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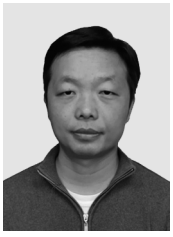
Anatomical Structure of *Gelsemium elegans* Benth and the Antibacterial Action of Alkaloids



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Abstract: In the present study, the microstructure of *Gelsemium elegans* vegetative organs was observed, and the alkaloids in plants were localized. Also, the antibacterial activity of the alkaloids was explored, and the antibacterial mechanism of the alkaloids was analyzed. The anatomical study of the structural characteristics of *G. elegans* vegetative organs was carried out with anatomical methods, and through histochemical staining, the alkaloids in the plant were localized. Microscopic observation results showed that the structure of *G. elegans* performed the typical characteristics of the dicotyledonous plants; the leaf of *G. elegans* was dorsiventral, and there were starch granules deposited in stem pith. The histochemical results showed that the vegetative organs of *G. elegans* contained alkaloids. Alkaloids were mainly distributed in the mesophyll tissue of leaves. In the stem, alkaloids were mainly distributed in stem epidermis, cortex cells and phloem parenchyma cells. Also, alkaloids were distributed in parenchyma cells of pith and ray cells. Yellow-brown sedimentation also could be observed in the cork cambium, the inner layer, the cortex, phloem parenchyma and ray cells of the rhizome. The antibacterial activity results showed that the *G. elegans* alkaloid had antibacterial activity against *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25923, *Staphylococcus aureus* CMCC26003, *Escherichia coli* ATCC25922, *Escherichia coli* O157, *Pseudomonas aeruginosa* ATCC27853, and *Pseudomonas aeruginosa* CMCC10104. With the increase of alkaloid concentration, the antibacterial effect was also enhanced. The inhibitory effect on *Listeria monocytogenes* was the most obvious. After treatment with its minimum inhibitory and lethal concentrations, the morphology and internal structure of *Staphylococcus aureus* ATCC25923 and *Escherichia coli* O157 showed that both two kinds of bacteria changed in different degrees, such as rough surface, depression, rupture of the cell membrane and cell wall, overflow of internal substance and even cell lysis. The morphology of *S. aureus* changed at the minimum inhibitory concentration and the cell wall of *E. coli* was stratified. This indicated that *G. elegans* alkaloid destroyed the permeability of the cell membrane, impeded the formation of the cell wall, and lysed the bacteria. The cell death at the minimum lethal concentration might be caused by the loss and cleavage of the mechanical strength and permeability of the wall.

Key word: *Gelsemium elegans* Benth; anatomical structure; alkaloid distribution; bacteriostatic effect; bacteriostatic mechanism

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钩吻解剖结构及钩吻生物碱抑菌作用研究

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摘 要: 对钩吻营养和繁殖器官进行解剖学方面研究, 并对植物体内的生物碱的分布位置进行定位, 此外, 还探索了钩吻生物碱的抑菌活性, 并对其抑菌机制进行分析研究。研究结果表明: 钩吻结构符合一般双子叶植物的典型特征, 叶为

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异面叶,茎圆形,表皮有气孔,髓中有淀粉粒沉积。组织化学染色研究显示,钩吻全株含有生物碱,生物碱类物质主要分布在叶的叶肉组织中,尤其栅栏组织含量较多,维管束中少或无;在茎中,生物碱类物质主要分布在茎的表皮、皮层细胞、韧皮薄壁细胞中,另外,在茎髓周围的薄壁细胞及髓射线细胞中也分布有生物碱类物质;在钩吻根状茎的木栓形成层、栓内层、皮层、韧皮部的薄壁细胞及射线细胞中均可观察到。通过滤纸片法、二倍微量稀释法测定钩吻生物碱的抑菌活性和最小抑菌浓度,结果显示,钩吻生物碱具有广谱抗菌性,对李斯特氏菌 ATCC19115、金黄色葡萄球菌 ATCC25923、金黄色葡萄球菌 CMCC26003、大肠杆菌 ATCC25922、大肠杆菌 O157、铜绿假单胞菌 ATCC27853、铜绿假单胞菌 CMCC10104 这7种供试菌都具有抑菌活性,且随着生物碱浓度的增加,抑菌效果也随之加强,其中对李斯特菌抑菌效果最明显。选择革兰氏阳性菌金黄色葡萄球菌 ATCC25923 和革兰氏阴性菌大肠杆菌 O157,测定出它们的最小致死质量浓度(MBC)分别为 500 和 750 g/L,利用透射电子显微镜观察钩吻生物碱在其最小抑菌浓度和最小致死浓度处理前后金黄色葡萄球菌 ATCC25923 和大肠杆菌 O157 菌体形态及内部结构的变化,结果发现2种菌体都发生了不同程度的改变,具体表现在菌体表面粗糙,出现凹陷,细胞膜和细胞壁发生破裂,内容物溢出甚至引发细胞裂解,发现在最小抑菌浓度下金黄色葡萄球菌形态发生改变,而大肠杆菌的细胞壁也出现分层现象,这表明生物碱破坏了菌体细胞壁的形成,因为细菌形状受到肽聚糖囊泡影响。在最小致死浓度下细胞死亡可能是由于壁的机械强度和渗透性的丧失并发生裂解所引起的。

关键词:钩吻;解剖结构;生物碱分布;抑菌作用;抑菌机制

Gelsemium elegans is evergreen vine of *Gelsemium*. *Gelsemium* was first attributed to the Apocynaceae. Later, according to the relationship between the number of chromosomes and phylogeny, it was revised to be attributed to Loganiaceae, and then it was separately formed into a family, the Gelsemiaceae[1-3]. *G. elegans* is highly toxic, and its main active ingredient is steroidal alkaloid, which can significantly inhibit central nervous system activity[4-6]. There are three species of *Gelsemium* plants in the world, including *G. elegans* in China and *G. sempervirens*, *G. rankinii* in North America[7-8]. China has a long history of the application of *G. elegans*, but it is mainly for external use, such as eliminating swelling[4]. At present, lots of studies have been conducted on the chemical composition, toxicology and pharmacological effects of *G. elegans*. The studies indicate that *G. elegans* can be applied to the treatment of diseases such as anti-tumor, analgesia, immune regulation and anti-anxiety[9-10]. *G. elegans* contains various secondary metabolites such as alkaloids, iridoids and triterpenoids. The main components are koumine, geliemine, gelsenicine, and gelsevirine, etc[5-6,11]. Moreover, many studies on secondary metabolites of various plants show that alkaloids have various functions such as anti-viral, inhibition of bacterial growth and anti-fungal[12].

Currently, the anatomical study of the vegetative organs of *G. elegans*, the distribution of alkaloids and the study on the inhibition of alkaloids have not been reported. In this study, the anatomical study of various organs of *G. elegans* was performed to explore the distribution of *G. elegans* alkaloids in plants, and the antibacterial action and mechanism of alkaloids.

1 Materials and Methods

1.1 Raw materials

The plant materials of this research were whole plants of wild *Gelsemium elegans* (Gardn, et champ.) Benth., which was identified as *G. elegans* Benth. of *Gelsemium*, Gelsemiaceae by Doc. Junxia Su, Shanxi Normal University. The samples were collected in July 2017 from the mountain roadside about 600 meters in Yulin City, Guangxi Province, China (110°17′ E, 22°63′ N). There were three gram-positive bacteria, *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25923, *Staphylococcus aureus* CMCC26003 and four gram-negative bacteria, *Escherichia coli* ATCC25922, *Escherichia coli* O157: H7NCTC12900, *Pseudomonas aeruginosa* ATCC27853 and *Pseudomonas aeruginosa* CMCC10104, all of which were from the China General Microbiological Culture Collection Center.

1.2 Analysis and test of plant materials

1.2.1 Paraffin section and histochemical detection After the plant raw materials were fixed by FAA

(formalin, acetic and alcohol with the volume ratio of 5∶5∶90), it was dehydrated with a graded series of ethanol, diaphanized in xylene, infiltrated with paraffin. The plant materials were embedded with a Leica EG1150H type paraffin embedding machine, and sectioned in a rotary microtome with the thickness of 6 – 8 μm. Then the sections were stained with safranine and fast green, and the Olympus FSX100 microscope was used to capture images. After the unstained sections were dewaxed, a modified solution of potassium bismuth iodide (Dragendorff) was added dropwise, and then they were stood at room temperature for 2 – 5 min. The color reaction of the sections was observed and the photomicrographs were obtained by using an Olympus FSX100 microscope.

1.2.2 Micromorphology analysis The plant materials for SEM analysis were dehydrated with a graded series of ethanol, transferred to isopentyl acetate, dried in a critical point dryer and coated with gold by using a sputter coater. Then they were inspected and photographed with an S-3400N scanning electron microscope.

In order to observe the ultrastructural changes of the strains after alkaloids treatment, we have carried out the observation on the strains at the ultrastructural level. After alkaloids treatment, *S. aureus* ATCC 25923 and *E. coli* O157 cultures cells were centrifuged at 5 000 r/min for 5 min. The resulting cell pellets were fixed by 2.5% gluteraldehyde-phosphate buffer saline(PBS) solution for 24 h at 4 ℃, followed by rinsed with PBS solution for three times. It was further fixed in osmium tetroxide solution(1 g osmium tetroxide dissolved in 100 mL PBS) for 24 h at 4 ℃. The bacterial samples were then dehydrated in gradient ethanol series(30%, 50%, 70%, 95% and 100%), each 10 min, followed by 10 min of acetone dehydration, SPI 812 resin (Electron Microscopy Sciences(EMS), Hatfield, PA) infiltration and embedding. Ultrathin sections(50 – 70 nm) were cut by using a ultra-microtome(Leica Microsystems UC 6), loaded onto the copper grid, and stained with 1% aqueous uranyl acetate for 20 min and Reynolds lead for 6 min. The images were observed on a Hitachi 7650 transmission electron microscope, operating at 200 kV.

1.3 Preparation of total alkaloids

G. elegans leaves and tender stems were dried and crushed, then they were soaked in 95% ethanol solution for 48 h. The extract was filtered and then distilled under reduced pressure. The concentrated solution was acidified with 2% hydrochloric acid to the pH value 2.0, and a small amount of petroleum ether was added to remove fat-soluble impurities. The pH value of the lower layer was adjusted to 9 – 10 with ammonium hydroxide, and the obtaining solution was extracted with chloroform and concentrated to obtain *G. elegans* alkaloids extract. It was dissolved with the aqueous solution containing 0.5% dimethyl sulfoxide to obtain *G. elegans* alkaloids solution with the mass concentration of 1 000 g/L.

1.4 Biological activity test of alkaloids

1.4.1 Test strain activation and bacterial suspension preparation 5 mL yeast extract liquid culture medium was placed in a sterilized test tube. The tested bacteria was added to the test tube and activated in a incubator at 37 ℃ for 8 h. Then the activated bacterial solutions were inoculated in 5 mL yeast extract liquid medium and cultivated in incubator at 37 ℃ for 14 h to prepare activated bacterial suspension. Sterile saline was used to dilute the concentration of bacterial solution to 10^6 – 10^7 CFU/mL for use.

1.4.2 Inhibition zone experiment The filter paper diffusion method was used to study the antibacterial effect of *G. elegans* alkaloids. 100 μL of bacterial suspension was added to the solid medium, and the bacterial suspension was spread evenly on the surface of the medium. Then a sterilized filter paper was placed on the surface of the medium, and 10 μL of *G. elegans* alkaloids solution was dripped on the filter paper. 0.5% dimethyl sulfoxide was used as the negative control, and saline was used as the blank control. Then *G. elegans* alkaloids with the mass concentration of 500, 250, 125, 62.5 g/L were put on the corresponding filter paper to explore the inhibition effect of *G. elegans* alkaloids on different bacteria, and each strain was repeated for 3

times. After placing the plate in incubator at 37 ℃ for 8.0 h, the diameters of the inhibition zones were measured. If the diameter of the inhibition zone is greater than the diameter of the filter paper(7.0 mm), it is evaluated as having a bacteriostatic effect. The saline and 0.5% dimethyl sulfoxide have no bacteriostatic effect.

1.4.3 Determination of the minimum inhibitory concentration(MIC), growth curve, the minimum lethal concentration(MBC) and lethal curve The MICs of *G. elegans* alkaloids to the strains were determined by the double microdilution method. 1.0 mL of activated bacterial suspension was taken and centrifuged for 2 min. The supernatant was discarded, and 1.0 mL of physiological saline was added and mixed well. 100 μL of Mueller-Hinton Agar(MHA) liquid medium was added to 96-well plates, 200 μL alkaloids solution was added to the outermost periphery as control. Then 100 μL of alkaloids solution with the mass concentration of 200 g/L was added to the first row of wells and mixed evenly. After that, 100 μL of the mixed solution was taken from the first row of wells and added to the second row of wells and mixed evenly, and so on until the last row. After being mixed well, half of the solution of each well was drawn out. Then 100 μL of the activated bacterial suspension was added to the wells with the mixture of alkaloid and medium and was placed in incubator at 37 ℃ for 16 – 18 h to determine the bacterial growth. 100 μL was drawn out from the liquid, spread on a solid medium and cultivated for 24 h to observe the colony growth, and determine the range of MIC of *G. elegans* alkaloid.

The growth curve was measured by using a microplate reader, and the absorbance of the 7 tested strains at different alkaloid concentrations at 600 nm wavelength was measured. The change in bacterial growth density with time was taken as an indicator, measured every 2 h, for a total of 24 h. The time and OD_{600} were respectively taken as the abscissa and ordinate to observe the inhibition effect of alkaloids on bacterial growth and evaluate the inhibitory effects of *G. elegans* alkaloids on the growth curve of the tested strains.

The minimum concentration sample solution in the culture medium with no turbidity and visible growth of the colonies after being cultured for 24 h in the incubator was selected from the 96-well plate for determining the MIC value of the bacterial species. 100 μL liquid was taken to spread evenly on the solid medium, and incubated at 37 ℃ for 24 h. MBC was defined as the minimum bactericidal concentration in the solid medium.

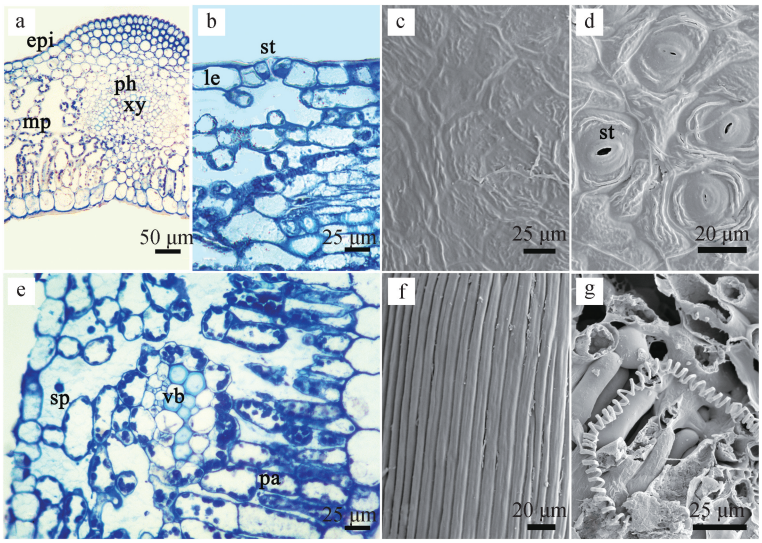
0.5% DMSO aqueous solution was used to formulate *G. elegans* total base into different concentrations. The activated suspension was washed in phosphate buffer saline(PBS) solution and inoculated into the corresponding *G. elegans* alkaloids solution at the proportion of 1∶100. Then it was mixed and incubated with aeration for 0, 15, 60, 120 min at 37 ℃. Finally, the survival of the bacterial species at different *G. elegans* alkaloids concentrations and incubation time was calculated by using the PBS serial dilution method, and the lethal curve was determined.

2 Results and Discussion

2.1 Anatomical structure and alkaloids distribution of *G. elegans*

G. elegans leaf structure was shown in Fig. 1. The leaf of *G. elegans* was mainly composed of epidermis, mesophyll and vein.

The epidermis was composed by a single layer of plumpness cells(Fig. 1(a)), and the stomata were observed only over the abaxial epidermis of the leaf(Fig. 1(b)). Both adaxial and abaxial epidermis were covered by thick cuticle and free of trichomes(Fig. 1(c)). The stomata were anomocytic type and the orifices are oval(Fig. 1(d)). The mesophyll tissues containing the parenchyma cells were below the epidermis(Fig. 1(e)). The midrib located in the center of the leaf, and the ridges protruded from the abaxial epidermal cells with striated lines on the surface(Fig. 1(f)). In addition, the spiral vessel elements were observed in vascular tissue(Fig. 1(g)).



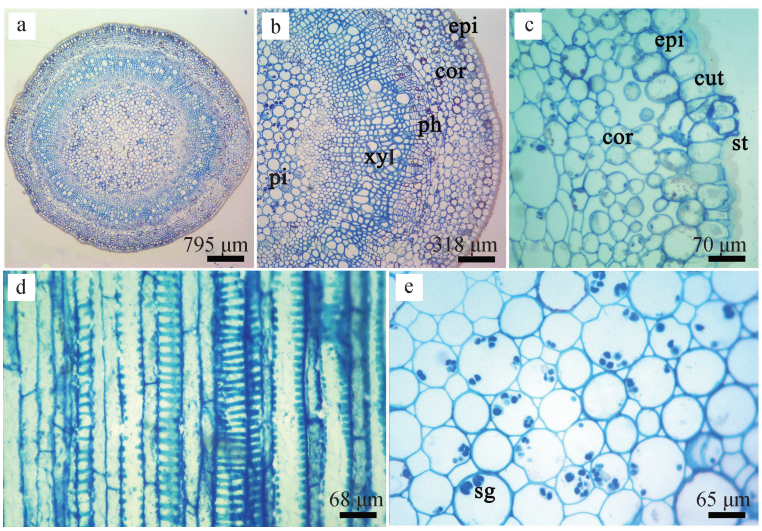
epi:epidermis; mp:mesophyll; xyl:xylem; ph:phloem; st:stoma, sp:sponge tissue; vb:vascular bundle; pa:palisade parenchyma

a. cross section; b. abaxial epidermis; c. adaxial epidermis covered by thick cuticle;

d. microstructure of stomata; e. mesophyll tissue; f. midrib; g. vascular tissue

Fig. 1　*G. elegans* leaf structure

The cross section of the stems was cylindrical(Fig. 2(a)), which was mainly composed of epidermis, parenchymatous cortex and the cylindrical vascular bundles that enclosed with the pith(Fig. 2(b)).



cor:cortex; pi:pith; cut:cuticle; st:stoma; pg:starch

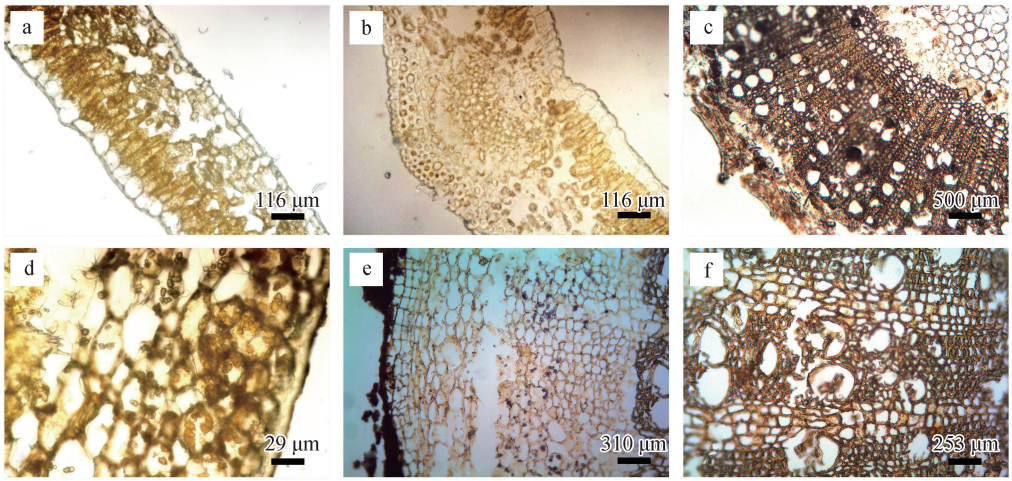
a. cross section; b. micro-structure tissues; c. stoma and cuticle; d. ongitudinal section; e. pith

Fig. 2　*G. elegans* stem structure

The epidermis consisted of one layer of cells with small, regular square shape with thicken cell wall, and the epidermis were covered with thick cuticle(Fig. 2(c)). The cortex consisted of 6 – 8 layers of cells(Fig. 2(c)). The stele containing pith, pith ray and vascular bundle with annulo spiral vessels was below the cortex (Fig. 2(d)). The pith was located in the center of the stems and consisted of parenchyma with abundant starch(Fig. 2(e)).

Histochemical analyses indicated a color reaction to tested alkaloid. The tawny color was shown by the reaction of bismuth potassium iodide and iodine potassium iodide solution and it proved the presence of alkaloids in tissues. The results showed that among *G. elegans* leaves, the tawny color was observed in the

palisade tissues and spongy tissues, and the dark color in the formed meant there was a high content of alkaloid in palisade tissues(Fig. 3(a)). However, the color was light or not in the vascular bundle of veins (Fig. 3(b)). Epidermis, cortex and phloem parenchyma cells in the stems were all dyed tawny color for the histochemical staining(Fig. 3(c)) and the color of the cortex was rather deep(Fig. 3(d)). Moreover, both pith ray and the parenchyma cells surrounding the pith showed distinct tawny color whereas the color of dyed the central of the pith parenchyma cells was pale or colorless(Fig. 3(c)). Alkaloids in the roots were distributed mainly in the cortical parenchyma cells, and no alkaloids could be detected in the vascular cylinder (Fig. 3(e)). In addition, tawny color could be found in cork cambium, phelloderm parenchyma cells, phloem parenchyma cells(Fig. 3(e)) and ray cells of the rhizome(Fig. 3(f)).



a. leaf; b. the vascular bundle of veins; c. micro-structure of the stem; d. stem cortex; e. root cortical parenchyma cells; f. ray cells of rhizome

Fig. 3 Identification of alkaloids of *G. elegans*

2.2 Determination of the antibacterial activity

2.2.1 Inhibition zone diameter The inhibition zone diameters determined by the filter paper diffusion method was taken as the index to evaluate the inhibitory effect of the alkaloids on the tested strains. As shown in Table 1, the *G. elegans* alkaloids had different degrees of inhibition effect against all the tested strains.

Table 1 Antibacterial effects of *G. elegans* alkaloids[1)]

strain	inhibition zone diameter of *G. elegans* alkaloids at different concn./mm			
	500 g/L	250 g/L	125 g/L	62.5 g/L
P. aeruginosa ATCC27853	11.00±0.23	10.00±0.23	9.00±0.00	7.00±0.00
P. aeruginosa CMCC10104	12.50±0.05	11.00±0.25	9.50±0.21	7.50±0.00
E. coli ATCC25922	13.20±0.00	13.00±0.05	11.00±0.00	7.50±0.25
E. coli O157	13.50±0.35	13.00±0.25	12.00±0.00	8.50±0.00
S. aureus ATCC25923	13.50±0.35	13.50±0.00	12.00±0.25	8.00±0.00
S. aureus CMCC26003	13.80±0.35	13.50±0.35	12.50±0.00	8.00±0.25
L. monocytogenes ATCC19115	15.00±0.05	14.00±0.21	13.00±0.12	9.00±0.26

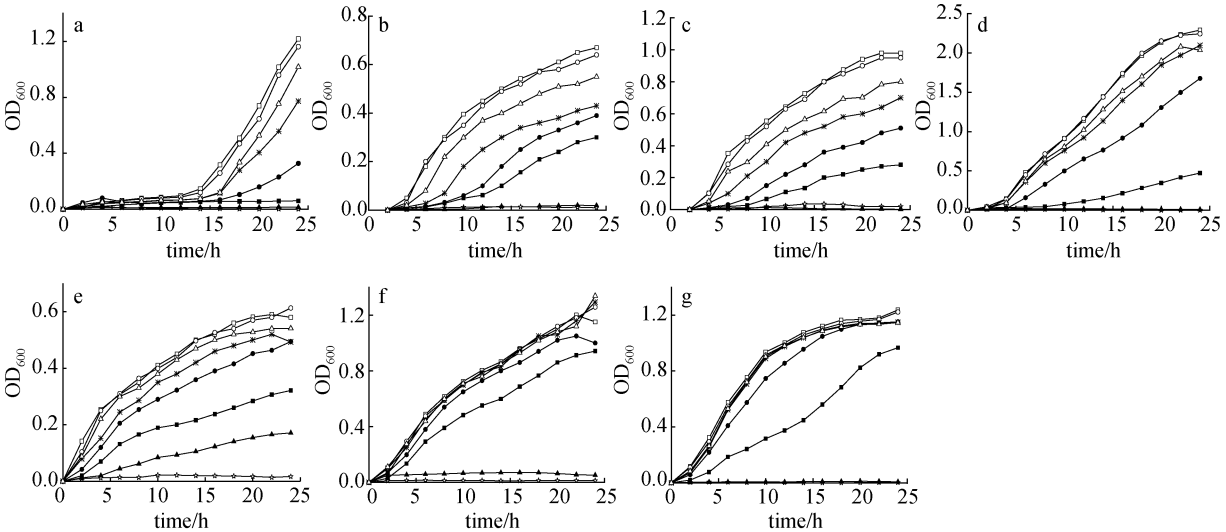
1) negative control(0.5% dimethyl sulfoxide):(7.00±0.00) mm; blank control(saline):(7.00±0.00) mm

Within the range of 62.5 – 500 g/L, the diameters of the inhibition zone increased significantly with the increase of the mass concentration of alkaloids($P<0.05$). This indicated that there was a positive correlation between the mass concentration of *G. elegans* alkaloids and the diameter of the inhibition zone. When the concentration of alkaloids was as low as 62.5 g/L, except for the *P. aeruginosa* ATCC27853, the inhibition zone diameters of other tested strains were greater than 7.00 mm, but the inhibition zone diameters of different strains under the alkaloids action of this concentration were different. This indicated that different strains had

different sensitivity to *G. elegans* alkaloids. With the increase of alkaloid concentration, i. e., the mass concentration of 125 and 250 g/L, the diameter of the inhibition zone increased, and the antibacterial effects were significantly improved. When the mass concentration of alkaloid was 500 g/L, the inhibition zone diameters against *P. aeruginosa* ATCC27853, *P. aeruginosa* CMCC10104, *E. coli* ATCC25922, *E. coli* O157, *S. aureus* ATCC25923, *S. aureus* CMCC26003, and *L. monocytogenes* ATCC19115 were 11.0, 12.5, 13.2, 13.5, 13.5, 13.8, and 15.0 mm, respectively. There was no inhibition zone in the solvent control group(0.5% DMSO) was 7.0 mm. This indicated that the solvent had no inhibitory effect on the tested strains, and the effect of the solvent on the experimental results was excluded. In general, the same concentration of *G. elegans* alkaloids had stronger inhibitory effect on *L. monocytogenes* ATCC19115, and the inhibition effects on *S. aureus* ATCC25923, *S. aureus* CMCC26003, *E. coli* ATCC25922 and *E. coli* O157 were equivalent. *G. elegans* alkaloids had poorer inhibition ability on *P. aeruginosa* CMCC10104. The *G. elegans* alkaloids had certain antibacterial abilities and different inhibitory effects on different strains. The inhibition zone experiment showed that the *G. elegans* alkaloids had antibacterial activities against the tested strains

2.2.2 Minimum inhibitory concentration(MIC) The minimum inhibitory concentrations of the alkaloids on seven tested strains were determined by using the double dilution method. The MIC is the minimum alkaloid concentration that can inhibit the growth of tested microorganisms and the main quantitative parameter for evaluating the sensitivity of pathogens to antimicrobial agents. The study showed that within the experimental range, the MIC of the alkaloids against *L. monocytogenes* ATCC19115 was 125 g/L, the MICs of *S. aureus* ATCC25923, *S. aureus* CMCC26003, *E. coli* ATCC25922, *E. coli* O157, *P. aeruginosa* CMCC10104 were 250 g/L, and the MIC of *P. aeruginosa* ATCC10104 was 500 g/L. They showed that *G. elegans* alkaloids could effectively inhibit the growth and reproduction of these bacteria.

2.2.3 Growth curve of bacteria The growth curves of the tested strains were determined by a microplate reader. It could be seen from Fig. 4 that when the *G. elegans* alkaloids were not added to the liquid medium, the growth curves of the strains had a normal lag phase, logarithmic phase and stability phase. The period was consistent with the growth of strains in the medium with 0.5% DMSO, indicating that the solvent did not affect the growth of the bacteria.



a. *L. monocytogenes* ATCC19115; b. *S. aureus* CMCC26003; c. *S. aureus* ATCC25923; d. *P. aeruginosa* CMCC10104; e. *P. aeruginosa* ATCC27853; f. *E. coli* O157; g. *E. coli* ATCC25922

Fig. 4 Effects of different concentrations of *G. elegans* alkaloids on the growth curves of tested strains

With the increase of the mass concentration of *G. elegans* alkaloids, the antibacterial activity also gradually increased. When the mass concentration of alkaloids was 500 g/L, it could effectively inhibit the growth of all tested strains for at least 24 h. When the alkaloids concentration was 250 g/L, the retardation period of *P. aeruginosa* ATCC27853 was effectively prolonged for nearly 4 h, and 6 kinds of other bacteria except *P. aeruginosa* ATCC27853 were inhibited. When the alkaloid concentration was 125 g/L, it could effectively inhibit the growth of *L. monocytogenes* ATCC19115 and delay the period of *L. monocytogenes* ATCC19115 for above 2 h. This obviously inhibited the bacteria from entering the logarithmic phase and the stability period. When the alkaloids concentration was 62.5 g/L, the alkaloids also effectively inhibited the number of all tested strains in the stability period, and delayed the *P. aeruginosa* CMCC10104, *S. aureus* CMCC26003, *S. aureus* ATCC25923, *L. monocytogenes* ATCC19115 into the logarithmic phase for 4 h. When the alkaloid concentration was 31.2 g/L, the antibacterial effect against *E. coli* ATCC25922 and *E. coli* O157 was weakened, and the growth curves were basically coincident with the control group, but the antibacterial effect on other bacteria was also limited. When the alkaloids concentration was 16.5 g/L, except for the gram-positive bacteria, the other four gram-negative bacteria coincided with the growth curve of the control group, indicating that the inhibitory effect of alkaloids on gram-positive bacteria was stronger than that on the gram-negative bacteria. In summary, the *G. elegans* alkaloids had significant inhibitory effects on the growth of these seven tested strains, and the *G. elegans* alkaloids had broad-spectrum antibacterial properties. The inhibition of the tested bacteria increased with the increase of alkaloids concentration and it was found that different strains showed different sensitivities to the same concentration of *G. elegans* alkaloids. *G. elegans* alkaloids had a higher inhibitory effect against gram-positive bacteria such as *L. monocytogenes* ATCC19115, but were not ideal for the bacteriostatic effect against gram-negative bacteria. In addition, the *G. elegans* alkaloids could significantly slow down the growth of the tested strains, prolong the duration of the lag phase, and significantly inhibit the growth of bacteria into the log phase.

2.2.4 MBC and lethal curve of *Gelsemium* alkaloids Studies showed that the MBC of the *G. elegans* alkaloids against *S. aureus* ATCC25923 was 500 g/L, and the MBC of *E. coli* O157 was 750 g/L. The survival curves of *E. coli* O157 and *S. aureus* ATCC25923 in different concentrations of alkaloids were shown in Fig. 5.

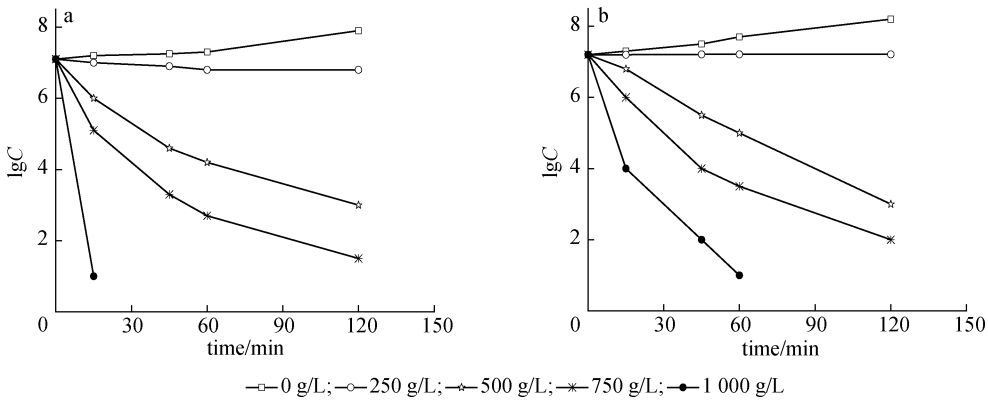


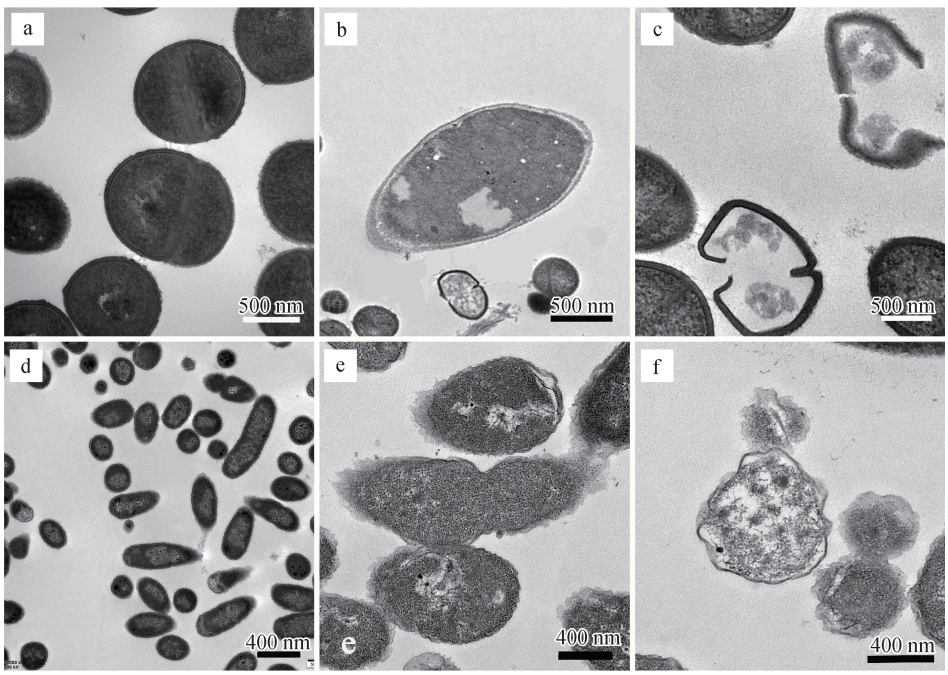
Fig. 5 Death curves of *S. aureus* ATCC25923 and *E. coli* O157 under different concentrations of alkaloids (*C* represented the number of bacteria)

The *G. elegans* alkaloids had a concentration-dependent effect on both bacteria. After the tested bacteria were cultured at 37 ℃ for 2 h in a solid medium without alkaloids, the number of both bacteria increased by one log compared to the initial one. The number of *E. coli* O157 in the medium containing 250 g/L alkaloids was almost unchanged within 2 h, while the number of *S. aureus* ATCC25923 decreased slightly. The 500 g/L

alkaloids showed bactericidal effect in 15 minutes for both bacteria. After 2 hours of 750 g/L alkaloids treatment, the numbers of *S. aureus* ATCC25923 and *E. coli* O157 decreased significantly. When the alkaloid concentration was 1 000 g/L, *S. aureus* could be killed within 30 min, and *E. coli* could be killed within 100 min.

2.3 Effects of alkaloids on the ultrastructure of *S. aureus* ATCC25923 and *E. coli* O157

In order to observe the ultrastructural changes of the strains, we have carried out the observation on the strains at the ultrastructural level. The ultrastructural study of the tested strains showed that the surface of the cells of untreated *S. aureus* was smooth and full, the cell wall and cell membrane were intact, and the intracellular electrolyte was rich and dense(Fig. 6(a)). However, the cell structure of the bacteria treated with alkaloids was significantly different. Under the action of the alkaloids with the minimum inhibitory concentration, the morphology of the bacteria changed, the cells became elliptical, the surface structure of the bacteria became rough, the walls separated, and some cytoplasm of the cells extravasated(Fig. 6(b)). Under the action of the MBC, the cells of the bacteria ruptured, and the content of the cells extravasated(Fig. 6(c)). After alkaloids treatment, the surface of *S. aureus* cells was seriously damaged. This might damage the cell wall and significantly change the permeability of the cell membrane.



a. untreated *S. aureus*; b. *S. aureus* treated with the alkaloids of the MIC; c. *S. aureus* treated with the alkaloids of the MBC; d. untreated *E. coli*; e. *E. coli* treated with the alkaloids of MIC; f. *E. coli* treated with the alkaloids of the MBC

Fig. 6　Ultrastructure of strains before and after treatment with *G. elegans* alkaloids

E. coli without alkaloid treatment was rod-shaped, the cells were intact, and the cytoplasm was even and dense(Fig. 6(d)). After alkaloids treatment with the minimum inhibitory concentration, the surface of the bacteria was rough, and there were obvious pores and sunkenness due to serious structural damage. Part of the cell electrolyte was extravasated and the cell wall structure was uneven(Fig. 6(e)). Under the action of MBC, the cell surface became rough, the cell wall was sunken and ruptured, the cell membrane was damaged, and the cytoplasm was almost completely lost(Fig. 6(f)). It could be seen that the *G. elegans* alkaloid had a significant effect on the cell structure of *E. coli* O157 and *S. aureus* ATCC25923. This might cause serious damage to the cell surface. After the cell surface was injured, the mechanical strength of the cell wall was lost, the permeability of the cell membrane changed, and the intracellular protoplasm might

extravasate. The viscosity of the protoplasm caused the cells to adhere to each other and agglomerate, and the cells underwent osmotic lysis. Studies on the antibacterial mechanism of plant bioactive substances showed that their antibacterial activity mainly focuseed on the damage to the cell wall and changed the cell membrane permeability[13-14]. They affected the activity of metabolic enzymes in the body, disturbed the metabolic mechanism of the bacteria and the normal function of the efflux pump, and affected the replication and transcription of the cells by acting on the topoisomerase[15]. They also affected the synthesis of related proteins by preventing the protein required for intracellular protein synthesis from entering the cells and misinterpreting the mRNA through binding to the 30S or 50S subunit of the prokaryotic ribosome[16]. This study indicated that when the *S. aureus* were treated with the minimum inhibitory concentration of *G. elegans* alkaloids, the morphology of the cells changed. All indicated that the alkaloids destroyed the formation of the cell wall, possibly because the shape of the bacteria was affected by peptidoglycan vesicles[13]. It was observed in this study that there was an uneven distribution of bacterial peptidoglycans, indicating that the *G. elegans* alkaloids might interfere with the membrane proteins EzrA and GpsB. During cell cycle progression, these proteins worked synergistically to form peptidoglycans in the cell wall (cell extension) and membrane walls (cell division). EzrA aggregated peptidoglycan synthase(penicillin-binding protein; PBP) to the cell division site, and GpsB directed these enzymes to the columnar portion of the cell after cell division[17]. Other possible targets included membrane-associated cytoskeletal proteins MreB and FtsZ. These proteins form the scaffolds were used for cell division and elongation, and topological information for the PBP enzyme[18-19]. In this study, the treatment of *S. aureus* with the mininum lethal concentration showed deformed cell walls with a small cytoplasmic electron density. These results indicated that high concentrations of alkaloids disrupted of the formation of the cell wall, thereby resulting in the loss of mechanical strength of the cell wall and osmotic lysis of cells. In general, the outer membrane and peptidoglycan layer of the cell wall of gram-negative bacteria were closely linked by Braun's lipoprotein, but the lipoprotein required a properly formed peptidoglycan layer to anchor itself[20]. After the *E. coli* treatment with the mininum inhibitory concentration, the cell wall structure was uneven. This proved that the *G. elegans* alkaloids destroyed the formation of peptidoglycan vesicles. Therefore, it was concluded that the disruption of peptidoglycan synthesis might cause osmotic lysis, and the *G. elegans* alkaloids could inhibit cell growth and cause cell death very rapidly. After the treatment with the mininum lethal concentration, the cell contents leaked, complete *E. coli* was rare, the cell membrane and intracellular electron density decreased, and a large number of extracellular fragments appeared. They were similar to the treatment results of *S. aureus*. These findings showed that the *G. elegans* alkaloids with high concentrations would reduce and destroy the mechanical strength of the cell wall. It became lower than the limit required to resist osmotic dissolution.

3 Conclusion

The anatomical structure of *G. elegans* showed the structural feature of typical dicotyledonous woody plants, with dorsiventral leaves and rounded stems. Histochemical studies showed that alkaloids were present in parenchyma cells of various parts of vegetative organs of *G. elegans*. The alkaloids had antibacterial properties against various bacteria. The antibacterial activity results showed that the *G. elegans* alkaloid had antibacterial activities against *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25923, *Staphylococcus aureus* CMCC26003, *Escherichia coli* ATCC25922, *Escherichia coli* O157, *Pseudomonas aeruginosa* ATCC27853, and *Pseudomonas aeruginosa* CMCC10104. And with the increase of alkaloid concentration, the antibacterial effect was also enhanced. Among them, the inhibitory effect on *Listeria monocytogenes* was the most obvious. After treatment with its minimum inhibitory and lethal concentrations,

studies on the ultrastructure of *S. aureus* ATCC25923 and *E. coli* O157 indicated that the *G. elegans* alkaloids under the MBC produced inhibition by disrupting the permeability of cell membrane, inhibiting cell wall synthesis and lysing the bacteria.

References:

[1]KLETT W. Umfang und inhalt der familie der Loganiaceae[J]. Botan Arch,1924,5:312-338.

[2]MOORE R J. Cytotaxonomic studies in the Loganiaceae. I:Chromosome numbers and phylogeny in the Loganiaceae[J]. American Journal of Botany,1947,34:527-538.

[3]STRUWE L,ALBERT V A,BREMER B. Cladistics and family level classification of the Gentianales[J]. Cladistics,1994,10(2):175-206.

[4]XU Y K,YANG L,LIAO S G,et al. Kou mine,humantenine,and yohimbane alkaloids from *Gelsemium elegans*[J]. Journal of Natural Products,2015,78(7):1511-1517.

[5]LEI W,JINFENG W,XIA M,et al. Gelsedine-type oxindole alkaloids from *Gelsemium elegans* and the evaluation of their cytotoxic activity[J]. Fitoterapia,2017,120:131-135.

[6]MINGXUE S,YAN C,YU L,et al. Indole alkaloids from *Gelsemium elegans*[J]. Phytochemistry,2019,162:232-240.

[7]ORNDUFF R. The systematics and breeding system of *Gelsemium*(Loganiaceae)[J]. Journal of the Arnold Arboretum,1970,51(1):1-17.

[8]WYATT B,BROYLES S B,HAMRICK J L,et al. Systematic relationships within *Gelsemium*(Loganiaceae):Evidence from isozymes and cladistics[J]. Systematic Botany,1993,18(2):345-355.

[9]XIONG B J,XU Y,JIN G L,et al. Analgesic effects and pharmacologic mechanisms of the *Gelsemium* alkaloid kou mine on a rat model of postoperative pain[J/OL]. Scientific Reports,2017,7(1):14269[2021-10-15]. https://doi.org/10.1038/s41598-017-14714-0.

[10]CHEUNG W L,LAW C Y,LEE H C H,et al. Gelsemium poisoning mediated by the non-toxic plant *Cassytha filiformis* parasitizing *Gelsemium elegans*[J]. Toxicon,2018,154:42-49.

[11]ZHANG W,ZHANG S Y,WANG G Y,et al. Five new kou mine-type alkaloids from the roots of *Gelsemium elegans*[J]. Fitoterapia,2017,118:112-117.

[12]QING Z X,YANG P,TANG Q,et al. Isoquinoline alkaloids and their antiviral,antibacterial,and antifungal activities and structure-activity relationship[J]. Current Organic Chemistry,2017,21(18):1920-1934.

[13]WALDEMAR V,DIDIER B,DE PEDRO M A. Peptidoglycan structure and architecture[J]. Fems Microbiology Reviews,2008,32(2):149-167.

[14]WU V C H,QIU X J,BUSHWAY A,et al. Antibacterial effects of American cranberry(*Vaccinium macrocarpon*) concentrate on foodborne pathogens[J]. LWT-Food Science and Technology,2008,41(10):1834-1841.

[15]VASCONCELOS N,CRODA J,SIMIONATTO S. Antibacterial mechanisms of cinnamon and its constituents:A review [J]. Microbial Pathogenesis,2018,120:198-203.

[16]ZACHARIAH S M,VISWANAD V,ALEYKUTTY N A,et al. Antimicrobial potential of herbal medicines[J]. International Journal of Pharmaceutical Sciences and Research,2011,2(7):1651-1658.

[17]DENNIS C,ROBYN E,HAMOEN L W,et al. Control of the cell elongation-division cycle by shuttling of PBP1 protein in Bacillus subtilis[J]. Molecular Microbiology,2010,68(4):1029-1046.

[18]ADAMS D W,JEFF E. Bacterial cell division:Assembly,maintenance and disassembly of the Z ring[J]. Nature Reviews Microbiology,2009,7(9):642-653.

[19]SHAEVITZ J W,ZEMER G. The structure and function of bacterial actin homologs[J]. Cold Spring Harbor Perspectives in Biology,2010,2(9):1-19.

[20]SINGLETON P. Bacteria in biology,biotechnology and medicine[J]. Journal of Hospital Infection,1999,34(4):409-410.