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Study on Anti-HBV Chemical Constituents from the Hypocotyls of Medicinal Mangrove Bruguiera (Postprint)

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Abstract

The hypocotyls of mangrove *Bruguiera gymnorhiza* are commonly used by the Jing ethnic group as a traditional herbal medicine for hepatitis B. To investigate the chemical constituents in *B. gymnorhiza* hypocotyls and their anti-hepatitis B virus (HBV) activity, this study first employed real-time quantitative PCR to evaluate the anti-HBV activity of different extraction fractions from *B. gymnorhiza* hypocotyls, then utilized modern chromatographic and spectroscopic methods to isolate and identify the chemical constituents from the active fractions, and finally tested the anti-HBV activity of the obtained compounds. The results demonstrated that the n-butanol extract of *B. gymnorhiza* hypocotyls exhibited anti-HBV activity. Eleven compounds were isolated and identified as uracil (1), thymine (2), adenosine (3), oryzalactam (4), n-butyl-O-D-fructopyranoside (5), nortetillapyrone (6), (4R,6S)-4-methoxyl-2,3-dihydroaquilegiolide (7), (4R,6S)-2-dihydromenisdaurilide (8), gallocatechin (9), 1-(4-hydroxy-3-meth-oxy)-phenyl-2-[4-(1,2,3-trihydroxypropyl)-2-methoxy]-phenoxy-1,3-propandiol (10), and ( )-lyoniresinol-9-O-β-D-xylopyranoside (11). Among these, compounds 4–5 and 7–8 were obtained from medicinal mangrove *B. gymnorhiza* for the first time. Compound 4 displayed anti-HBV activity. This study enriches the understanding of anti-HBV chemical constituents from *B. gymnorhiza* hypocotyls.

Full Text

Study on Anti-HBV Chemical Constituents from the Hypocotyl of Medicinal Mangrove *Bruguiera gymnorhiza*

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Abstract

The hypocotyl of *Bruguiera gymnorhiza* is a traditional marine herbal medicine used by the Jing people for treating hepatitis B. To investigate its chemical constituents and anti-hepatitis B virus (HBV) activity, this study first employed real-time quantitative PCR to evaluate the anti-HBV activity of different extracts from the hypocotyl. Modern chromatographic and spectroscopic methods were then used to isolate and identify chemical constituents from the active fraction, followed by testing their anti-HBV activity. The results demonstrated that the n-butanol fraction of *B. gymnorhiza* hypocotyl exhibited anti-HBV activity. Eleven compounds were isolated and identified as uracil (1), thymine (2), adenosine (3), oryzalactam (4), n-butyl-O-D-fructopyranoside (5), nortetillapyrone (6), (4R,6S)-4-methoxyl-2,3-dihydroaquilegiolide (7), (4R,6S)-2-dihydromenisdaurilide (8), gallocatechin (9), 1-(4-hydroxy-3-methoxy)-phenyl-2-[4-(1,2,3-trihydroxypropyl)-2-methoxy]-phenoxy-1,3-propandiol (10), and ( )-lyoniresinol-9-O-β-D-xylopyranoside (11). Among these, compounds 4–5 and 7–8 were obtained from medicinal *B. gymnorhiza* for the first time. Compound 4 displayed anti-HBV activity. This study enriches our understanding of the anti-HBV chemical constituents of *B. gymnorhiza* hypocotyl.

Keywords: *Bruguiera gymnorhiza* hypocotyl; chemical constituents; isolation and purification; structural identification; anti-HBV activity

Introduction

Hepatitis B virus (HBV) is a prevalent virus that causes hepatitis B and belongs to the Hepadnaviridae family. Currently, 240 million people worldwide are infected with HBV, including 93 million in China, of whom 20 million are chronic hepatitis B patients. HBV infection and its complications have become a major public health challenge in China. Clinically available drugs such as lamivudine (3TC) and interferon have significant drawbacks including toxic side effects and drug resistance (Zheng, 2012). Therefore, developing novel agents that inhibit HBV replication represents an urgent challenge in hepatitis B therapy.

The Jing people are an ethnic minority residing in Guangxi and represent China' s only maritime ethnic group (He, 2013). Through their long history of development, the Jing have established a distinctive medicinal system emphasizing marine-derived herbal medicines. *Bruguiera gymnorhiza* serves as a traditional marine medicine in Jing culture, with documented use in treating hepatitis B (Ning et al., 2013; Zhang et al., 2016). Elucidating the material basis of this traditional Jing medicine using modern technological approaches holds significant scientific importance and potential application value. Our research group previously isolated seven nitrile compounds from *B. gymnorhiza* hypocotyl that could inhibit HBV DNA replication in hepatitis B cells (Yi et al., 2015). Continuing our investigation, we subsequently isolated a new alkaloid, Gymnorrhizin A, which showed inhibitory effects on hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) (Chen et al., 2016). However, analysis of HPLC chromatograms revealed additional unreported chemical constituents in *B. gymnorhiza* hypocotyl extracts, hindering the development of this resource for hepatitis B therapeutics. Therefore, to comprehensively elucidate the material basis of *B. gymnorhiza* hypocotyl and identify more anti-HBV compounds, we continued our research to expand the library of marine-derived anti-HBV compounds and establish a foundation for developing novel anti-hepatitis B drugs from this source.

1. Materials and Methods

1.1 Instruments and Materials

The following instruments were used: Waters E2695 semi-preparative HPLC system (Waters, USA) with a Welch Ultimate XB-C18 semi-preparative column [10 mm I.D. × 250 mm, 5 m, Welch Materials (Shanghai) Co., Ltd.]; analytical HPLC column Welch Ultimate XB-C18 (4.6 mm I.D. × 250 mm, 5 m, Welch Materials); Sepacore medium-pressure preparative chromatography (Buchi, Switzerland); EYELA N-1300V-WB rotary evaporator (Shanghai Ailang Instrument Co., Ltd.); WFH-203B darkroom UV analyzer (Hangzhou Qiwei Instrument Co., Ltd.); SW-CJ-2F clean bench (Suzhou Antai Air Technology Co., Ltd.); TAdvanced 96 PCR amplifier (Biometra, Germany); ZWYP-2102 constant temperature incubator shaker (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.); Infinite M200PRO multi-mode microplate reader (Tecan, Switzerland); LightCycler 480 II real-time fluorescence quantitative PCR system (Roche, Switzerland); and HR1500-IIB2 biosafety cabinet (Qingdao Haier Biomedical Co., Ltd.).

Materials included: silica gel for column chromatography (200–300 mesh, Qingdao Marine Chemical Factory); TLC silica gel plates (Yantai Chemical Industry Research Institute); Sephadex LH-20 gel column chromatography (40–70 m, GE Healthcare, USA); fetal bovine serum (Beijing Solarbio Science & Technology Co., Ltd.); DMEM medium (Beijing Solarbio); PBS buffer (Beijing Solarbio); MTT (Beijing Solarbio); trypsin (Beijing Solarbio); lamivudine (Shanghai Macklin Biochemical Co., Ltd.); and HBV DNA quantitative detection kit (Hunan Sansure Biotech Co., Ltd.).

Bruguiera gymnorhiza hypocotyl samples were collected in May 2019 from the Beilun River Estuary Mangrove Nature Reserve in Guangxi (108°12 E, 21°36 N) and authenticated by researcher GAO Chenghai from the Institute of Marine Drugs, Guangxi University of Chinese Medicine, as the hypocotyl of *B. gymnorhiza* (Rhizophoraceae). Voucher specimens (No. GXIMD M20190517) are deposited at the Institute.

1.2 Extraction and Separation

Fresh *B. gymnorhiza* hypocotyl samples (wet weight ~62.0 kg) were extracted three times with 95% industrial ethanol (solid-liquid ratio 1:3) for 7 days each time. The filtrates were combined and concentrated under reduced pressure to obtain a crude extract. The extract was sequentially partitioned with equal volumes of petroleum ether, ethyl acetate, and n-butanol (three times each) to yield petroleum ether (127.2 g), ethyl acetate (178.06 g), n-butanol (626.0 g), and aqueous (1,214.0 g) fractions.

1.3 Anti-HBV Active Fraction Screening

The anti-HBV activity of different extracts was determined by measuring HBV DNA content in the supernatant of cultured HepG2.2.15 cells. The procedure was as follows: (1) Preparation of test solutions: Each crude extract (1 mg) was precisely weighed, dissolved in appropriate DMSO, and diluted with complete cell culture medium to concentrations of 500, 250, and 125 g $\cdot$ mL^{-1}. (2) Cytotoxicity evaluation: MTT assay was used to assess the cytotoxicity of crude extracts and fractions to determine appropriate non-toxic concentrations for antiviral testing. HepG2.2.15 cells in logarithmic growth phase were seeded at 5,000 cells per well in 96-well plates. After adherence, different concentrations of each fraction were added, with negative and lamivudine (3TC) positive control groups. After 72 h incubation, the supernatant was removed, 50 L of 5 mg $\cdot$ mL^{-1} MTT solution was added, and incubation continued for 4 h. After removing the medium, 100 L DMSO was added to each well, mixed thoroughly, and absorbance was measured at 490 nm to calculate cell viability: Cell viability (%) = (A_{490} of test group / A_{490} of negative control) × 100. (3) Anti-HBV activity evaluation: Using quantitative real-time PCR, HepG2.2.15 cells in logarithmic phase were seeded at 5×10^5 cells $\cdot$ mL^{-1} in 24-well plates. After 24 h adherence, the medium was replaced with drug-containing medium (test groups) or complete medium (blank group). On day 6, cell supernatants were collected, high-purity HBV DNA was extracted using a viral DNA preparation kit, and HBV DNA levels were quantified using a hepatitis B nucleic acid detection kit. HBV DNA inhibition rate (%) = (HBV DNA copies in negative control - HBV DNA copies in test group) / HBV DNA copies in negative control × 100.

1.4 Chemical Component Separation

The n-butanol extract (626.0 g) was mixed with silica gel and subjected to silica gel column chromatography with a $CHCl_3$-MeOH gradient system (10:0, 10:1, 5:1, 20:7, 0:10, v/v) to collect 13 fractions (Z1:1; Z2:2–3; Z3:4–8; Z4:9; Z5:10–20; Z6:21–28; Z7:29–33; Z8:34–37; Z9:38–43; Z10:44–57; Z11:58–63; Z12:64–82; Z13:83). Fraction Z3 was further separated by silica gel column chromatography using $CHCl_3$-MeOH (10:0, 10:1, 10:2, 10:4, 10:10, 0:10, v/v) to obtain six subfractions (d1–d6). Subfraction d3 was purified by semi-preparative HPLC (MeOH:H_2O = 10:90, v/v) to yield compounds 7 (2.3 mg, t_R = 13.56 min), 8 (1.6 mg, t_R = 19.18 min), and 4 (1.1 mg, t_R = 21.89 min). Fraction Z4 was separated by silica gel column chromatography with $CHCl_3$-MeOH (10:0, 10:1, 10:2, 10:4, 10:8, 0:10, v/v) to give seven subfractions (e1-e7). Subfraction e4 was purified by semi-preparative HPLC: compound 10 (3.2 mg, t_R = 21.52 min) was obtained at MeOH:H_2O = 20:80 (v/v), and compound 11 (2.4 mg, t_R = 30.52 min) at MeOH:H_2O = 40:60 (v/v). Fraction Z6 spontaneously crystallized upon standing; after repeated recrystallization and semi-preparative HPLC analysis, a pure compound was obtained as compound 5 (10.2 mg). Fraction Z10 was subjected to Sephadex LH-20 gel chromatography with methanol as eluent, and fractions were combined based on TLC analysis to yield 11 subfractions (a1-a11). Subfraction a4 was purified by semi-preparative HPLC (MeOH:H_2O = 95:5, v/v) to obtain compounds 1 (10.09 mg, t_R = 7.08 min), 2 (9.14 mg, t_R = 17.32 min), and 6 (6.64 mg, t_R = 9.79 min). Subfraction a5 was purified by semi-preparative HPLC (MeOH:H_2O = 95:5, v/v) to yield compound 3 (4.23 mg, t_R = 13.05 min). Fraction Z13 (wet weight 15 g) was subjected to macroporous resin column chromatography (eluted sequentially with water, 50% methanol, and 100% methanol, 5 column volumes each) to obtain three subfractions (b1-b3). Subfraction b3 was purified by semi-preparative HPLC (MeOH:H_2O = 80:20, v/v) to yield compound 9 (2.01 mg, t_R = 23.45 min).

1.5 Anti-HBV Activity Evaluation of Monomeric Compounds

For MTT assays, HepG2.2.15 cells were seeded at 5,000 cells per well in 96-well plates. After adherence, isolated compounds were diluted to 100 $\mu g \cdot mL^{-1}$, with 100 $\mu g \cdot mL^{-1}$ lamivudine (3TC) as positive control and a negative control group. After 72 h treatment, absorbance was measured at 490 nm using a microplate reader, and cell viability was calculated as: Cell viability (%) = (A_{490} of test group / A_{490} of negative control) × 100.

For quantitative real-time PCR, HepG2.2.15 cells were seeded at 5×10^5 cells per well in 24-well plates. After adherence, compounds were diluted to 100 $\mu g \cdot mL^{-1}$ with 100 $\mu g \cdot mL^{-1}$ 3TC as positive control and a negative control group. HBV DNA content in cell supernatants was quantified on day 6 using a hepatitis B nucleic acid detection kit.

2. Results

2.1 Active Fraction Screening Results

High drug concentrations can cause cell deformation and death, leading to reduced secretion of HBV markers and confounding assessment of antiviral activity (Liu and Lin, 1995). Therefore, it is essential to determine non-toxic drug concentrations for HBV experiments. MTT assays were used to evaluate the effects of crude extract and various fractions on HepG2.2.15 cell proliferation to ensure subsequent anti-HBV experiments were conducted under essentially non-toxic conditions. As shown in , compared with the negative control group, the crude extract and all fractions except the aqueous fraction significantly inhibited cell proliferation at 500 and 250 $\mu g \cdot mL^{-1}$ ($P < 0.05$). At 125 $\mu g \cdot mL^{-1}$, the crude extract and other fractions showed no cytotoxicity to HepG2.2.15 cells, making this concentration suitable for subsequent activity assays.

HBV-DNA serves as the fundamental basis for viral replication and a direct indicator for monitoring HBV replication. Research indicates that the infectivity of chronic HBV infection correlates closely with HBV DNA levels, and pathological manifestations of hepatitis B disease vary with changes in HBV DNA replication levels. Quantitative HBV DNA detection is the most direct method for determining viral replication (Wang et al., 2022). The anti-HBV activity of *B. gymnorhiza* crude extract and fractions was evaluated by measuring their effects on HBV DNA levels in HepG2.2.15 cell supernatants. As shown in [Figure 1: see original paper], compared with the blank control group, the petroleum ether fraction showed no inhibitory effect on HBV DNA levels. The crude extract, aqueous fraction, and n-butanol fraction all significantly reduced HBV DNA levels ($P < 0.05$), with the n-butanol fraction demonstrating extremely significant inhibition ($P < 0.01$) and stronger effects than the crude extract. Therefore, the n-butanol fraction was selected for further chemical investigation.

2.2 Chemical Structure Identification

The structures of isolated compounds are shown in [Figure 2: see original paper].

Compound 1 was obtained as white crystals. ESI-MS m/z: 244.7 $[M+H]^+$, molecular formula: $C_9H_{12}N_2O_6$. ^1H NMR (500 MHz, DMSO-d_6) δ_H: 11.31 (1H, s, H-3), 7.89 (1H, d, J = 8.07 Hz, H-6), 5.78 (1H, d, J = 5.46 Hz, H-1), 5.65 (1H, d, J = 8.04 Hz, H-5), 4.02 (1H, t, J = 5.34 Hz, H-2), 3.96 (1H, t, J = 4.53 Hz, H-3), 3.84 (1H, q, J = 3.44 Hz, H-4), 3.62 (1H, dt, J = 3.06, 12.11 Hz, H-5), 3.55 (1H, dt, J = 3.01, 12.20 Hz, H-6); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 150.8 (C-2), 163.2 (C-4), 101.8 (C-5), 140.8 (C-6), 87.7 (C-1), 69.9 (C-2), 73.6 (C-3), 84.9 (C-4), 60.9 (C-5). The spectroscopic data matched literature reports (Wang et al., 2010), identifying compound 1 as uracil.

Compound 2 was obtained as a white powder. ESI-MS m/z: 243.1 $[M+H]^+$, molecular formula: $C_{10}H_{14}N_2O_5$. ^1H NMR (500 MHz, DMSO-d_6) δ_H: 11.27 (1H, s, H-3), 7.70 (1H, d, J = 1.51 Hz, H-6), 6.17 (1H, dd, J = 6.13, 7.63 Hz, H-1), 4.24 (1H, dt, J = 2.98, 5.86 Hz, H-3), 3.76 (1H, q, J = 3.72 Hz, H-4), 3.56 (2H, m, H-5), 2.07 (2H, m, H-2), 1.77 (3H, d, J = 1.18 Hz, -CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 150.5 (C-2), 163.8 (C-4), 109.4 (C-5), 136.2 (C-6), 83.7 (C-1), 70.4 (C-3), 87.3 (C-4), 61.3 (C-5), 12.3 (5-CH_3). The spectroscopic data matched literature reports (Yao et al., 2018), identifying compound 2 as thymine.

Compound 3 was obtained as a white powder. ESI-MS m/z: 268.1 $[M+H]^+$, molecular formula: $C_{10}H_{13}N_5O_4$. ^1H NMR (DMSO-d_6, 500 MHz) δ_H: 8.35 (1H, s, H-8), 8.13 (1H, s, H-2), 7.34 (2H, s, NH_2), 5.87 (1H, d, J = 6.24 Hz, H-1), 4.61 (1H, m, H-2), 4.14 (1H, dd, J = 4.97, 2.95 Hz, H-3), 3.96 (1H, m, H-4), 3.67 (1H, dd, J = 12.19, 3.66 Hz, H-5), 3.55 (1H, dd, J = 12.17, 3.66 Hz, H-5); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 152.4 (C-2), 149.1 (C-4), 119.4 (C-5), 156.2 (C-6), 139.9 (C-8), 87.9 (C-1), 73.4 (C-2), 70.7 (C-3), 85.9 (C-4), 61.7 (C-5). The spectroscopic data matched literature reports (Kun et al., 1991), identifying compound 3 as adenosine.

Compound 4 was obtained as a yellow powder. ESI-MS m/z: 238.2 $[M+H]^+$, molecular formula: $C_{11}H_{11}NO_5$. ^1H NMR (DMSO-d_6, 500 MHz) δ_H: 6.76 (1H, t, J = 1.55 Hz, H-3), 6.58 (2H, d, J = 1.49 Hz, H-1,4), 6.05 (1H, s, H-6), 3.41 (3H, s, 12-CH_3), 2.89 (2H, d, J = 10.83 Hz, 8-CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 114.9 (C-1), 152.3 (C-2), 112.0 (C-3), 109.8 (C-4), 72.9 (C-5), 177.6 (C-7), 41.3 (C-8), 134.0 (C-9), 131.8 (C-10), 169.0 (C-11), 51.1 (C-12). The spectroscopic data matched literature reports (Wang et al., 2014), identifying compound 4 as oryzalactam.

Compound 5 was obtained as colorless needle crystals. ESI-MS m/z: 235.2 $[M-H]^-$, molecular formula: $C_{10}H_{20}O_6$. ^1H NMR (MeOH-d_4, 500 MHz) δ_H: 3.91 (1H, d, J = 9.90 Hz, H-3), 3.87 (1H, dt, J = 3.35, 1.65 Hz, H-5), 3.76 (3H, m, H-1 ,4), 3.67 (2H, m, H-6), 3.51 (2H, m, 1-CH_2), 1.56 (2H, dqd, J = 8.60, 6.64, 2.50 Hz, 2-CH_2), 1.40 (2H, m, 3-CH_2), 0.94 (3H, t, J = 7.38 Hz, 4-CH_3); ^{13}C NMR (125 MHz, MeOH-d_4) δ_C: 61.6 (C-1), 33.3 (C-2), 20.5 (C-3), 14.3 (C-4), 63.5 (C-1), 101.6 (C-2), 70.6 (C-3), 71.6 (C-4), 71.1 (C-5), 65.2 (C-6). The spectroscopic data matched literature reports (An et al., 2006), identifying compound 5 as n-butyl-O-D-fructopyranoside.

Compound 6 was obtained as a white powder. ESI-MS m/z: 227.2 $[M-H]^-$, molecular formula: $C_{10}H_{12}O_6$. ^1H NMR (500 MHz, DMSO-d_6) δ_H: 7.85 (1H, d, J = 8.09 Hz, H-4), 6.15 (1H, m, H-7), 5.63 (1H, d, J = 8.08 Hz, H-3), 4.23 (1H, dq, J = 3.10, 6.34 Hz, H-9), 3.78 (1H, q, J = 3.58 Hz, H-11), 3.55 (2H, m, H-11), 2.08 (2H, m, H-8); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 163.2 (C-2), 101.8 (C-3), 140.6 (C-4), 150.5 (C-6), 84.1 (C-7), 39.7 (C-8), 70.4 (C-9), 87.4 (C-10), 61.3 (C-11). The spectroscopic data matched literature reports (Watanadilok et al., 2001), identifying compound 6 as nortetillapyrone.

Compound 7 was obtained as a colorless powder. ESI-MS m/z: 169.1 $[M+H]^+$, molecular formula: $C_9H_{12}O_3$. ^1H NMR (DMSO-d_6, 500 MHz) δ_H: 5.86 (1H, d, J = 1.97 Hz, H-9), 4.92 (1H, ddd, J = 11.67, 6.18, 1.58 Hz, H-6), 3.75 (1H, m, H-4), 3.58 (3H, s, CH_3), 2.75 (1H, ddd, J = 14.23, 4.73, 2.16 Hz, H-2), 2.33 (1H, tdd, J = 14.00, 5.60, 1.98 Hz, H-2), 2.04 (1H, dtd, J = 12.34, 3.87, 2.03 Hz, H-5), 1.19 (3H, m, H-5, H-3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 172.4 (C-1), 34.7 (C-2), 23.7 (C-3), 79.4 (C-4), 42.3 (C-5), 65.1 (C-6), 173.0 (C-8), 111.9 (C-9), 51.3 (CH_3). The spectroscopic data matched literature reports (Otsuka et al., 1993), identifying compound 7 as (4R,6S)-4-methoxy-2,3-dihydroaquilegiolide.

Compound 8 was obtained as a colorless powder. ESI-MS m/z: 155.1 $[M+H]^+$, molecular formula: $C_8H_{10}O_3$. ^1H NMR (DMSO-d_6, 500 MHz) δ_H: 5.86 (1H, d, J = 1.92 Hz, H-9), 5.04 (1H, m, H-6), 4.12 (1H, p, J = 2.87 Hz, H-4), 2.64 (2H, m, H-2), 2.48 (1H, dd, J = 6.32, 3.17 Hz, H-5), 1.96 (1H, ddq, J = 13.37, 5.30, 2.38 Hz, H-3), 1.46 (1H, tdd, J = 13.34, 5.20, 2.34 Hz, H-3), 1.31 (1H, td, J = 11.95, 2.39 Hz, H-5); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 173.1 (C-1), 32.7 (C-2), 40.3 (C-3), 63.9 (C-4), 22.5 (C-5), 78.7 (C-6), 111.4 (C-7), 173.4 (C-8). The spectroscopic data matched literature reports (Otsuka et al., 1993), identifying compound 8 as (4R,6S)-2-dihydromenisdaurilide.

Compound 9 was obtained as a yellow oil. ESI-MS m/z: 305.2 [M-H]$^-$, molecular formula: $C_{15}H_{14}O_7$. ^{1}H NMR (DMSO-d$_6$, 500 MHz) δ_H: 6.24 (2H, s, H-2 ,6), 5.88 (1H, d, J = 2.34 Hz, H-6), 5.69 (1H, d, J = 2.26 Hz, H-8), 4.42 (1H, d, J = 7.04 Hz, H-2), 3.78 (1H, ddd, J = 12.44, 7.48, 5.04 Hz, H-3), 2.61 (1H, m, H-4), 2.34 (1H, m, H-4); ^{13}C NMR (125 MHz, DMSO-d$_6$) δ_C: 81.0 (C-2), 66.3 (C-3), 27.4 (C-4), 156.4 (C-5), 95.0 (C-6), 156.2 (C-7), 93.8 (C-8), 155.3 (C-9), 98.9 (C-10), 129.8 (C-1), 106.0 (C-2 , C-6), 145.6 (C-3 , C-5), 132.5 (C-4). The spectroscopic data matched literature reports (Xiao et al., 2015), identifying compound 9 as gallocatechin.

Compound 10 was obtained as a colorless powder. ESI-MS m/z: 411.2 [M+H]$^+$, molecular formula: $C_{20}H_{26}O_9$. ^{1}H NMR (MeOH-d$_4$, 500 MHz) δ_H: 6.97 (1H, d, J = 2.07 Hz, H-2), 6.93 (1H, m, H-5), 6.91 (1H, s, H-2), 6.76 (2H, ddt, J = 14.21, 7.87, 1.70 Hz, H-6,6), 6.65 (1H, dd, J = 8.19, 1.23 Hz, H-5), 4.80 (1H, m, H-7), 4.47 (1H, d, 5.84 Hz, H-7), 4.18 (1H, m, H-8), 3.78 (3H, s, OCH$_3$), 3.72 (3H, s, OCH$_3$), 3.56 (1H, m, H-8), 3.39 (1H, dd, J = 11.94, 5.37 Hz, H-9), 3.27 (1H, m, H-9), 1.80 (s, 1H), 1.25 (d, J = 6.75 Hz, 2H), 1.19 (s, 2H); ^{13}C NMR (125 MHz, MeOH-d$_4$) δ_C: 132.4 (C-1), 110.3 (C-2), 147.5 (C-3), 147.4 (C-4), 117.2 (C-5), 119.2 (C-6), 72.6 (C-7), 85.8 (C-8), 60.5 (C-9), 136.5 (C-1), 110.8 (C-2), 150.1 (C-3), 145.8 (C-4), 114.4 (C-5), 119.3 (C-6), 73.7 (C-7), 76.0 (C-8), 62.8 (C-9), 55.1 (OCH$_3$), 54.9 (OCH$_3$). The spectroscopic data matched literature reports (Greca et al., 1998), identifying compound 10 as 1-(4-hydroxy-3-methoxy)-phenyl-2-[4-(1,2,3-trihydroxypropyl)-2-methoxy]-phenoxy-1,3-propandiol.

Compound 11 was obtained as a yellow powder. ESI-MS m/z: 551.5 [M-H]$^-$, molecular formula: $C_{27}H_{36}O_{12}$. ^{1}H NMR (MeOH-d$_4$, 500 MHz) δ_H: 6.57 (1H, s, H-2), 6.43 (2H, s, H-2,6), 4.38 (1H, d, J = 6.63 Hz, H-7), 4.21 (1H, d, J = 7.55 Hz, H-1), 3.84 (6H, m, 3 ,5 -CH$_3$), 3.74 (6H, s, 3,5-CH$_3$), 3.64-3.16 (9H, 3 , H-9,9 , 2 -5), 2.71 (1H, dd, J = 15.20, 4.59 Hz, H-7), 2.63 (1H, dd, J = 15.16, 11.56 Hz, H-7), 2.05 (1H, dddd, J = 10.70, 6.80, 4.08, 2.64 Hz, H-8), 1.70 (1H, m, H-8); ^{13}C NMR (125 MHz, MeOH-d$_4$) δ_C: 138.9 (C-1), 134.4 (C-4), 149.0 (C-3,5), 106.9 (C-2,6), 43.0 (C-7), 46.7 (C-8), 70.9 (C-9), 56.8 (3,5-OCH$_3$), 130.1 (C-1), 107.8 (C-2), 148.6 (C-3), 139.4 (C-4), 147.6 (C-5), 126.4 (C-6), 33.9 (C-7), 40.5 (C-8), 66.0 (C-9), 56.6 (3 -OCH$_3$), 105.5 (C-1), 75.0 (C-2), 78.0 (C-3), 71.3 (C-4), 67.0 (C-5), 60.0 (5 -OCH$_3$). The spectroscopic data matched literature reports (Wu and Li, 2011), identifying compound 11 as ( )-lyoniresinol-9-O-β-D-xylopyranoside.

2.3 Anti-HBV Activity of Monomeric Compounds

At 100 µg · mL^{-1}, compounds 1 and 10 significantly reduced HepG2.2.15 cell viability by 14.05% and 11.10%, respectively, compared with the blank control group ($P < 0.05$). The remaining compounds showed no significant cytotoxicity, as shown in . Therefore, compounds 2-9 and 11 were selected for anti-HBV activity evaluation at 100 $\mu g \cdot mL^{-1}$.

Compared with the negative control group, compound 4 reduced HBV DNA levels in HepG2.2.15 cell supernatants with an inhibition rate of 23.59% ($P > 0.05$), while other compounds showed no significant reduction in HBV DNA secretion ($P > 0.05$), as shown in [Figure 3: see original paper].

3. Discussion and Conclusion

The Jing people refer to mangroves as “Hailanshan” (sea olive mountain). Due to the lack of clean drinking water and a diet primarily consisting of fish, shrimp, and crabs, hepatitis has been a prevalent disease among the Jing community. *Bruguiera gymnorhiza* possesses heat-clearing and detoxifying properties and is traditionally used by the Jing people to treat hepatitis B. The hypocotyl represents a renewable biological resource whose harvest does not damage the ecological environment or mangrove resources. This study confirmed the anti-HBV activity of the n-butanol fraction from *B. gymnorhiza* hypocotyl, thereby validating the scientific basis of traditional Jing medicine.

Eleven compounds were obtained from the active n-butanol fraction, including four compounds isolated from medicinal *B. gymnorhiza* for the first time: oryzalactam (4), n-butyl-O-D-fructopyranoside (5), (4R,6S)-4-methoxyl-2,3-dihydroaquilegiolide (7), and (4R,6S)-2-dihydromenisdaurilide (8), thus enriching the chemical profile of this species. Previous studies have identified various structural classes from *B. gymnorhiza* including diterpenoids, triterpenoids, phenolics, sulfur-containing compounds, and alkaloids (Gao et al., 2022). The current study expanded this chemical diversity with alkaloid (compound 4), glycoside (compound 5), and lactone (compounds 7-8) structures.

Four alkaloid compounds were isolated: uracil (1), thymine (2), adenosine (3), and oryzalactam (4). Alkaloids are important natural products exhibiting antiviral activity against various viruses including influenza A, HBV, HCV, HSV, HIV, Zika virus, Coxsackie virus, and tobacco mosaic virus (Wang et al., 2022). This study reports for the first time that the alkaloid oryzalactam (4) possesses anti-HBV activity, in addition to strong $ABTS^{+}$ and DPPH radical scavenging capacity (Wang et al., 2014). We hypothesize that the anti-HBV activity of oryzalactam may be related to its potent antioxidant properties, where antioxidant functional groups may synergistically or antagonistically modulate physiological factors to enhance expression of beneficial factors, thereby manifesting significant anti-HBV activity at non-cytotoxic concentrations. Previous studies have shown that alkaloids exert anti-HBV activity by directly inhibiting HBV-DNA and HBV-cccDNA production, while others such as sophoridine act indirectly by reducing protein kinase (p38 MAPK) levels (Liang et al., 2021). The strong antioxidant activity of compound 4 may antagonize HBV-DNA factor expression, consistent with our hypothesis. Further research on anti-HBV constituents from hypocotyl extracts is warranted. Based on the UV absorption characteristics of compound 4, future work will employ HPLC methods to directionally obtain more analogs, providing lead compounds for developing novel marine-derived anti-HBV agents and enhancing the medicinal value of *B. gymnorhiza* hypocotyl.

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