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Original Research Articles

Screening of 35 Chinese herbal medicines and metabolomic analysis of cortex moutan against *Streptococcus agalactiae* in tilapia

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Streptococcosis caused by *Streptococcus agalactiae* is a major bacterial disease affecting tilapia aquaculture worldwide, necessitating the development of safe and effective alternatives to conventional antibiotics. In this study, 35 Chinese herbal medicines were screened for antibacterial activity against *S. agalactiae* using the disk diffusion and broth dilution methods. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined, and untargeted metabolomics combined with multivariate analysis was performed to investigate the metabolic regulatory mechanisms of the most effective herbal extract. The results showed that Cortex Moutan (the root bark of *Paeonia* × *suffruticosa* Andrews), Folium Sennae, and Fructus Foeniculi exhibited in vitro antibacterial activity against *S. agalactiae*, with Cortex Moutan being the most effective, showing an inhibition zone diameter of 15.63 ± 0.52 mm, n=3, an MIC of 0.125 g/mL, and an MBC of 0.5 g/mL, as determined from three independent biological replicates. Notably, Folium Sennae and Fructus Foeniculi have been less studied against this pathogen, and their activity, observed here, represents a secondary contribution of this study. Metabolomics and VIP analyses revealed that several plant-derived compounds (e.g., emodin and graveoline) were detected at higher levels in the treatment group, and that key metabolic pathways, including the TCA cycle, amino acid metabolism, and the electron transport chain, may be affected. These findings provide preliminary in vitro evidence. Further in vivo validation is required before any aquaculture application.

INTRODUCTION

Tilapia is one of the most economically important freshwater fish species in China, owing to its rapid growth rate, strong environmental adaptability, and favorable processing characteristics (e.g., absence of intramuscular bones). It is widely cultivated in southern China.[1] However, the rapid expansion of intensive aquaculture has been accompanied by an increasing prevalence of bacterial diseases. Among these, streptococcosis caused by *Streptococcus agalactiae* is considered one of the most serious threats to tilapia farming, often resulting in mass mortality and causing substantial economic losses.[2]

Currently, disease management for streptococcosis in tilapia mainly relies on improved aquaculture practices, environmental optimization, and immunoprophylaxis. Chinese herbal medicines have attracted considerable attention as promising candidates for aquatic disease control, owing to their abundant resources, relatively lower toxicity than synthetic antibiotics, and lower risk of inducing bacterial resistance. However, it should be noted that many medicinal plants contain bioactive compounds with substantial toxicity, and safety is compound-, dose-, species-, and exposure-dependent.[3] Their antibacterial mechanisms are generally characterized as multi-target and multi-pathway in nature, including disruption of bacterial cell wall and membrane integrity, inhibition of nucleic acid and protein synthesis, interference with energy metabolism, suppression of biofilm formation, and disruption of quorum sensing systems.[4-9] Despite these promising features, systematic screening of Chinese herbal medicines specifically against tilapia-derived *S. agalactiae* remains limited, and the underlying metabolic regulatory mechanisms are still poorly understood.

Cortex Moutan, the root bark of Paeonia × suffruticosa Andrews, is a widely used traditional Chinese medicine with a long history of use for treating inflammatory and infectious diseases.[10] Its major bioactive constituents include paeonol and paeoniflorin, which exhibit antibacterial, anti-inflammatory, and antioxidant activities. Paeonol, in par-

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ticular, has been reported to inhibit several Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Escherichia coli*. However, studies on the antibacterial activity of Cortex Moutan against aquatic pathogens, especially *S. agalactiae*, remain limited.[11]

Traditional approaches to studying the antibacterial activity of herbal medicines have largely been confined to phenotypic screening (e.g., inhibition zone diameter and MIC determination), which provide limited insight into their mechanisms of action. As a key component of systems biology, metabolomics enables the comprehensive detection of global changes in small-molecule metabolites, thereby facilitating the elucidation of drug–pathogen interaction networks. This approach has been successfully applied to investigate the mechanisms of various herbal medicines against aquatic pathogens.[10] However, to the best of our knowledge, based on a comprehensive literature search of PubMed, Web of Science, and CNKI, the metabolomic regulatory mechanism of Cortex Moutan against S. agalactiae has not been reported previously.

In the present study, 35 commonly used Chinese herbal medicines were screened for in vitro antibacterial activity against *S. agalactiae* isolated from tilapia. The most effective extract was further investigated using untargeted metabolomics to elucidate its metabolic regulatory mechanisms. We hypothesized that Cortex Moutan exerts its antibacterial activity against *S. agalactiae* through both direct antimicrobial action of its bioactive constituents and disruption of central carbon and amino acid metabolism. This study aims to provide preliminary in vitro evidence and candidate compounds for the future development of plant-derived alternatives to antibiotics in tilapia aquaculture.

MATERIALS AND METHODS

TEST STRAIN AND REAGENTS

The *S. agalactiae* strain used in this study was isolated from naturally infected Nile tilapia, then identified by biochemical and molecular characterization, and stored in the laboratory. *S. agalactiae* was grown in brain-heart infusion agar (BHIA) (Qingdao Hope Bio-Technology Co., Ltd., China) at 30 °C for 48 h. Sterile drug-sensitive tablets have a diameter of 6mm, a thickness of 0.7mm, and a maximum liquid absorption of 20 μl (Changde Bimanke Biotechnology Co., Ltd., China). All herbal materials (Table 1) were purchased from Beijing Tongrentang, Chongzuo City, Guangxi, China. The botanical identity of each herb was authenticated according to the Chinese Pharmacopoeia. The 35 herbal medicines were selected based on three criteria: (i) traditional use in Chinese medicine for treating infectious or inflammatory diseases; (ii) reported antibacterial activity against aquatic or other bacterial pathogens; and (iii) availability in local markets in Guangxi, China. This selection strategy aimed to balance ethnopharmacological rationale with practical applicability in tilapia aquaculture.

PREPARATION OF LIQUID MEDICINE

Each herbal powder (20 g) was soaked in 500 mL of distilled water for 60 min, boiled for 60 min, and filtered through four-layer gauze. The residue was re-decocted, and the combined filtrates were concentrated to 20 mL (1 g crude drug/mL), then autoclaved at 121°C for 20 min and stored at 4°C.

PREPARATION OF BACTERIAL SUSPENSION

A single colony of *S. agalactiae* was inoculated into 1.5 mL BHI broth and incubated overnight at 28°C with shaking (180 r/min). A 5 μL aliquot was transferred into 5 mL fresh BHI broth and incubated for 6 h under the same conditions.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The Kirby–Bauer disk diffusion method was used. Bacterial suspension (1×10^8 CFU/mL, 100 μL) was spread onto BHI agar plates. Blank disks (6 mm) served as negative controls. Plates were incubated at 28°C for 24 h. Inhibition zones were measured with a vernier caliper in triplicate. Susceptibility criteria: >20 mm (extremely sensitive), 15–20 mm (highly sensitive), 10–15 mm (moderately sensitive), ⩽10 mm (weakly sensitive), no zone (resistant).

ANTIMICROBIAL SUSCEPTIBILITY TESTING OF HERBAL EXTRACTS

The disk diffusion method was used for herbal extracts. BHI agar plates were prepared. *S. agalactiae* suspension (OD_{600} = 1.0, ≈1×10^9 CFU/mL) was swabbed onto plates. After drying (3–5 min), sterile paper disks impregnated with 20 μL herbal decoction (1 g/mL) were placed on the agar. Sterile water served as negative control. Plates were incubated at 28°C for 24 h. All antibacterial assays were performed in three independent biological replicates. Inhibition zones are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, USA). Differences among groups were analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test. $P < 0.05$ was considered statistically significant.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of selected herbal extracts

Selected herbal extracts were evaluated by broth dilution. Two-fold serial dilutions of stock solution (1 g/mL) were prepared in BHI broth, yielding concentrations from 0.5 to 0.001953 g/mL. Positive (no herbal) and negative (no bacteria) controls were included. Each tube received 3 μL bacterial suspension (~1×10^6 CFU/mL) and was incubated at 28°C for 18–24 h. MIC was the lowest concentration with no visible turbidity. For MBC, 100 μL aliquots from clear tubes were spread on BHI agar and incubated at 28°C for 18 h. MBC was the lowest concentration yielding <5 colonies. MIC and MBC values were determined from three independent biological replicates.

Metabolomics sample preparation

S. agalactiae was cultured to log phase, then treated with

Table 1. Information of crude herbal medicines used in this study

Name	Botanical source	Part used
Banzia (Pinelliae Rhizoma)	*Pinellia ternata* (Thunb.) Breit.	Tuber
Beimu (Fritillariae Thunbergii Bulbus)	*Fritillaria thunbergii* Miq.	Bulb
Binglang(Arecae Semen)	*Areca catechu* L.	Seed
Buxuzhi (Psoraleae Fructus)	*Cullen corylifolium* (L.) Medik.	Fruit
Bohe (Menthae Haplocalycis Herba)	*Mentha haplocalyx* Briq.	Aerial part
Chuanxinlian (Andrographis Herba)	*ndrographis paniculata* (Burm.f.) Nees	Aerial part
Dahuang (Rhei Radix et Rhizoma)	*Rheum officinale* Baill.	Root and rhizome
Daqingye (Isatidis Folium)	*Isatis indigotica* Fortune	Leaf
Dingxiang(Caryophylli Flos)	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Flower bud
Duzhong(Eucommiae Cortex)	*Eucommia ulmoides* Oliv.	Bark
Danggui (Angelicae Sinensis Radix)	*Angelica sinensis* (Oliv.) Diels	Root
Fanxieye(Sennae Folium)	*Senna alexandrina* Mill.	Leaflet
Fuling(Poria)	*Wolfiporia cocos* (F.A.Wolf) Ryvarden & Gilb.	Sclerotium
Gancao (Glycyrrhizae Radix et Rhizoma)	*Glycyrrhiza uralensis* Fisch.	Root and rhizome
Guangxi Ezhu (Curcumae Kwangsiensis Rhizoma)	*Curcuma kwangsiensis* S.G.Lee & C.F.Liang	Rhizome
Houpo(Magnoliae Officinalis Cortex)	*Magnolia officinalis* Rehder & E.H.Wilson	Bark
Huangbai (Phellodendri Chinensis Cortex)	*Phellodendron chinense* Schneid.	Bark
Huanglian (Coptidis Rhizoma)	*Coptis chinensis* Franch.	Rhizome
Huangqin (Scutellariae Radix)	*Scutellaria baicalensis* Georgi	Root
Honghua (Carthami Flos)	*Carthamus tinctorius* L.	Flower
Jigucao(Abri Herba)	*Abrus cantoniensis* Hance	Whole plant
Jixueteng (Spatholobi Caulis)	*Spatholobus suberectus* Dunn	Stem
Liangmianzhen (Zanthoxyli Radix)	*Zanthoxylum nitidum* (Roxb.) DC.	Root
Mahuang(Ephedrae Herba)	*Ephedra sinica* Stapf	Herbaceous stem
Mudanpi(Cortex Moutan)	*Paeonia × suffruticosa* Andrews	Root bark
Renshen (Ginseng Radix)	*Panax ginseng* C.A.Mey.	Root
Rougui (Cinnamomi Cortex)	*Cinnamomum cassia* (L.) J.Presl	Bark
Shanzhuyu (Corni Fructus)	*Cornus officinalis* Siebold & Zucc.	Fruit
Shandougen(Sophorae Tonkinensis Radix et Rhizoma)	*Sophora tonkinensis* Gagnep.	Root and rhizome
Sharen (Amomi Fructus)	*Amomum villosum* Lour.	Fruit
Tianhuafen (Trichosanthis Radix)	*Trichosanthes kirilowii* Maxim.	Root
Wubeizi (Galla Chinensis)	*Galla chinensis* (insect gall on Rhus chinensis Mill., induced by Melaphis chinensis (Bell))	Insect gall
Xiaohuixiang(Foeniculi Fructus)	*Foeniculum vulgare* Mill.	Fruit
Yangjinhua (Daturae Flos)	*Datura metel* L.	Flower
Zhuling (Polyporus)	*Polyporus umbellatus* (Pers.) Fr.	Sclerotium

Cortex Moutan extract at MIC or equal volume PBS (control), and incubated at 28°C, 180 r/min for 10 h. Three biological replicates (50 mL/tube) were collected. Bacterial pellets were obtained by centrifugation (4500 r/min, 10 min), quenched in liquid nitrogen, and stored at −80°C.

METABOLITE EXTRACTION AND UPLC-Q-TOF-MS ANALYSIS

Each pellet (50 mg) was extracted with 500 µL ice-cold 80% methanol, vortexed, sonicated, and incubated at −20°C for 30 min. After centrifugation (4°C, 20,000 × g, 10 min), the supernatant was collected and centrifuged again for 5 min. Equal aliquots were pooled as QC samples.

Chromatography used an ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 µm; Waters). Mobile phase: (A) 5 mM ammonium acetate + 5 mM acetic acid in water, (B) acetonitrile. Gradient: 0–0.8 min (2% B), 0.8–2.8 min (2–70% B), 2.8–5.0 min (70–90% B), 5.0–5.5 min (90–100% B), 5.5–7.5 min (100% B), 7.5–7.6 min (100–2% B), 7.6–10.0 min (2% B). Flow rate: 0.35 mL/min, injection: 4 µL, column temperature: 40°C.

Mass spectrometry used ESI in positive and negative modes. Ion source: 350°C. Capillary voltages: +3.8 kV (positive), −3.4 kV (negative). Gas flows: purge (1 Arb), auxiliary (15 Arb), sheath (50 Arb). Data acquisition: full scan (70–1050 Da, resolution 60,000) with DDA. Top four ions (>5,000 intensity) were selected for MS/MS (resolution 15,000). Dynamic exclusion: 4 s.

DATA PROCESSING

Raw data were converted to mzML format using ProteoWizard. Peak alignment, retention time correction, and peak area extraction were performed using XCMS (parameters: centWave m/z = 20 ppm, peakwidth = 5–25 s, snthresh = 6, prefilter = c(4, 5000), noise = 1000; grouping: binSize_R = 0.4, gapInit = 0.3, binSize_G = 0.015, bw = 5, minfrac = 0.5). Metabolites with missing values >50% in QC or >80% in experimental samples were removed. Remaining missing values were imputed by KNN, outliers removed, and PQN normalization applied. Data from positive and negative ESI modes were processed separately and then combined for multivariate analysis. Redundant annotations across modes were removed by matching retention time and m/z within 10 ppm and 0.1 min. Only unique features were retained for downstream analysis. Differential metabolites were selected using VIP ⩾ 1.0 and T-test P < 0.05. KEGG pathway enrichment analysis was performed using a hypergeometric test with FDR correction (Q-value ⩽ 0.05).

RESULTS

IN VITRO ANTIBACTERIAL ACTIVITY OF 35 CHINESE HERBAL EXTRACTS AGAINST *S. AGALACTIAE*

The inhibition zones of all 35 herbal extracts are summarized in Table 2. Only three extracts produced detectable inhibition zones: Cortex Moutan (15.63 ± 0.52 mm), Folium Sennae (14.38 ± 0.45 mm), and Fructus Foeniculi (14.75 ± 0.12 mm). The remaining 32 extracts produced no detectable inhibition zone. The negative control (sterile water) showed no inhibition zone (0 ± 0 mm).

MIC AND MBC OF SELECTED HERBAL EXTRACTS AGAINST *S. AGALACTIAE*

Based on the inhibition zone diameters obtained from the disk diffusion assay, three herbal extracts—Cortex Moutan, Folium Sennae, and Fructus Foeniculi—that exhibited the most potent antibacterial activity against *S. agalactiae* were selected for the determination of MIC and MBC using the broth dilution method. The results are presented in Table 3. The MIC values of Cortex Moutan, Folium Sennae, and Fructus Foeniculi were 0.125, 0.125, and 0.25 g/mL, respectively. All three extracts exhibited an MBC of 0.5 g/mL.

QUALITY ASSESSMENT OF METABOLOMICS DATA

PCA was performed for an initial assessment of data quality and overall metabolic patterns (Figure 1). PC1 and PC2 explained 30.2% and 17.8% of the total variance, respectively. The treatment group (Cortex Moutan aqueous extract) was separated from the control group along the PC1 axis. However, PCA separation alone requires further validation. QC samples clustered tightly around the origin. Biological replicates within each group also showed good clustering.

MULTIVARIATE STATISTICAL ANALYSIS

OPLS-DA was performed to further discriminate the metabolic profiles between the control and treatment groups (Figure 2). The OPLS-DA model showed a separation between the control and treatment groups. The three biological replicates within each group clustered tightly, demonstrating good reproducibility. However, given the small sample size (n = 3 per group), the OPLS-DA results should be interpreted with caution.

SCREENING AND IDENTIFICATION OF DIFFERENTIAL METABOLITES

A total of 23,541 features were detected across both positive and negative ESI modes. Using the criteria of VIP ⩾ 1.0 and T-test P < 0.05, 330 differential metabolites were identified between the control and treatment groups (Figure 3), including 187 up-regulated and 143 down-regulated metabolites. The slightly higher number of up-regulated metabolites may suggest that the treatment induced both metabolic inhibition and stress responses in *S. agalactiae*.

The volcano plot (Figure 4) displayed the distribution of these metabolites, with the x-axis representing $\log_2$(fold change) and the y-axis representing $-\log_{10}$(P-value). The metabolic profile of *S. agalactiae* was extensively altered, with fold changes ranging from −6 to +6 and the highest significance at $-\log_{10}(P) = 3.8$ ($P \approx 1.6 \times 10^{-4}$). Down-regulated metabolites (left) showed larger changes and greater significance, suggesting stronger inhibitory effects, whereas up-regulated metabolites (right) were more numerous but less significant, possibly reflecting bacterial stress responses.

VIP ANALYSIS

The top 15 metabolites contributing to the group separation are shown in the VIP bar plot (Figure 5). The highest VIP scores were observed for 5-hexyltetrahydro-2-furanoctanoic acid (VIP = 22), graveoline (VIP = 18), piperolactam A (VIP = 16), erythro-isoleucine (VIP = 14), phenylalanine (VIP = 10), and emodin (VIP = 9). Based on MS/MS spectral matching and database comparison, graveoline, piperolactam A, and emodin were tentatively identified and detected

Table 2. Susceptibility of the isolated *Streptococcus agalactiae* strain to 35 herbal extracts

Chinese name	Botanical source	Inhibition zone diameter (mm)	Susceptibility level
Sterile water (negative control)	/	0± 0[a]	Resistant
Banzia(Pinelliae Rhizoma)	*Pinellia ternata* (Thunb.) Breit.	—	Resistant
Beimu (Fritillariae Thunbergii Bulbus)	*Fritillaria thunbergii* Miq.	—	Resistant
Binglang(Arecae Semen)	*Areca catechu* L.	—	Resistant
Buxuzhi (Psoraleae Fructus)	*Cullen corylifolium* (L.) Medik.	—	Resistant
Bohe (Menthae Haplocalycis Herba)	*Mentha haplocalyx* Briq.	—	Resistant
Chuanxinlian (Andrographis Herba)	*ndrographis paniculata* (Burm.f.) Nees	—	Resistant
Dahuang (Rhei Radix et Rhizoma)	*Rheum officinale* Baill.	—	Resistant
Daqingye (Isatidis Folium)	*Isatis indigotica* Fortune	—	Resistant
Dingxiang(Caryophylli Flos)	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	—	Resistant
Duzhong(Eucommiae Cortex)	*Eucommia ulmoides* Oliv.	—	Resistant
Danggui (Angelicae Sinensis Radix)	*Angelica sinensis* (Oliv.) Diels	—	Resistant
Fanxieye(Sennae Folium)	*Senna alexandrina* Mill.	14.38 ± 0.45[b]	Moderately sensitive
Fuling(Poria)	*Wolfiporia cocos* (F.A.Wolf) Ryvarden & Gilb.	—	Resistant
Gancao (Glycyrrhizae Radix et Rhizoma)	*Glycyrrhiza uralensis* Fisch.	—	Resistant
Guangxi Ezhu (Curcumae Kwangsiensis Rhizoma)	*Curcuma kwangsiensis* S.G.Lee & C.F.Liang	—	Resistant
Houpo(Magnoliae Officinalis Cortex)	*Magnolia officinalis* Rehder & E.H.Wilson	—	Resistant
Huangbai (Phellodendri Chinensis Cortex)	*Phellodendron chinense* Schneid.	—	Resistant
Huanglian (Coptidis Rhizoma)	*Coptis chinensis* Franch.	—	Resistant
Huangqin (Scutellariae Radix)	*Scutellaria baicalensis* Georgi	—	Resistant
Honghua (Carthami Flos)	*Carthamus tinctorius* L.	—	Resistant
Jigucao(Abri Herba)	*Abrus cantoniensis* Hance	—	Resistant
Jixueteng (Spatholobi Caulis)	*Spatholobus suberectus* Dunn	—	Resistant
Liangmianzhen (Zanthoxyli Radix)	*Zanthoxylum nitidum* (Roxb.) DC.	—	Resistant
Mahuang(Ephedrae Herba)	*Ephedra sinica* Stapf	—	Resistant
Mudanpi(Cortex Moutan)	*Paeonia × suffruticosa* Andrews	15.63 ± 0.52[b]	Highly sensitive
Renshen (Ginseng Radix)	*Panax ginseng* C.A.Mey.	—	Resistant
Rougui (Cinnamomi Cortex)	*Cinnamomum cassia* (L.) J.Presl	—	Resistant
Shanzhuyu (Corni Fructus)	*Cornus officinalis* Siebold & Zucc.	—	Resistant
Shandougen(Sophorae Tonkinensis Radix et Rhizoma)	*Sophora tonkinensis* Gagnep.	—	Resistant
Sharen (Amomi Fructus)	*Amomum villosum* Lour.	—	Resistant
Tianhuafen (Trichosanthis Radix)	*Trichosanthes kirilowii* Maxim.	—	Resistant
Wubeizi (Galla Chinensis)	*Galla chinensis* (insect gall on Rhus chinensis Mill., induced by Melaphis chinensis (Bell))	—	Resistant
Xiaohuixiang(Foeniculi Fructus)	*Foeniculum vulgare* Mill.	14.75 ± 0.12[b]	Moderately sensitive
Yangjinhua (Daturae Flos)	*Datura metel* L.	—	Resistant
Zhuling (Polyporus)	*Polyporus umbellatus* (Pers.) Fr.	—	Resistant

*Data are presented as mean ± SD (n = 3). Inhibition zones were measured in three independent biological replicates. "—" indicates no detectable inhibition zone. Susceptibility criteria: >20 mm (extremely sensitive), 15–20 mm (highly sensitive), 10–15 mm (moderately sensitive), ≤10 mm (weakly sensitive). Different lowercase letters (a, b) indicate significant differences among groups (Kruskal–Wallis test followed by Dunn's post hoc test, $P < 0.05$).

at higher levels in the treatment group, while isoleucine and phenylalanine were detected at lower levels.

METABOLITE SET ENRICHMENT ANALYSIS (MSEA) OF DIFFERENTIAL METABOLITES

To further explore the biological functions of the identified differential metabolites, metabolite set enrichment analysis (MSEA) was performed, and the results are presented as a clustered bar chart (Figure 6). As shown in Figure 6,

Table 3. MIC and MBC of three selected herbal extracts against the isolated *S. agalactiae* strain

Chinese name	Botanical source	MIC (g/mL)	MBC (g/mL)
Fanxieye(Sennae Folium)	*Senna alexandrina* Mill.	0.125	0.5
Mudanpi(Cortex Moutan)	*Paeonia × suffruticosa* Andrews	0.125	0.5
Xiaohuixiang(Foeniculi Fructus)	*Foeniculum vulgare* Mill.	0.25	0.5

*Data are presented as single representative values determined from three independent biological replicates (n = 3).

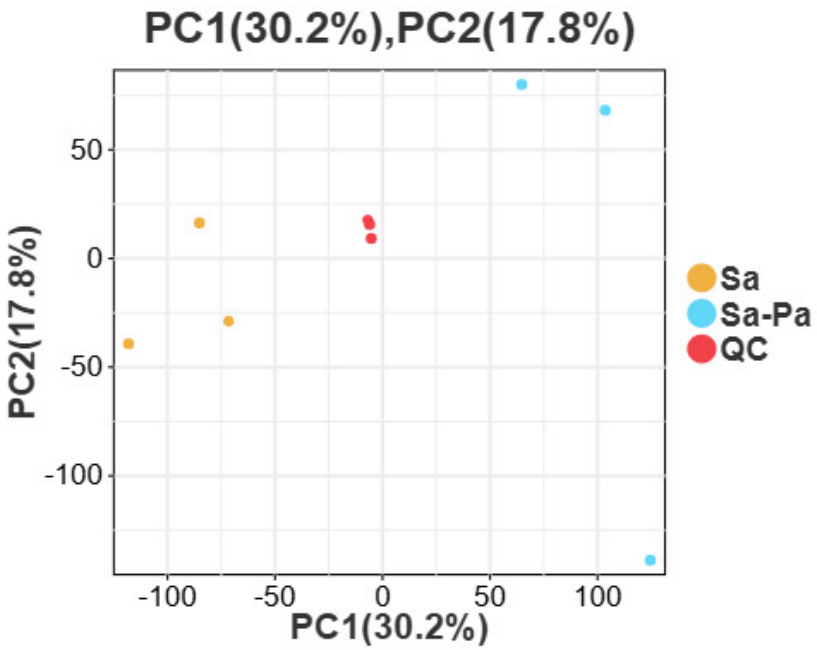


Figure 1. PCA score plot of the control and Cortex Moutan aqueous extract treatment groups.

the differential metabolites were significantly enriched in multiple metabolic pathways, including valine, leucine and isoleucine degradation ($P = 2.0 \times 10^{-4}$), glutamate metabolism, butyrate metabolism, arginine and proline metabolism, ketone body metabolism, the electron transport chain (ETC), and the tricarboxylic acid (TCA) cycle. These pathways were primarily associated with branched-chain amino acid (BCAA) catabolism, core energy metabolism (TCA cycle and butyrate oxidation), and nitrogen metabolism. In addition, purine metabolism, riboflavin metabolism, and tryptophan metabolism exhibited moderate enrichment trends, although they did not reach statistical significance ($P = 0.07$). In contrast, pathways such as α-linolenic acid metabolism and glycine/serine metabolism showed no significant enrichment ($P > 0.05$).

DISCUSSION

In this study, 35 Chinese herbal medicines were screened against *S. agalactiae*. Cortex Moutan, Folium Sennae, and Fructus Foeniculi showed inhibitory effects, with Cortex Moutan exhibiting the strongest activity.

Among the 35 herbal extracts tested, only three showed detectable activity against *S. agalactiae*, with Cortex Moutan being the most effective. This low hit rate contrasts with some previous screening studies and may reflect differences in extraction methods, bacterial strains, and testing conditions. In particular, the aqueous decoction used here may have extracted fewer lipophilic antibacterial compounds than organic solvents, which could explain why several herbs previously reported to be active against *S. agalactiae* showed no activity in our assay.

Cortex Moutan contains paeonol and paeoniflorin as its major active components, which exhibit antibacterial, anti-inflammatory, and antioxidant activities.[11] However, its activity against *S. agalactiae* has not been previously reported. To the best of our knowledge, based on a comprehensive literature search of PubMed, Web of Science, and CNKI, this is the first report of the antibacterial activity of Cortex Moutan specifically against *S. agalactiae*. Folium Sennae contains anthraquinones such as sennosides and emodin, while Fructus Foeniculi contains volatile oils such as anethole and anisaldehyde.[12,13] Studies on the application of these two herbs against aquatic pathogens remain limited, and the present study expands their potential use in aquaculture.

Notably, most of the tested herbal extracts (32 out of 35) showed no activity against *S. agalactiae*, which con-

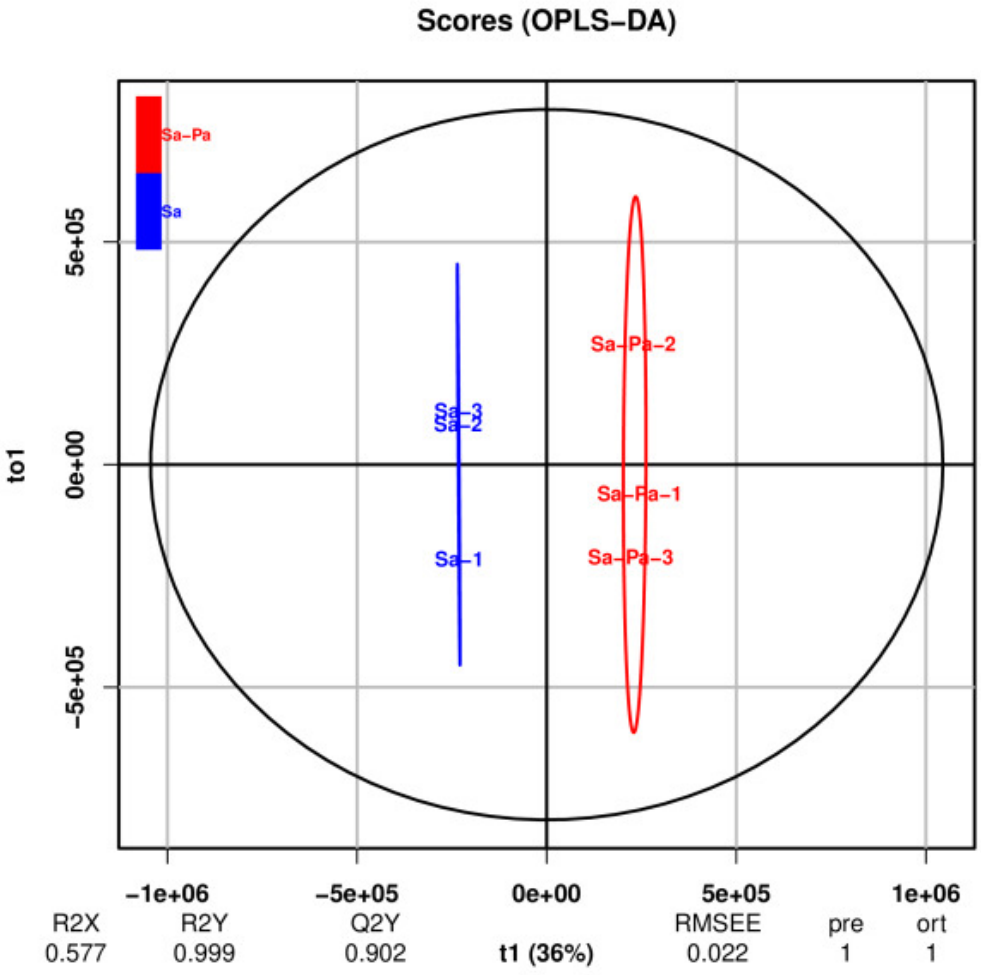


Figure 2. OPLS-DA score plot of the control and Cortex Moutan aqueous extract treatment groups.

trasts with previous findings. For example, Wang reported that Sappan wood and Phellodendron chinense exhibited good antibacterial activity against *S. agalactiae*.[3] Several factors may explain this discrepancy. First, strain variation may play a role, as different *S. agalactiae* isolates may have distinct susceptibility profiles.[14] Second, the discrepancy with Wang et al. may be partly explained by the extraction method. Organic solvents are generally more efficient than water at extracting lipophilic antibacterial compounds such as alkaloids and terpenoids, whereas the aqueous decoction used here mainly extracts polar compounds.[15] Third, the concentration of the herbal extracts (1 g/mL) may be suboptimal for some herbs.[16] Fourth, differences in culture conditions, such as medium, pH, and temperature, may also influence the results.[17]

Based on MS/MS spectral matching and database comparison, graveoline (VIP = 18), piperolactam A (VIP = 16), and emodin (VIP = 9) were tentatively identified and detected at higher levels in the treatment group. Among these, emodin has been reported to possess anti-Gram-positive bacterial activity by disrupting bacterial cell membranes and inhibiting nucleic acid synthesis.[11,18]Additionally, graveoline exhibits a broad spectrum of biological activities, including antibacterial and anti-inflammatory effects. These compounds may be derived from the Cortex Moutan aqueous extract; however, it remains unclear whether they were taken up by bacterial cells or simply remained associated with the extracellular extract.[19] Furthermore, 5-hexyltetrahydro-2-furanoctanoic acid (VIP = 22) was identified as the metabolite with the highest VIP score among all differential metabolites. However, its biological function remains largely unclear. It is hypothesized that this metabolite may be associated with bacterial fatty acid metabolism or stress responses, warranting further investigation.

On the other hand, bacterial metabolism was significantly disrupted. Isoleucine (VIP = 14) and phenylalanine (VIP = 10) were down-regulated in the treatment group. BCAAs and aromatic amino acids are essential for protein synthesis and serve as TCA cycle substrates. MSEA showed significant enrichment of TCA cycle, ETC, and valine/leucine/isoleucine degradation ($P = 2.0 \times 10^{-4}$). Succinate, a TCA cycle intermediate, was also down-regulated. These results may suggest that Cortex Moutan inhibits bacterial growth by disrupting energy metabolism and amino acid synthesis. However, these interpretations are speculative and require further validation.

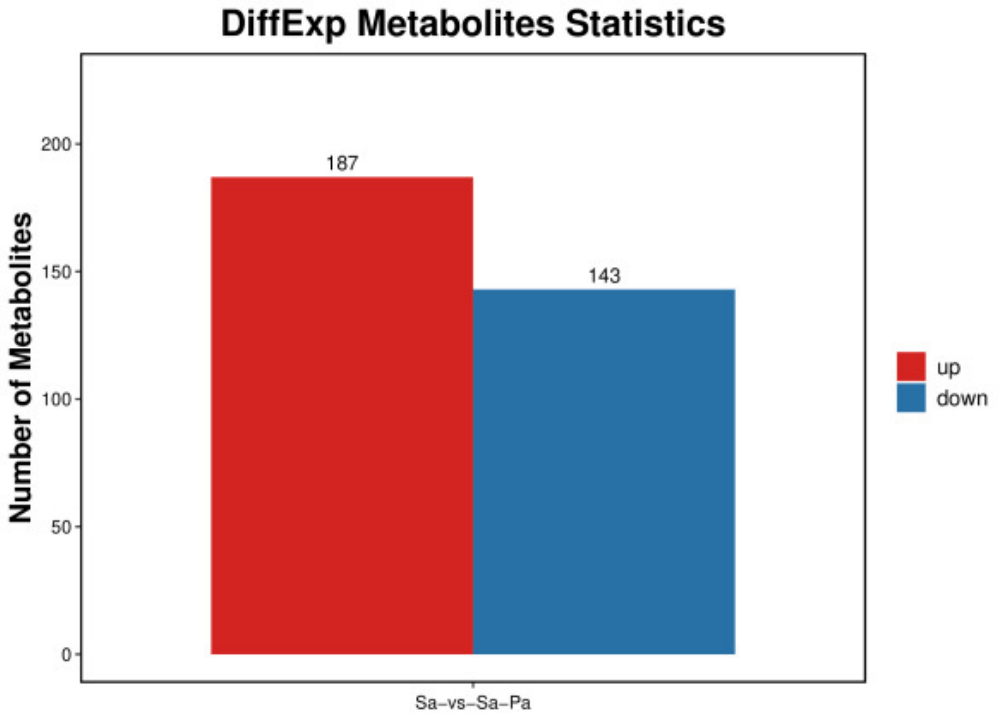


Figure 3. Number of differential metabolites identified between the control and Cortex Moutan aqueous extract treatment groups (up-regulated: 187; down-regulated: 143).

In conclusion, Cortex Moutan, Folium Sennae, and Fructus Foeniculi exhibited in vitro antibacterial activity against *S. agalactiae*, with Cortex Moutan being the most effective. Integrative metabolomics and VIP analyses may suggest that its antibacterial action arises from both the activity of its bioactive metabolites and the suppression of bacterial central carbon and amino acid metabolism. However, these interpretations require further validation. These findings provide preliminary mechanistic insights for exploring plant-derived alternatives to conventional antibiotics in aquaculture. Future studies should extend this work in several directions: testing multiple *S. agalactiae* isolates, increasing the metabolomics sample size, confirming compound identities with authentic standards, including an extract-only control and intracellular quantification, and evaluating the cytotoxicity and safety of Cortex Moutan extracts in tilapia host tissues.

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DECLARATION OF COMPETING INTERESTS

The authors declare no conflict of interest.

ETHICAL CONDUCT APPROVAL

All procedures were carried out in accordance with the Institutional Animal Care and Use Committee Guide at Guangxi Vocational and Technical College of Agricultural Engineering.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All data generated or used during the study appear in the submitted article.

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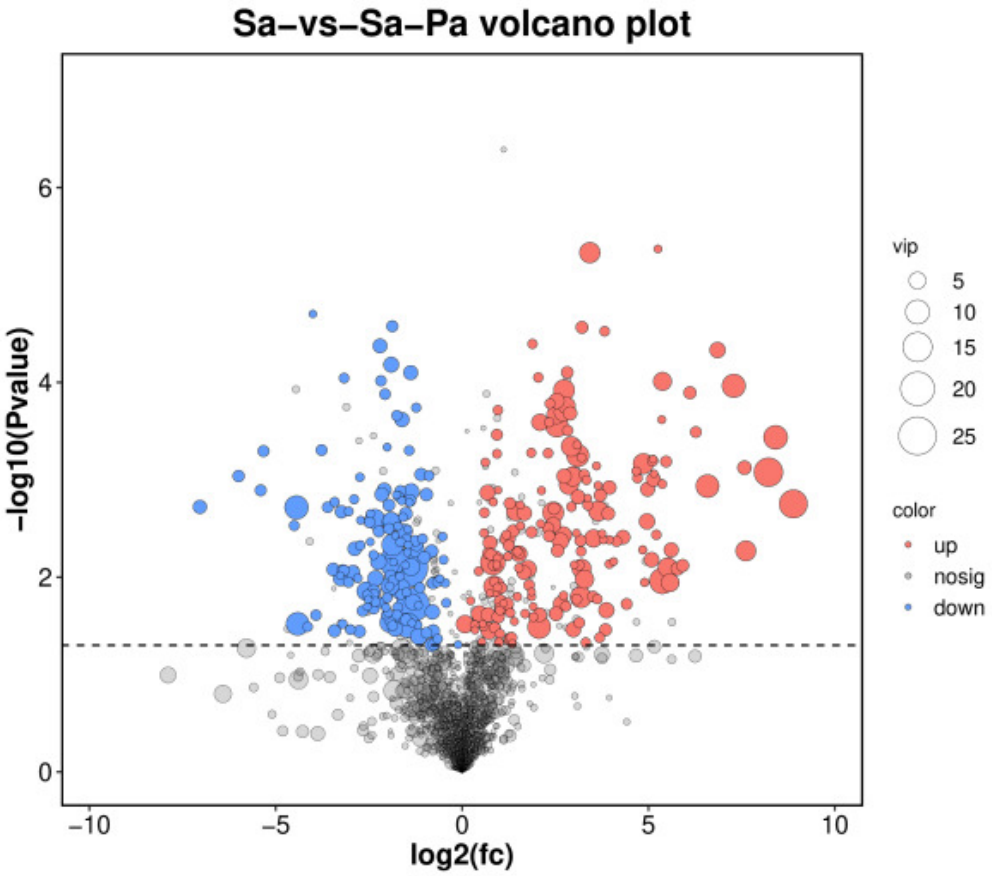


Figure 4. Volcano plot of differential metabolites between the control and Cortex Moutan aqueous extract treatment groups.

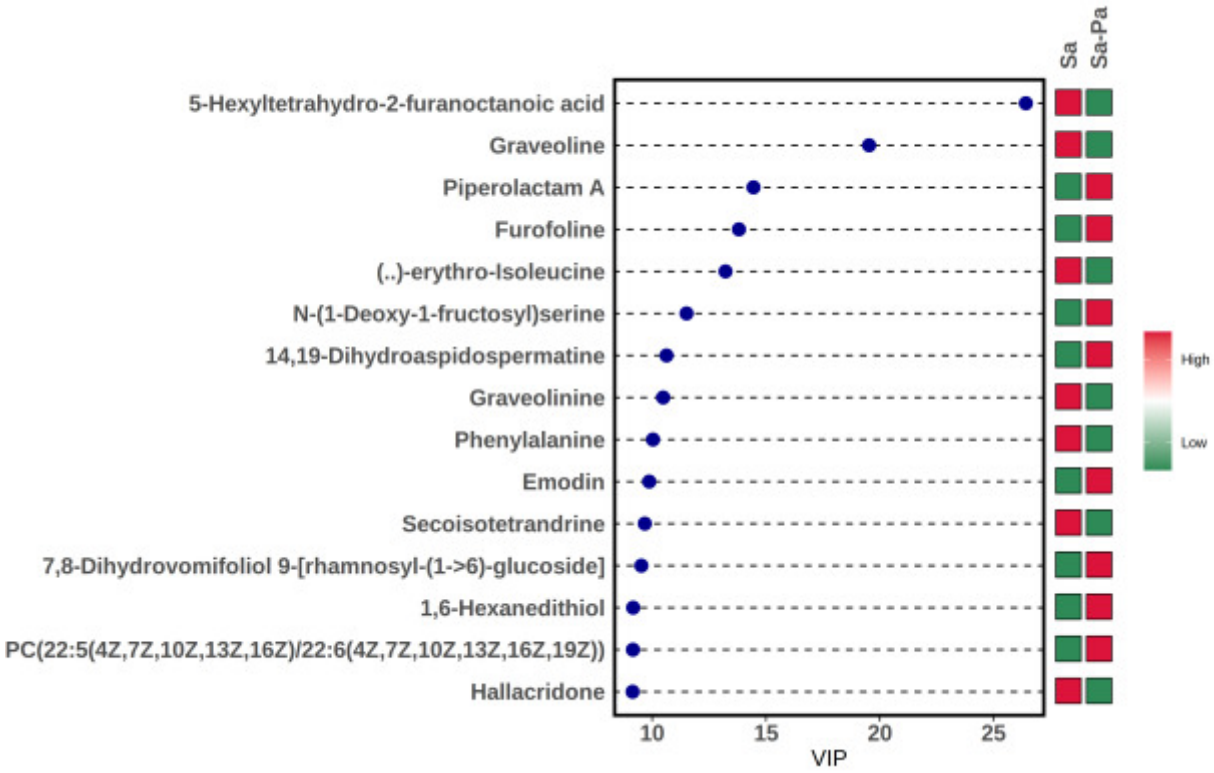


Figure 5. VIP bar plot of the top 15 differential metabolites between the control and Cortex Moutan aqueous extract treatment groups.

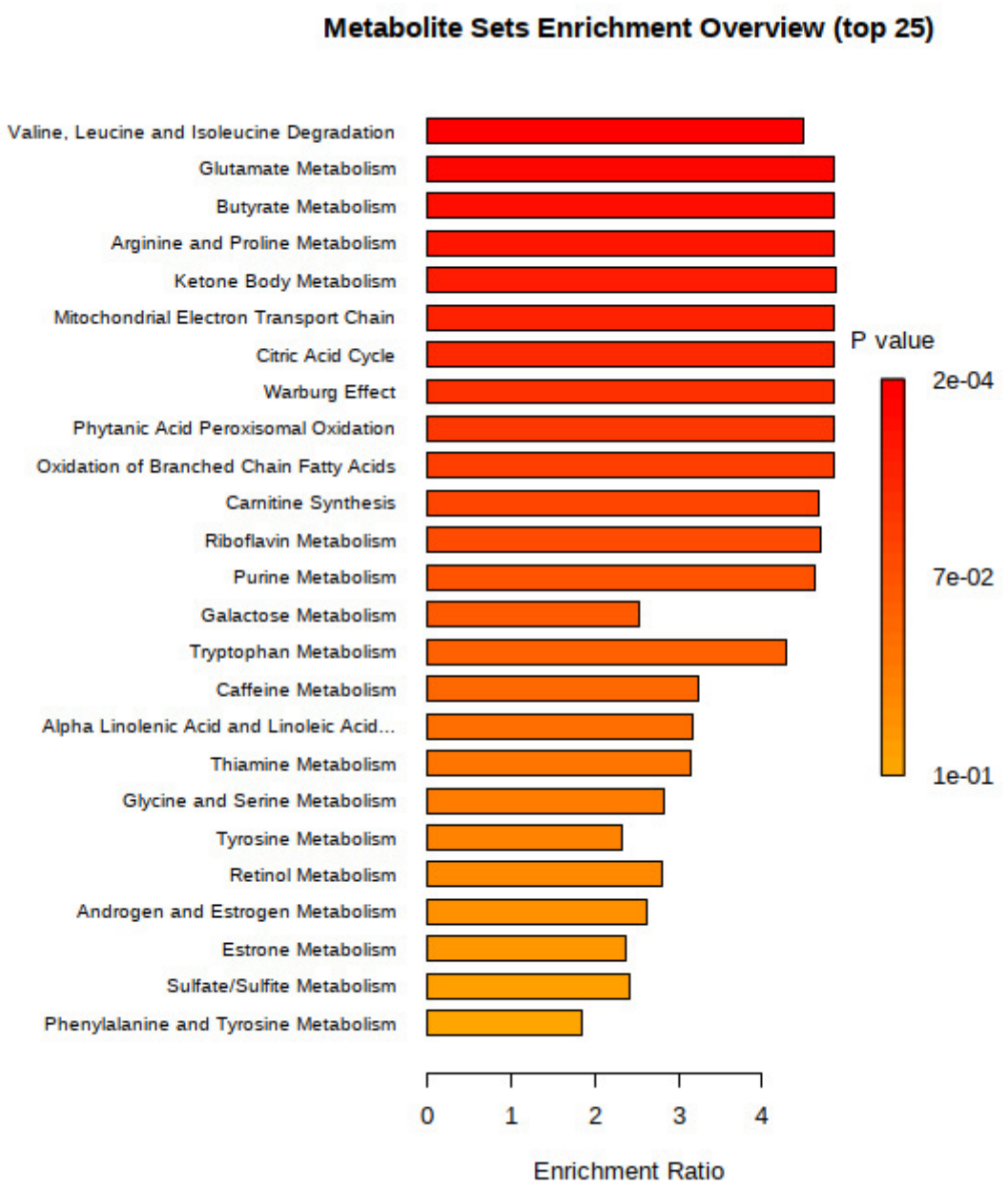


Figure 6. MSEA of differential metabolites between the control and Cortex Moutan aqueous extract treatment groups (top 25 pathways ranked by P-value). The annotation of the electron transport chain pathway is based on the MSEA database and does not directly demonstrate respiratory chain activity in *S. agalactiae*.

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