An universal cause of contamination.*Biocatalysis and Agricultural Biotechnology* , **7** , 56-66.

Shi, J., Wang, Y., Wei, H., Hu, J., Gao, M.-T. 2020. Structure analysis of condensed tannin from rice straw and its inhibitory effect on Staphylococcus aureus. *Industrial Crops and Products* ,**145** , 112130.

Singh, S., Numan, A., Somaily, H.H., Gorain, B., Ranjan, S., Rilla, K., Siddique, H.R., Kesharwani, P. 2021. Nano-enabled strategies to combat methicillin-resistant Staphylococcus aureus. *Mater Sci Eng C Mater Biol Appl* , **129** , 112384.

Sun, j., Zhu, Y., Wang, H., Niu, X., Zhang, J., Gao, Q. 2021. Comparison of stability between heterologous expressed indigoidine in Escherichia coli and indigo. *Microbiology China* , **48** (01), 1-13.

Takenaka, S., Iwaku, M., Hoshino, E. 2001. Artificial Pseudomonas aeruginosa biofilms and confocal laser scanning microscopic analysis.*J Infect Chemother* , **7** (2), 87-93.

Wang, H., Wang, Z., Wei, C., Wang, J., Xu, Y., Bai, G., Yao, Q., Zhang, L., Chen, Y. 2021. Anticancer potential of indirubins in medicinal chemistry: Biological activity, structural modification, and structure-activity relationship. *Eur J Med Chem* , **223** , 113652.

Wang, Y.z., Yang, J., Wei, H., Hou, R., Shi, J., Jin, Z., Yang, F., Hu, J., Gao, M.-T. 2020. Reduction of Fermentation-Associated Stresses by Straw-Based Soluble Saccharides for Enhancing Ethanol Production.*Journal of Agricultural and Food Chemistry* , **68** (21), 5863-5872.

Wei, H., Jin, Z., Wang, Y., Yang, F., Xiao, Y., Jiang, Y., Hu, J., Gao, M.T. 2022. Antibacterial Effect of Phenolic Acids Derived from Rice Straw and in Combination with Antibiotics Against Escherichia coli.*Appl Biochem Biotechnol* .

Wei, H., Wang, Y., Jin, Z., Yang, F., Hu, J., Gao, M.T. 2021. Utilization of straw-based phenolic acids as a biofugicide for a green agricultural production. *J Biosci Bioeng* , **131** (1), 53-60.

Xu, Z., Cai, Y., Chen, Y., Zhao, Z., Ma, Q., Yang, D., Xu, X. 2021. Chemical Constituents from the Leaves of Baphicacanthus cusia.*Journal of Chinese Medicinal Materials* (08), 1876-1880.

Yang, L., He, C., Sudunabuqi, Bao, X., Wang, X., Ao, W. 2020. Alkaloids from the leaves of Isatis indigotica fortune and their chemotaxonomic significance. *Biochemical Systematics and Ecology* , **93** , 104163.

Yu, H., Li, T.-n., Ran, Q., Huang, Q.-w., Wang, J. 2021. Strobilanthes cusia (Nees) Kuntze, a multifunctional traditional Chinese medicinal plant, and its herbal medicines: A comprehensive review. *Journal of Ethnopharmacology* , **265** , 113325.

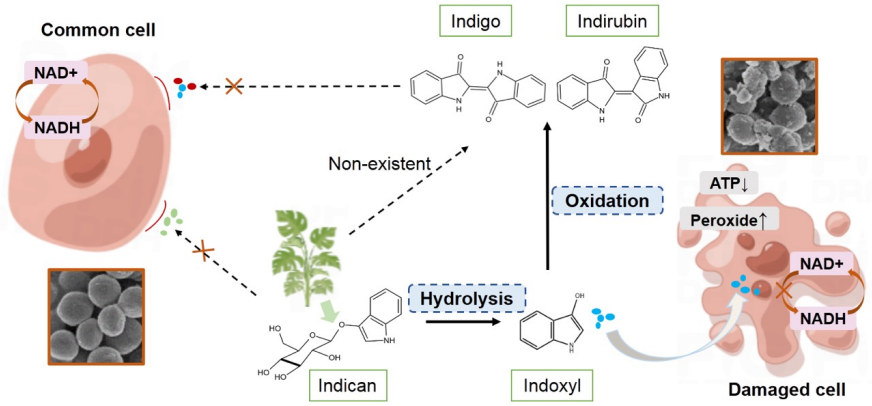
Yu, J., Zhang, Y., Ning, S., Ye, Q., Tan, H., Chen, R., Bu, Q., Zhang, R., Gong, P., Ma, X., Zhang, L., Wei, D. 2019. Molecular cloning and metabolomic characterization of the 5-enolpyruvylshikimate-3-phosphate synthase gene from Baphicacanthus cusia. *BMC Plant Biol* ,**19** (1), 485.

Zhang, L., Yang, H., Wang, Y., Zhuang, H., Chen, W., Lin, Z., Xu, J., Wang, Y. 2021. Blue footprint: Distribution and use of indigo-yielding plant species Strobilanthes cusia (Nees) Kuntze. *Global Ecology and Conservation* , **30** .

Zhang, Y., Huang, Y., Wan, H., Li, Z., Sun, W., Wu, S. 2020. Advances in biosynthesis of indigo in plants. *CHINA JOURNAL OF CHINESE MATERIA MEDICA* , **45** (03), 491-496.

Zhao, R.H., Gao, W., Qiu, L., Chen, X., Dong, X., Li, C., Huang, J. 2020. A staining method for detection of Enterocytozoon hepatopenaei (EHP) spores with calcofluor white. *J Invertebr Pathol* ,**172** , 107347.

Zhou, B., Yang, Z., Feng, Q., Liang, X., Li, J., Zanin, M., Jiang, Z., Zhong, N. 2017. Aurantiamide acetate from baphicacanthus cusia root exhibits anti-inflammatory and anti-viral effects via inhibition of the NF-kappaB signaling pathway in Influenza A virus-infected cells. *J Ethnopharmacol* , **199** , 60-67.



Graphical Abstract