Studies on Cytotoxic Activity against HepG-2 Cells of Naphthoquinones from Green Walnut Husks of *Juglans mandshurica* Maxim

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**Abstract:** Twenty-seven naphthoquinones and their derivatives, including four new naphthalenyl glucosides and twenty-three known compounds, were isolated from green walnut husks, which came from *Juglans mandshurica* Maxim. The structures of four new naphthalenyl glucosides were elucidated based on extensive spectroscopic analyses. All of these compounds were evaluated for their cytotoxic activities against the growth of human cancer cells lines HepG-2 by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay. The results were shown that most naphthoquinones in an aglycone form exhibited better cytotoxicity in vitro than naphthalenyl glucosides with IC₅₀ values in the range of 7.33–88.23 μM. Meanwhile, preliminary structure-activity relationships for these compounds were discussed.

**Keywords:** *Juglans mandshurica* Maxim; green walnut husks; naphthoquinones; cytotoxic activity; structure-activity relationships
1. Introduction

With the increased use of natural product-based cancer chemotherapy, exploring the cytotoxic activity of phytochemicals for anticancer drug design has gained extensive attention worldwide [1]. *Juglans mandshurica* Maxim is a well-known member of the *Juglandaceae* family which is widely distributed throughout urban and rural areas in northeast China [2–4]. A few distribute in Russia, Korea and Japan. It is one of the most important medicinal plants of which the green husks, leaf, root and bark all can be medically used [5–8]. Its green husks have been used as a folk medicine for treatment of gastric ulcers, uterine prolapse, leukopenia, diarrhea and dysentery for many years in China [9]. In recent years, many studies showed that green walnut husks have obvious advantages in tumor treatment like liver cancer [9–11].

Based on our interest in natural antitumor sources prompted us to continue investigating the phytochemicals and cytotoxicity of this plant [12–16]. A number of extracts and compounds obtained from medical materials have been identified as in vitro tumor inhibitors [7,9]. These beneficial effects have largely been ascribed to the presence of naphthoquinones. Naphthoquinones are the most important and widely distributed chemical class in the quinone family. Their derivatives have exhibited a variety of biological responses which include antiallergic, antibacterial, antifungal, anti-inflammatory, anti-thrombotic, antiplatelet, antiviral, apoptosis, lipoxygenase, radical scavenging, and anti-ringworm activities. Many studies have shown that naphthoquinones have biological activities specifically against pathogenic protozoa and cancer cells owing to their privileged structures in medicinal chemistry [17]. In this study, we obtained a series of naphthoquinone aglycones and glucosides, and then presented the isolation in vitro from medical materials have been identified as tumor inhibitors [7,9]. These beneficial effects were required for cytotoxic activity through the determination of the ability of 27 naphthoquinones to inhibit proliferation of cancer cells. The aim of this work was to define the key naphthoquinone structural elements that were required for cytotoxic activity through the determination of the ability of 27 naphthoquinones belonging to main structural subtypes such as naphthoquinone, tetralone, and naphthols.
2. Results and Discussion

2.1. Isolation and Characterization of Compounds 18, 25–27

The compounds were isolated using silica gel columns and semi-preparative HPLC chromatography from 30% ethanol extract of fresh green husks of *Juglans mandshurica* Maxim. The structures of four new naphthalenyl glucosides were elucidated based on extensive mass and spectroscopic analyses including HR-ESI-MS, IR, ¹H-NMR, ¹³C-NMR, DEPT, HSQC, HMBC, and CD. Their structures, ¹H- and ¹³C-NMR data, and HMBC correlations are shown in Figures 1 and 2 and Table 1.

![Chemical structures of compounds 1–27.](image)

Figure 1. The chemical structures of compounds 1–27.

Compound 18 was a red amorphous powder. The molecular formula C₁₉H₂₂O₁₀ was determined from HR-ESI-MS and ¹³C-NMR data. There were two major differences between 18 and 25–27: two methylene groups located at C-2 and C-3, respectively, at δC 33.0–35.0 and 30.0–31.5 in compounds 25–27 were replaced by methenyl groups at δC 109.9 and 105.8 in compound 18, indicating no presence of a hydrogenated position. Furthermore, the independent existence of the glucopyranosyl moiety was not together with p-hydroxybenzoyl on the basis of 1D-, 2D-NMR data. Noise-decoupled ¹³C-NMR and the distortionless enhancement by polarization transfer (DEPT) spectrum of 18 showed 19 carbon peaks, including one methyl, two methylenes, nine methynes, and seven quaternary carbons. There were 10 carbons due to the naphthalene ring, six carbons due to the glucose, and a carbonyl ketone at...
δc 171.8 correlated with one ethyl group, which was assigned to acetyl group. In the ¹H-NMR spectrum, there were ABC-spin aromatic proton signals at δH 6.99 (dd, J = 1.0, 7.8 Hz, H-5), 7.40 (t, J = 7.8 Hz, H-6), and 7.86 (dd, J = 1.0, 7.8 Hz, H-7), which couple among themselves. Moreover, one isolated proton signal due to H-2 at δH 7.72 and one double-peak signal due to an anomic proton at δH 4.99 were distinct. In the HMBC spectrum of 18 (Figure 2), the correlation peak between the anomic proton and C-1 at δc 148.0 was observed. The results implied that the glucopyranosyl was linked to C-1 of the aglycone (Table 1, Figure 2). Thus, the structure of 18 was elucidated as 1,4,8-trihydroxy-3-naphthalenecarboxylic acid 1-O-β-D-glucopyranoside ethyl ester.

Figure 2. Key HMBC correlations of compounds 18, 25–27.

Compound 25 was obtained as a yellow amorphous powder and the molecular formula was assigned as C₂₃H₂₄O₉ from its HR-ESI-MS and ¹³C-NMR data. ¹H-NMR and ¹³C-NMR spectra revealed that 25 contained a typical β-D-glucopyranosyl (δH 4.42 (d, J = 7.6 Hz, H-1'); δc 103.7, 75.2, 78.1, 72.2, 75.5, 65.0), which was confirmed by acid hydrolysis and co-chromatography in comparison with an authentic sample. Moreover, the remaining 17 carbon signals, which respectively belong to the tetralone moiety and a p-hydroxybenzoyl group, were attributable to two methylenes, nine methines, four olefinic quaternary carbons, and two quaternary carbonyl groups. To ascertain the structure of the aglycone and the glycosidic connection, a complete ¹H- and ¹³C-NMR spectral assignment was carried out utilizing a combination of DEPT, HSQC, HMBC, and CD experiments. To be specific, the ¹H-NMR spectrum of 25 showed two methylenes of tetralone at δH 2.87 (ddd, J = 4.5, 8.9, 17.5 Hz, Hax-2) and δH 2.41 (ddd, J = 4.5, 6.5, 17.5 Hz, Heq-2); 2.34 (ddd, J = 2.2, 4.5, 8.9, 13.4 Hz, Hax-3) and 2.28 (ddd, J = 3.8, 4.5, 6.5, 13.4 Hz, Heq-3), corresponding to C-atom signals at δc 35.5 and 31.5 in the HSQC spectrum. In the ¹H-NMR spectrum, there was a set of correlation signals at δH 7.65 (br.d, J = 7.6 Hz, H-5), 7.52 (dt, J = 1.2, 7.6 Hz, H-6), 7.43 (dt, J = 1.2, 7.6 Hz, H-7), and 7.93 (dd, J = 1.2, 7.6 Hz, H-8) due to an
ortho-disubstituted aromatic ring. All above data implied that 25 was an α-tetralone derivative. Hydrolysis of 25 yielded glucose, which was identified on a thin layer chromatography (TLC) plate by comparison with a reference sample. Moreover, a suggestive correlation was observed between the anomic proton signal of glucose and a methane carbon signal at δC 75.9 (C-4) in the HMBC spectrum (Figure 2), indicating that the sugar moiety was linked at the C-4 position. The β-anomic configuration for glucopyranose was determined from the JH1,H2 value (7.6 Hz). At the same time, it was also observed that the δH 4.66 (dd, J = 2.2, 11.8 Hz, H-6′a) and 4.47 (dd, J = 8.8 Hz, H-3″, 5″), and δC132.9 (C-2″, 6″), 116.3 (C-3″, 5″) in the 1H- and 13C-NMR spectrum, indicating the presence of p-hydroxybenzoyl. To determine the absolute configuration of the chiral center at the C-4 position, 25 was hydrolyzed to give the aglycone, which was identified to be S configuration by comparing its NMR data with those of the reference [18,19] and the circular dichroism CD spectrum, where a negative Cotton effect at 236 nm was observed. On the basis of the above evidence, the structure of 25 was established as (4S)-4-hydroxy-α-tetralone 4-O-β-D-(6′-O′-4″-hydroxybenzoyl) glucopyranoside.

Compound 26, a yellow amorphous powder, was assigned as C23H24O10 on the basis of its HR-ESI-MS and 13C-NMR data. The 1D- and 2D-NMR spectrographic data were similar as compound 25 except for the aryl ring moiety of the tetralone. The 1H-NMR spectrum of 26 showed a set of proton signals that was in accordance with the ABC-type aromatic proton signals, indicating the presence of a hydroxyl group at the C-5 position on the aromatic ring. The position of the hydroxyl group was also deduced to the C-5 position by observation of the correlations between δH 5.37 (H-4) and δC 157.0 (C-5) in the HMBC spectrum (Figure 2). The C-6, C-8, and C-10 located in the para-position of C-5 were different from compound 25 due to the influence of the hydroxyl group. Moreover, the absolute configuration of 26 was determined as 4S from the CD spectrum of its aglycon [18], which had a negative Cotton effect. Thus, the structure of 26 was established as (4S)-4,5-dihydroxy-α-tetralone-4-O-β-D-(6′-O′-4″-hydroxybenzoyl) glucopyranoside.

Compound 27 was isolated as a yellow powder, which had the molecular formula C23H24O11, established in HR-ESI-MS. Hydrolysis of 27 was similar to 25 and 26. Glucose was further confirmed by 1H-, 13C-NMR, and the DEPT spectrum (δH 4.81 (d, J = 7.5 Hz, H-1′); δC 104.4, 75.2, 78.0, 72.0, 75.8, 64.8). The correlation position between the aglycone and glucose was different from compounds 25 and 26, which was deduced to transfer to δC 148.3 (C-5), implying the connection at the aryl ring of the tetralone by the HMBC spectrum. The relative configuration of the glucopyranose moiety was determined as β by the coupling constant (J = 7.5 Hz) of the anomic proton. Furthermore, the 1H-NMR spectrum showed the AB-type aromatic proton signals at δH 7.40 (d, J = 9.1 Hz, H-6) and 6.67 (d, J = 9.1 Hz, H-7) in this aryl ring. It was also observed that a new quaternary carbon signal appeared at δC 159.3 due to the C-8 position in DEPT spectrum. It was also worth noting that the carbon signal at δC 116.2 (C-3″, 5″) not only had connections with H-3″, 5″ and H-2″, 6″, but also related with H-7 and H-4 (Figure 2) in HMBC. So we deduced that C-9 and C-3″, 5″ occurred in the same position. The absolute configuration of the chiral center at the C-4 position was deduced to be S by CD spectrum analysis of its aglycon [18]. Thus, the structure of 27 was determined to be (4S)-4,5,8-thihydroxy-α-tetralone 5-O-β-D-(6′-O′-4″-hydroxybenzoyl) glucopyranoside.
Table 1. $^1$H-(400 MHz) and $^{13}$C-(100 MHz) NMR data of 18, 25–27 in CD$_3$OD.

| No. | δ$_H$ (J in Hz) | δ$_C$ | δ$_H$ (J in Hz) | δ$_C$ | δ$_H$ (J in Hz) | δ$_C$ | δ$_H$ (J in Hz) | δ$_C$ |
|-----|-----------------|-------|-----------------|-------|-----------------|-------|-----------------|-------|
| 1   | —               | 148.0 | —               | —     | 200.0           | —     | 200.9           | —     |
| 2   | 7.72, s         | 109.9 | Hax: 2.87, dddd (4.5, 8.9, 17.5) | 35.5 | Hax: 3.03, dddd (5.0, 13.4, 17.0) | 33.9 | Hax: 3.01, ddd (5.9, 12.9, 17.6) | 33.5 |
|     | 105.8           |       | Hax: 2.41, dddd (4.5, 6.5, 17.5) | 31.5 | Hax: 2.48, dddd (1.3, 3.2, 4.7, 12.6) | 30.0 | Hax: 2.10, tt (4.2, 12.6) | 30.3 |
|     | 155.1           | 4.97, dd (3.6, 6.5) | 75.9 | 5.37, t (2.9) | 69.9 | 5.32, t (3.1) | 61.3 |
| 4   | 6.99, dd (1.0, 7.8) | 116.0 | 7.65, brd (7.6) | 130.0 | —               | 157.0 | —               | 148.3 |
| 5   | 7.40, t (7.8) | 128.6 | 7.52, dt (1.2, 7.6) | 134.8 | 7.08, dd (0.8, 8.0) | 122.3 | 7.40, d (9.1) | 128.9 |
| 7   | 7.86, dd (1.0, 7.8) | 116.1 | 7.43, dt (1.2, 7.6) | 129.4 | 7.27, t (8.0) | 130.6 | 6.67, d (9.1) | 118.9 |
| 8   | —               | 158.0 | 7.93, dd (1.2, 7.6) | 127.9 | 7.46, dd (0.8, 8.0) | 118.9 | —               | 159.3 |
| 9   | —               | 120.2 | —               | 132.9 | —               | 134.4 | —               | 116.2 |
| 10  | —               | 131.1 | —               | 143.9 | —               | 129.6 | —               | 135.5 |
| 1’  | 4.99, d (7.6) | 105.5 | 4.42, d (7.6) | 103.7 | 4.60, d (7.8) | 103.8 | 4.81, d (7.5) | 104.4 |
| 2’  | 3.50, m         | 78.9  | 3.34, m         | 75.2  | 3.2, m          | 75.3  | 3.53, dd (8.8, 16.5) | 75.2 |
| 3’  | 3.53, m         | 75.0  | 3.36, dd (2.5, 7.0) | 78.1  | 3.38, m         | 78.0  | 3.49, t (8.6) | 78.0 |
| 4’  | 3.43, m         | 71.3  | 3.36, dd (2.5, 7.0) | 72.2  | 3.38, m         | 72.1  | 3.43, dd (10.6, 16.2) | 72.0 |
| 5’  | 3.48, m         | 78.2  | 3.62, m         | 75.5  | 3.65, m         | 75.7  | 3.68, dt (2.2, 8.4) | 75.8 |
| 6’a | 3.96, dd (2.1, 12.0) | 62.5 | 4.66, dd (2.2, 11.8) | 65.0 | 4.64, dd (2.2, 11.8) | 65.0 | 4.61, dd (2.2, 11.8) | 64.8 |
| 6’b | 3.77, dd (5.5, 12.0) | 4.47, dd (7.2, 11.8) | 4.43, dd (6.8, 11.8) | 4.40, dd (7.4, 11.8) | 4.40, dd (7.4, 11.8) | 4.40, dd (7.4, 11.8) | 4.40, dd (7.4, 11.8) | 4.40, dd (7.4, 11.8) | 4.40, dd (7.4, 11.8) |
| 1'' | —               | 171.8 | —               | 122.2 | —               | 121.9 | —               | 122.1 |
| 2'' | 4.44, dq (3.6, 17.7) | 62.8 | 7.95, d (8.8) | 132.9 | 7.95, dt (2.6, 8.8) | 133.0 | 7.82, dt (2.7, 8.8) | 132.9 |
| 3'' | 1.43, t (7.2) | 14.5  | 6.84, d (8.8) | 116.3 | 6.83, dt (2.6, 8.8) | 116.2 | 6.81, dt (2.7, 8.8) | 116.2 |
| 4'' | —               | —     | —               | 163.7 | —               | 163.6 | —               | 163.7 |
| 5'' | —               | —     | 6.84, d (8.8) | 116.3 | 6.83, dt (2.6, 8.8) | 116.2 | 6.81, dt (2.7, 8.8) | 116.2 |
| 6'' | —               | —     | 7.95, d (8.8) | 132.9 | 7.95, dt (2.6, 8.8) | 133.0 | 7.82, dt (2.7, 8.8) | 132.9 |
| 7'' | —               | —     | —               | 167.9 | —               | 168.1 | —               | 167.8 |

2.2. Cytotoxic Activity

It was reported that green husks of *Juglans mandshurica* Maxim had an obvious effect on liver cancer. HepG-2 is a kind of human liver cancer cells which are often applied to evaluate cytotoxic activity in vitro [20,21]. Therefore, we tested the cytotoxicity of compounds 1–27 against HepG-2 by the MTT method and compared with references for some compounds [22–24].
The results were shown that most naphthoquinones in an aglycone form exhibited better cytotoxicity in vitro than naphthalenyl glucosides with IC\textsubscript{50} values in the range of 7.33–88.23 \mu M. None of them had better IC\textsubscript{50} values than cisplatin itself, but some naphthoquinone aglycones including juglone (1) and 3,5-dihydroxy-1,4-naphthoquinone (6) had obvious inhibition effects similar with cisplatin. The IC\textsubscript{50} value of juglone was $8.14 \pm 1.95$, and that of 3,5-dihydroxy-1,4-naphthoquinone was $7.33 \pm 0.52$ at 24 h of MTT assay, respectively (Table 2). Furthermore, these naphthoquinone aglycones with the structural features of 2,3-unsaturated moieties showed better and stronger cytotoxicity effects compared to other tetralones with a partial saturated aryl ring.

## Table 2. Cytotoxicities of compound 1–27 from J. mandshurica Maxim on HepG-2 cells lines.

| Compd. | Structural Features | IC\textsubscript{50} (\mu M) \textsuperscript{a} | SD \textsuperscript{b} | Compd. | Structural Features | IC\textsubscript{50} (\mu M) \textsuperscript{a} | SD \textsuperscript{b} |
|--------|---------------------|------------------|----------------|--------|---------------------|------------------|----------------|
| 1      |                    | 8.14             | 1.95           | 15     |                    | NA               | 3.21           |
| 2      |                    | 68.72            | 1.50           | 16     |                    | NA               | -              |
| 3      | 16.11              | 3.54             | 17             | 83.32  | 4.54               |
| 4      | 18.83              | 2.98             | 18             | NA     | -                  |
| 5      | 15.37              | 1.63             | 19             | 78.61  | 2.38               |
| 6      |                    | 7.33             | 0.52           | 20     |                    | NA               | -              |
| 7      |                    | 43.54            | 0.15           | 21     |                    | NA               | -              |
| 8      |                    | 22.38            | 0.66           | 22     |                    | NA               | -              |
| 9      |                    | 30.42            | 2.48           | 23     |                    | NA               | -              |
| 10     |                    | 32.51            | 0.46           | 24     |                    | NA               | -              |
| 11     |                    | 34.80            | 0.33           | 25     |                    | NA               | -              |
| 12     |                    | 56.87            | 4.27           | 26     |                    | NA               | -              |
| 13     |                    | 67.95            | 3.22           | 27     |                    | NA               | -              |
| 14     | \textsuperscript{R} \textsuperscript{H} (CH\textsubscript{3}) | 88.23            | 1.90           | PC \textsuperscript{c} | metal complex | 4.51            | 0.38           |

\textsuperscript{a} IC\textsubscript{50}, concentration required for inhibiting growth of HepG-2 by 50\% (in \mu M). These results are average results of three experiments; \textsuperscript{b} SD, standard deviation; \textsuperscript{c} PC, positive control (cisplatin); NA = not active.

Above results were merely obtained from the distinction of the mother nucleus structure. The different nature of the substituent in the naphthoquinone also seemed to influence the cytotoxicity activity. One or two phenolic hydroxyl groups without other substituents, which were introduced to a set of analogues (compounds 1, 3, 4, 5 and 6), were responsible for the lower IC\textsubscript{50} value and better inhibition effect. However, it was worth noting that the position and number of the hydroxyl group had a limited or negligible effect on HepG-2 inhibitory activities. For example, the IC\textsubscript{50} value of compound 1 with one hydroxyl group was similar with compound 6 with two hydroxyl groups; the IC\textsubscript{50} value of compound 3 which was substituted at the 5, 8-position was similar to compound 5 which was substituted at the 2, 5-position. In addition, the introduction of the methoxy or ethyoxyl group to some naphthoquinones with 2, 3-unsaturated moieties resulted in a slight decrease in the inhibition effect, including compounds 7, 8, 9 and 10. Among of them, compound 2 had the worst effect on the inhibition of HepG-2 cells.

A majority of naphthoquinone glycosides exhibited no activity against HepG-2 cells. These results were in accordance with previous reports \cite{24} that the most active compounds were those without the linkage of saccharide. Some glycosides like compound 17 and 19, of which aglycone was the integrated...
conjugation structure assigned to the naphthols, possessed slight cytotoxicity in vitro with IC\textsubscript{50} values of 83.3 ± 4.54 and 78.61 ± 2.38, respectively. However, some naphthols substituted with more than one saccharide or other groups, except for phenolic hydroxyl groups, had no cytotoxic activity against HepG-2 cells. The results indicated that there were differences in cytotoxic activity between these naphthoquinone glycosides according to the way of substitution and the type of aglycone.

3. Experimental Section

3.1. General Information

High resolution-electron spray ionization (HR-ESI) mass spectra were obtained on a micromass LCT spectrometer. \textsuperscript{1}H-, \textsuperscript{13}C-NMR, DEPT, HSQC, HMBC were obtained on Bruker DPX 400 NMR instrument (Bruker, Rheinstetten, Germany). Chemical shifts (δ) are expressed in parts per million (ppm) using tetramethyl-silane (TMS) as an internal standard. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), dd (double doublet), and m (multiplet). The UV spectra were recorded on a Thermo Scientific Evolution 300 UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Optical rotations were recorded using an Anton Paar-MCP 600 polarimeter r (Anton Paar, Graz, Austria). GC was run on Agilent 7890A Gas Chromatograph System (Agilent Technologies, Santa Clara, CA, USA). CD spectra were obtained on a Bio-Logic MOS-450 CD spectrometer. The IR spectra were obtained on a Shimadzu FTIR-8400S spectrometer (Shimadzu, Kyoto, Japan). Melting points are uncorrected and were obtained on a Hoover capillary melting point. HPLC chromatograms were obtained with an Agilent Technologies 1260 infinity HPLC system (Agilent Technologies, Germany) and semi-preparative HPLC (Waters, 515-2414, Milford, MA, USA) was performed using a Hypersil-ODS II column (300 mm × 20 mm i.d., 10 μm, Ylite, Dalian, China). De-ionized water was prepared with a Milli-Q system (Milford, MA, USA). HepG-2 cell line obtained from Institute of Biochemistry and Cell Biology (Shanghai, China) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Hyclone, NRH0020), supplemented with 5% fetal bovine serum and 1% antibiotic mixture comprising penicillin-streptomycin, in a humidified atmosphere at 37 °C with 5% CO\textsubscript{2}. A multiscan microplate reader (Thermo Labystems, Helsinki, Finland) was used for the MTT assays. The solvents used for open column isolation, such as ethyl acetate, methanol, acetonitrile, and chloroform were purchased from Merck (Darmstadt, Germany). MTT and Dulbecco’s Modified Dagle’s Medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. Plant Material

The green husks of \textit{J. mandshurica} were collected in late July from the Changbai Mountains (Jilin, China), and identified by the professor Zhen-Yue Wang. The dried samples were grounded into fine powder (60 mesh), and dried thoroughly in an oven at 40 °C for 3 days.

3.3. Extraction and Isolation

The air-dried parts of materials (10.0 kg) were powdered and soaked in 80 L of CHCl\textsubscript{3} for 7 days. The extraction was repeated three times and then concentrated under reduced pressure to afford the CHCl\textsubscript{3} extract (350 g). CHCl\textsubscript{3} extract was subjected to silica gel (200–300 mesh) column chromatography
(CC), eluted with PE:EtOAc (40:1→1:1, v/v), to create twelve fractions (Fr1-Fr12). Fraction 3 (15.50 g) was subjected to silica gel (200–300 mesh) CC, eluted with PE:EtOAc (40:1→1:1, v/v), to give fractions 3a–3c. Compounds 1 (245.4 mg), 3 (12.1 mg), 4 (33.8 mg), and 8 (11.8 mg), were isolated from fraction 3a by repeated column chromatography over silica gel, eluted with PE:EtOAc (40:1→1:1, v/v), to give fractions 3a–3c. Compounds 1 (245.4 mg), 3 (12.1 mg), 4 (33.8 mg), and 8 (11.8 mg), were isolated from fraction 3a by repeated column chromatography over silica gel, eluted with PE:EtOAc (40:1→10:1, v/v). Fraction 6 (12.80 g) was subjected to silica gel (200–300 mesh) CC, eluted with PE:EtOAc (20:1→5:1, v/v) to obtain compounds 5 (15.5 mg), 7 (22.0 mg), 9 (25.3 mg), 10 (15.2 mg), 12 (75.0 mg), and 13 (23.4 mg). Fraction 8 (8.92 g) was subjected to silica gel (200–300 mesh) CC, eluted with PE:EtOAc (5:1→1:1, v/v) to obtain 2 (5.8 mg), 6 (7.7 mg), 11 (6.8 mg), and 14 (12.8 mg).

The residue of materials were reflux extracted three times with 60 L EtOH (95% v/v), then concentrated under reduced pressure to afford the EtOH extract (750 g). The EtOH extract was subjected to Macroporous Resin AB-8 CC, sequentially eluted with H2O, 30% EtOH, and 95% EtOH. Compounds 15–27 were isolated from 30% EtOH fraction. Next, the isolation procedure of these compounds was explained. The 30% EtOH elution fraction was evaporated and concentrated to yield a crude residue (98 g). The residue was further purified by octadecyl silane (ODS) CC with MeOH/H2O (2:8→1:0) to give eleven fractions (Fr1-Fr11). Fraction 2 (6.50 g) was fractionated by ODS CC with MeOH/H2O (2:8→1:0) to afford a number of subfractions: 2a, 2b, 2c. Subfraction 2c (1.0 g) was subjected to semi-preparative HPLC chromatography (MeOH/H2O 35:65, v/v, flow rate 3 mL/min) to yield compounds 15 (5.3 mg, tR = 21 min), 16 (6.1 mg, tR = 23 min), and then subjected to semi-preparative HPLC chromatography (MeOH/H2O 45:55, v/v, flow rate 3 mL/min) to yield compound 19 (3.2 mg, tR = 22 min). Fraction 5 (9.40 g) was fractionated twice by ODS CC with MeOH/H2O (2:8→1:0) to afford a number of subfractions: 5a, 5b, 5c, 5d and 5e. Subfraction 5b (0.84 g) was purified by semi-preparative HPLC chromatography (MeOH/H2O 45:55, v/v, flow rate 3 mL/min) to yield compound 24 (4.7 mg, tR = 32 min) and purified by semi-preparative HPLC chromatography (MeOH/H2O 55:45, v/v, flow rate 3 mL/min) to obtain compound 17 (4.3 mg, tR = 25 min). Similarly, Subfraction 5c was purified by semi-preparative HPLC chromatography (MeOH/H2O 70:30, v/v, flow rate 3 mL/min, tR = 25 min) to yield compounds 18 (4.5 mg, tR = 13 min), 26 (2.8 mg, tR = 38 min), 25 (3.1 mg, tR = 40 min), and 27 (5.9 mg, tR = 42 min). Fraction 8 (5.70 g) was subjected to silica gel (200–300 mesh) CC, eluted with CH2Cl2:MeOH (5:1→0:1, v/v) to afford compounds 20 (7.5 mg), 21 (5.3 mg), 22 (4.6 mg), 23 (4.4 mg).

3.4. Spectral Data

**Juglone** (1). Orange needle crystal, 1H-NMR (CDCl3, 400 MHz) δ (ppm): 6.99 (2H, d, J = 12.2 Hz, H-2, 3), 7.27 (1H, dd, J = 1.9, 7.7 Hz, H-6), 7.65 (1H, t, J = 7.6 Hz, H-7), 7.63 (1H, dd, J = 7.6, 7.7 Hz, H-8), 11.90 (1H, s, 5-OH). 13C-NMR (CDCl3, 100 MHz) δ (ppm): 190.3 (C-1), 138.7 (C-2), 139.6 (C-3), 184.3 (C-4), 161.5 (C-5), 136.6 (C-6), 131.8 (C-7), 119.2 (C-8), 115.1 (C-9), 138.1 (C-10).

**5-Methoxy-1,4-naphthoquinone** (2). Light yellow powder, 1H-NMR (CDCl3, 400 MHz) δ: 7.58 (1H, d, J = 7.6 Hz, H-2), 7.48 (1H, d, J = 7.6 Hz, H-3), 6.73 (1H, br.d, J = 7.7 Hz, H-6), 7.36 (1H, t, J = 7.7 Hz, H-7), 7.42 (1H, br.d, J = 7.7 Hz, H-8), 3.83 (3H, s, 5-OCH3), 11.82 (1H, s, 5-OH). 13C-NMR (CDCl3, 100 MHz) δ (ppm): 190.4 (C-1), 138.3 (C-2), 139.3 (C-3), 186.3 (C-4), 161.2 (C-5), 119.2 (C-6), 124.3 (C-7), 136.1 (C-8), 131.4 (C-9), 138.1 (C-10).
5,8-Dihydroxy-1,4-naphthoquinone (3). Light yellow powder, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 7.13 (4H, s, H-2, 3, 6, 7). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 173.1 (C-1, 4, 5, 8), 134.4 (C-2, 3, 6, 7), 112.0 (C-9, 10).

2-Hydroxy-1, 4-naphthoquinone (4). Light yellow powder, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 7.38 (1H, s, H-3), 8.13 (2H, d, $J = 7.3$ Hz, H-5, 8), 7.82 (1H, m, H-6), 7.74 (1H, m, H-7). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 182.2 (C-1), 156.5 (C-2), 110.7 (C-3), 185.0 (C-4), 135.3 (C-5), 126.8 (C-6), 126.7 (C-7), 133.4 (C-8), 130.0 (C-9), 129.5 (C-10).

2,5-Dihydroxy-1, 4-naphthoquinone (5). Light yellow powder, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 6.12 (1H, s, H-3), 7.23 (1H, d, $J = 8.2$ Hz, H-6), 7.57 (1H, m, H-7), 7.40 (1H, d, $J = 7.6$ Hz, H-8), 12.10 (1H, s, 5-OH). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 180.3 (C-1), 160.2 (C-2), 110.4 (C-3), 191.7 (C-4), 159.3 (C-5), 124.2 (C-6), 135.3 (C-7), 118.2 (C-8), 130.3 (C-9), 113.7 (C-10).

3,5-Dihydroxy-1, 4-naphthoquinone (6). Light yellow powder, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 6.14 (1H, s, H-3), 7.26 (1H, d, $J = 7.8$ Hz, H-6), 7.66 (1H, t, $J = 7.8$ Hz, H-7), 7.43 (1H, d, $J = 7.8$ Hz, H-8), 12.02 (1H, s, 5-OH). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 183.0 (C-1), 160.0 (C-2), 111.4 (C-3), 185.3 (C-4), 160.3 (C-5), 122.2 (C-6), 136.0 (C-7), 117.0 (C-8), 132.5 (C-9), 114.2 (C-10).

3-Methoxy juglone (7). Orange powder, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 6.12 (1H, s, H-2), 7.27 (1H, d, $J = 7.5$, 1.2 Hz, H-6), 7.62 (1H, t, $J = 7.5$ Hz, H-7), 7.67 (1H, d, $J = 7.5$, 1.2 Hz, H-8), 3.90 (3H, s, OCH₃), 11.75 (1H, s, OH-5). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 184.9 (C-1), 162.0 (C-5), 123.9 (C-6), 132.5 (C-7), 116.9 (C-8), 131.1 (C-9), 114.3 (C-10), 56.6 (3-OCH₃).

2-Methoxy juglone (8). Orange-red needle crystal, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 6.11 (1H, s, H-2), 7.28 (1H, d, $J = 7.5$, 1.2 Hz, H-6), 7.58 (1H, t, $J = 8$ Hz, H-7), 7.67 (1H, d, $J = 8$, 1.2 Hz, H-8), 3.93 (3H, s, OCH₃), 11.75 (1H, s, OH-5). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 184.9 (C-1), 161.1 (C-2), 109.5 (C-3), 190.8 (C-4), 161.1 (C-5), 125.2 (C-6), 135.4 (C-7), 119.5 (C-8), 131.1 (C-9), 114.3 (C-10), 56.6 (3-OCH₃).

3-Ethoxy juglone (9). Orange powder, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 6.13 (H, s, H-2), 7.24 (1H, dd, $J = 7.5$, 1.2 Hz, H-6), 7.62 (1H, t, $J = 7.5$ Hz, H-7), 7.67 (1H, dd, $J = 7.5$, 1.2 Hz, H-8), 4.11 (3H, q, $J = 6.9$ Hz, H-1a), 1.60 (2H, t, $J = 6.9$ Hz, H-2a), 11.78 (1H, s, OH-5). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 185.1 (C-1), 110.8 (C-2), 159.3 (C-3), 184.1 (C-4), 161.9 (C-5), 123.8 (C-6), 118.8 (C-7), 137.1 (C-8), 132.0 (C-9), 114.3 (C-10), 65.6 (C-1a), 13.9 (C-2a).

2-Ethoxy juglone (10). Light yellow flaky crystal, $^1$H-NMR (CDCl₃, 400 MHz) δ (ppm): 6.08 (1H, s, H-3), 7.67 (1H, dd, $J = 7.5$, 1.2 Hz, H-6), 7.59 (1H, t, $J = 7.5$ Hz, H-7), 7.26 (1H, dd, $J = 7.5$, 1.2 Hz, H-8), 4.12 (2H, q, $J = 7.2$ Hz, -OCH₂), 1.53 (3H, t, $J = 7.2$ Hz, -CH₃). $^{13}$C-NMR (CDCl₃, 100 MHz) δ (ppm): 190.0 (C-1), 160.1 (C-2), 109.3 (C-3), 179.2 (C-4), 161.0 (C-5), 114.6 (C-6), 135.3 (C-7), 125.0 (C-8), 131.1 (C-9), 119.0 (C-10), 65.5 (C-1'), 13.8 (C-2').
Engelharquinone (11). Yellow needle crystal, $^1$H-NMR (CDCl$_3$, 400 MHz) δ (ppm): 6.92 (1H, d, $J = 8.4$ Hz, H-2), 7.48 (1H, dd, $J = 7.4$, 8.4 Hz, H-3), 7.14 (1H, d, $J = 7.4$ Hz, H-4), 7.25 (1H, dd, $J = 7.6$, 1.9 Hz, H-8), 7.59 (1H, dd, $J = 7.6$, 7.4 Hz, H-9), 7.65 (1H, dd, $J = 7.6$, 1.9 Hz, H-10), 4.23 (1H, d, $J = 3.1$ Hz, H-12), 3.08 (1H, dd, $J = 3.8$, 10.6 Hz, H-14a), 3.04 (1H, brd, $J = 3.8$, 10.6 Hz, H-14b), 11.50 (1H, s, 1-OH), 4.80 (1H, s, 5-OH), 11.52 (1H, s, 7-OH). $^{13}$C-NMR (CDCl$_3$, 100 MHz) δ (ppm): 198.1 (C-13), 188.8 (C-6), 180.5 (C-11), 163.5 (C-1), 162.1 (C-7), 154.4 (C-5a), 148.7 (C-11a), 146.3 (C-4a), 137.3 (C-9), 136.8 (C-3), 132.6 (C-11a), 125.2 (C-8), 120.4 (C-10), 119.4 (C-2), 115.3 (C-6a), 110.5 (C-13a), 82.0 (C-5), 54.1 (C-14), 52.6 (C-12).

(S)-Regiolone (12). White powder, $^1$H-NMR (DMSO-$d_6$, 400 MHz) δ (ppm): 2.74 (2H, m, H-2), 2.20 (1H, m, H-3a), 2.00 (1H, m, H-3b), 4.76 (1H, m, H-4), 7.06 (1H, d, $J = 8.0$ Hz, H-5), 7.53 (1H, t, $J = 8.0$, 8.2 Hz, H-6), 6.83 (1H, d, $J = 8.2$ Hz, H-7), 5.60 (H, s, OH-4), 12.42 (1H, s, OH-8). $^{13}$C-NMR (DMSO-$d_6$, 100 MHz) δ (ppm): 205.5 (C-1), 35.5 (C-2), 31.8 (C-3), 66.6 (C-4), 117.8 (C-5), 137.0 (C-6), 116.1 (C-7), 162.0 (C-8), 149.4 (C-9), 115.3 (C-10).

(4S)-4-Hydroxy-a-tetralone (13). Claybank oil substance, $^1$H-NMR (CDCl$_3$, 400 MHz) δ (ppm): 2.53 (1H, ddd, $J = 17.8$, 9.6, 4.8 Hz, H-2a), 2.86 (1H, ddd, $J = 17.8$, 7.5, 4.6 Hz, H-2), 2.15 (1H, m, H-3a), 2.37 (1H, m, H-3b), 4.95 (1H, dd, $J = 8.1$, 3.9 Hz, H-4), 7.52 (1H, m, H-5, 6), 7.38 (1H, m, H-7), 7.98 (1H, brd, $J = 7.8$ Hz, H-8). $^{13}$C-NMR (CDCl$_3$, 100 MHz) δ (ppm): 197.8 (C-1), 35.2 (C-2), 32.3 (C-3), 67.7 (C-4), 126.9 (C-5), 134.2 (C-6), 127.3 (C-7), 128.5 (C-8), 130.8 (C-9), 145.5 (C-10).

(4S)-5-Hydroxy-4-methoxy-a-tetralone (14). White amorphous powder, $^1$H-NMR (CDCl$_3$, 400 MHz) δ (ppm): 2.63 (1H, m, H-2), 2.87 (1H, m, H-2), 2.11 (1H, m, 3a), 2.24 (1H, m, H-3b), 4.98 (1H, m, H-4), 7.07 (1H, dd, $J = 8.0$, 1.0 Hz, H-6), 7.58 (1H, dd, $J = 8.0$, 1.0 Hz, H-7), 7.33 (1H, t, $J = 8.0$ Hz, H-8), 8.55 (1H, s, 5-OH), 3.57 (3H, s, 4-OCH$_3$). $^{13}$C-NMR (CDCl$_3$, 100 MHz) δ (ppm): 196.2 (C-1), 35.6 (C-2), 27.3 (C-3), 80.2 (C-4), 156.3 (C-5), 122.32 (C-6), 129.6 (C-7), 119.3 (C-8), 132.5 (C-9), 126.7 (C-10), 55.3 (C-11).

1,4,5-Trihydroxynaphthalene-1,4-di-O-β-D-glucopyranoside (15). Yellow amorphous powder, $^1$H-NMR (CD$_3$OD, 400 MHz) δ (ppm): 7.16 (1H, d, $J = 8.8$ Hz, H-2), 7.30 (1H, d, $J = 8.8$ Hz, H-3), 6.85 (1H, dd, $J = 7.6$, 1.2 Hz, H-6), 7.34 (1H, dd, $J = 8.4$, 7.6 Hz, H-7), 7.89 (1H, dd, $J = 8.4$, 1.2 Hz, H-8), 5.04 (1H, d, $J = 7.7$ Hz, H-1'), 3.63 (1H, dd, $J = 9.0$, 7.7 Hz, H-2'), 3.53 (1H, dd, $J = 9.0$, 8.8 Hz, H-3'), 3.45 (1H, m, H-4'), 3.44 (1H, m, H-5'), 3.73 (1H, dd, $J = 11.8$, 5.8 Hz, H-6'a), 3.89 (1H, brd, $J = 11.8$ Hz, H-6'b), 5.06 (1H, d, $J = 7.8$ Hz, H-1''), 3.58 (1H, dd, $J = 9.0$, 7.8 Hz, H-2''), 3.50 (1H, dd, $J = 9.0$, 8.5 Hz, H-3''), 3.43 (1H, m, H-4''), 3.46 (1H, m, H-5''), 3.74 (1H, dd, $J = 12.4$, 5.2 Hz, H-6''a), 3.94 (1H, dd, $J = 12.4$, 1.8 Hz, H-6''b). $^{13}$C-NMR (CDCl$_3$, 100 MHz) δ (ppm): 150.9 (C-1), 111.3 (C-2), 112.2 (C-3), 151.0 (C-4), 155.1 (C-5), 112.5 (C-6), 128.3 (C-7), 114.8 (C-8), 130.4 (C-9), 117.5 (C-10), 103.0 (C-1'), 75.0 (C-2'), 78.5 (C-3'), 71.4 (C-4'), 78.5 (C-5'), 62.4 (C-6'), 105.4 (C-1''), 78.4 (C-3''), 71.4 (C-4''), 78.9 (C-5''), 62.5 (C-6'').

1,4,5-Trihydroxynaphthalene-1,5-di-O-β-D-glucopyranoside (16). Yellow amorphous powder, $^1$H-NMR (CD$_3$OD, 400 MHz) δ (ppm): 7.20 (1H, d, $J = 8.2$ Hz, H-2), 6.72 (1H, d, $J = 8.2$ Hz, H-3), 7.41 (1H, dd, $J = 7.8$, 1.6 Hz, H-6), 7.36 (1H, dd, $J = 8.0$, 7.8 Hz, H-7), 8.13 (1H, dd, $J = 8.0$, 1.6 Hz, H-8), 4.92 (1H,
1,4,8-Trihydroxynaphthalene-1-\(\beta\)-d-glucopyranoside (17). Yellow amorphous powder, \(^1\)H-NMR (CD\(_3\)OD, 400 MHz) \(\delta\) (ppm): 6.70 (1H, d, \(J = 8.6\) Hz, H-2), 7.22 (1H, d, \(J = 8.6\) Hz, H-3), 6.81 (1H, dd, \(J = 7.7, 1.1\) Hz, H-6), 7.26 (1H, dd, \(J = 8.8, 7.7\) Hz, H-7), 7.64 (1H, dd, \(J = 8.8, 1.1\) Hz, H-8), 4.98 (1H, \(d, J = 7.8\) Hz, H-1'), 3.53 (1H, dd, \(J = 9.0, 7.8\) Hz, H-2'), 3.49 (1H, m, H-3'), 3.42 (1H, m, H-4'), 3.49 (1H, m, H-5'), 3.74 (1H, dd, \(J = 12.0, 5.8\) Hz, H-6'), 3.94 (1H, dd, \(J = 12.0, 2.1\) Hz, H-6'). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz) \(\delta\) (ppm): 150.8 (C-1'), 108.5 (C-2'), 113.2 (C-3'), 148.8 (C-4'), 154.9 (C-5'), 112.6 (C-6'), 127.1 (C-7'), 114.7 (C-8'), 129.2 (C-9'), 117.9 (C-10), 105.2 (C-1'), 75.2 (C-2'), 78.3 (C-3'), 71.5 (C-4'), 78.8 (C-5'), 62.8 (C-6').

1,4,8-Trihydroxy-3-naphthalencarboxylic acid-1-\(\beta\)-d-glucopyranoside ethyl ester (18). Red powder, mp 136-138 °C; [\(\alpha\)]\(_D\)\(^{25}\) = -12.3 (c 0.50, MeOH); \(\lambda_{max}^{UV} = 243\) nm; IR (KBr) \(\nu_{max} = 3401, 2956, 1646, 1506\) cm\(^{-1}\); \(^1\)H-NMR and \(^{13}\)C-NMR data see Table 1; HR-ESI-MS (positive): \(m/z = 433.1375 [M + Na]^+\) (calcd for C\(_{19}\)H\(_{22}\)NaO\(_{10}\), 433.1381).

1,4,8-Trihydroxynaphthalene-1-\(\beta\)-d-[6′-O-(3″,4″,5″-trihydroxybenzoyl)] glucopyranoside (19). Yellow amorphous powder, \(^1\)H-NMR (CD\(_3\)OD, 400 MHz) \(\delta\) (ppm): 6.50 (1H, d, \(J = 8.4\) Hz, H-2), 7.18 (1H, d, \(J = 8.4\) Hz, H-3), 7.68 (1H, dd, \(J = 8.0, 3.1\) Hz, H-5), 7.23 (1H, dd, \(J = 8.0, 7.8\) Hz, H-6), 6.80 (1H, dd, \(J = 8.2, 3.1\) Hz, H-7), 4.96 (1H, d, \(J = 7.8\) Hz, H-1'), 3.58 (1H, m, H-2'), 3.53 (1H, m, H-3'), 3.29 (1H, m, H-4'), 3.84 (1H, m, H-5'), 4.53 (1H, dd, \(J = 12.0, 6.9\) Hz, H-6′a), 4.66 (1H, dd, \(J = 12.0, 2.1\) Hz, H-6′b), 7.18 (2H, s, H-2″, H-6″). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz) \(\delta\) (ppm): 148.8 (C-1″), 108.6 (C-2″), 113.3 (C-3″), 150.7 (C-4″), 114.7 (C-5″), 127.1 (C-6″), 112.2 (C-7″), 154.8 (C-8″), 117.9 (C-9″), 128.9 (C-10″), 105.4 (C-1″), 78.2 (C-2″), 75.1 (C-3″), 72.0 (C-4″), 76.2 (C-5″), 65.1 (C-6″), 168.6 (C-7″), 121.6 (C-1″), 110.4 (C-2″, C-6″), 146.7 (C-3″, C-5″), 140.3 (C-4″).

(4S)-4-Hydroxy-a-tetralone-4-\(\beta\)-d-glucopyranoside (20). White amorphous powder, \(^1\)H-NMR (CD\(_3\)OD, 400 MHz) \(\delta\) (ppm): 3.05 (1H, dddd, \(J = 17.8, 9.6, 4.8\) Hz, H-2a), 2.62 (1H, dddd, \(J = 17.8, 6.8, 4.8\) Hz, H-2b), 2.45 (1H, dddd, \(J = 13.2, 9.6, 4.8, 3.4\) Hz, H-3a), 2.38 (1H, dddd, \(J = 13.2, 6.8, 6.2, 4.8\) Hz, H-3b), 5.10 (1H, dd, \(J = 6.2, 3.4\) Hz, H-4), 7.71 (1H, dd, \(J = 7.8, 1.6\) Hz, H-5), 7.63 (1H, td, \(J = 7.8, 1.6\) Hz, H-6), 7.47 (1H, td, \(J = 7.8, 1.6\) Hz, H-7), 7.97 (1H, dd, \(J = 7.8, 1.6\) Hz, H-8), 4.38 (1H, d, \(J = 7.8\) Hz, H-1″), 3.24 (1H, dd, \(J = 8.5, 7.8\) Hz, H-2′″), 3.32 (1H, m, H-3′″), 3.31 (1H, m, H-4′″), 3.32 (1H, m, H-5′″), 3.71 (1H, dd, \(J = 11.8, 5.3\) Hz, H-6′a), 3.95 (1H, dd, \(J = 11.8, 1.0\) Hz, H-6′b). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz) \(\delta\) (ppm): 200.2 (C-1), 35.6 (C-2), 31.6 (C-3), 75.1 (C-4), 130.4 (C-5), 135.1 (C-6), 129.9 (C-7), 128.2 (C-8), 133.1 (C-9), 144.1 (C-10), 103.3 (C-1′″), 75.4 (C-2′″), 78.2 (C-3′″), 71.9 (C-4′″), 78.1 (C-5′″), 63.0 (C-6″).
(4S)-4,5-Dihydroxy-a-tetralone-4-O-ß-D-glucopyranoside (21). White amorphous powder, $^1$H-NMR (CD$_3$OD, 400 MHz) δ (ppm): 3.12 (1H, ddd, J = 17.6, 14.0, 5.1 Hz, H-2a), 2.49 (1H, dt, J = 17.6, 3.3 Hz, H-2b), 2.19 (1H, tt, J = 14.0, 3.3 Hz, H-3a), 2.56 (1H, ddt, J = 14.0, 5.1, 3.3 Hz, H-3b), 5.41 (1H, t, J = 3.3 Hz, H-4), 7.12 (1H, dd, J = 7.9, 1.1 Hz, H-6), 7.30 (1H, t, J = 7.9 Hz, H-7), 7.48 (1H, dd, J = 7.9, 1.1 Hz, H-8), 4.62 (1H, d, J = 7.8 Hz, H-1'), 3.19 (1H, dd, J = 8.7, 7.8 Hz, H-2'), 3.35 (1H, m, H-3'), 3.36 (1H, m, H-4'), 3.36 (1H, m, H-5'), 3.75 (1H, dd, J = 12.2, 4.8 Hz, H-6'a), 3.90 (1H, dd, J = 12.2, 1.8 Hz, H-6'b). $^{13}$C-NMR (CD$_3$OD, 100 MHz) δ (ppm): 201.2 (C-1), 34.0 (C-2), 30.3 (C-3), 70.1 (C-4), 156.8 (C-5), 122.3 (C-6), 130.7 (C-7), 119.2 (C-8), 134.7 (C-9), 130.0 (C-10), 104.1 (C-1'), 75. 6 (C-2'), 78.1 (C-3'), 71.6 (C-4'), 78.1 (C-5'), 62.9 (C-6').

(4S)-4,6-Dihydroxy-a-tetralone-4-O-ß-D-glucopyranoside (22). White amorphous powder, $^1$H-NMR (CD$_3$OD, 400 MHz) δ (ppm): 2.97 (1H, ddd, J = 17.7, 9.7, 5.1 Hz, H-2a), 2.52 (1H, ddd, J = 17.7, 6.4, 4.8 Hz, H-2b), 2.38 (1H, dddd, J = 13.6, 9.6, 4.8, 3.5 Hz, H-3a), 2.30 (1H, dddd, J = 13.6, 6.4, 6.2, 5.1 Hz, H-3b), 5.02 (1H, dd, J = 6.2, 3.5 Hz, H-4), 7.04 (1H, d, J = 2.2 Hz, H-5), 6.85 (1H, dd, J = 8.4, 2.2 Hz, H-7), 7.88 (1H, d, J = 8.4 Hz, H-8), 4.38 (1H, d, J = 7.8 Hz, H-1'), 3.26 (1H, dd, J = 8.7, 7.8 Hz, H-2'), 3.33 (1H, t, J = 8.7 Hz, H-3'), 3.30 (1H, m, H-4'), 3.28 (1H, m, H-5'), 3.70 (1H, dd, J = 11.9, 5.8 Hz, H-6'a), 3.93 (1H, dd, J = 11.9, 1.6 Hz, H-6'b). $^{13}$C-NMR (CD$_3$OD, 100 MHz) δ (ppm): 199.6 (C-1), 35.3 (C-2), 31.5 (C-3), 75.1 (C-4), 116.0 (C-5), 164.4 (C-6), 117.0 (C-7), 131.1 (C-8), 125.5 (C-9), 146.4 (C-10), 103.0 (C-1'), 75.3 (C-2'), 78.2 (C-3'), 71.9 (C-4'), 78.1 (C-5'), 63.2 (C-6').

(4S)-4,5,8-Trihydroxy-a-tetralone-4-O-ß-D-glucopyranoside (23). White amorphous powder, $^1$H-NMR (CD$_3$OD, 400 MHz) δ (ppm): 3.22 (1H, ddd, J = 18.0, 13.8, 4.8 Hz, H-2a), 2.52 (1H, dt, J = 18.0, 3.8 Hz, H-2b), 2.17 (1H, tdd, J = 13.8, 3.8, 3.0 Hz, H-3a), 2.48 (1H, m, H-3b), 5.40 (1H, t, J = 3.0 Hz, H-4), 7.11 (1H, d, J = 9.1 Hz, H-6), 6.81 (1H, d, J = 9.1 Hz, H-7), 4.52 (1H, d, J = 7.8 Hz, H-1'), 3.17 (1H, dd, J = 8.9, 7.8 Hz, H-2'), 3.32 (1H, m, H-3'), 3.32 (1H, m, H-4'), 3.32 (1H, m, H-5'), 3.72 (1H, dd, J = 12.1, 4.8 Hz, H-6'a), 3.91 (1H, dd, J = 12.1, 1.8 Hz, H-6'b). $^{13}$C-NMR (CD$_3$OD, 100 MHz) δ (ppm): 207.2 (C-1), 34.3 (C-2), 29.9 (C-3), 69.3 (C-4), 148.6 (C-5), 127.0 (C-6), 119.6 (C-7), 157.3 (C-8), 116.9 (C-9), 127.5 (C-10), 103.4 (C-1'), 75.4 (C-2'), 78.2 (C-3'), 71.7 (C-4'), 78.1 (C-5'), 62.8 (C-6').

(4S)-4,5,8-Trihydroxy-a-tetralone-5-O-ß-D-[6'-O-(3''4''5''-trihydroxybenzoyl)] glucopyranoside (24). Light yellow amorphous powder, $^1$H-NMR (CD$_3$OD, 400 MHz) δ (ppm): 2.49 (1H, dd, J = 17.6, 3.5 Hz, H-2a), 3.03 (1H, ddd, J = 17.6, 11.8, 6.5 Hz, H-2b), 2.15 (1H, m, H-3), 5.34 (1H, t, J = 3.2 Hz, H-4), 7.42 (1H, d, J = 9.2 Hz, H-6), 6.76 (1H, d, J = 9.2 Hz, H-7), 4.78 (1H, d, J = 7.7 Hz, H-1'), 3.55 (1H, t, J = 8.0 Hz, H-2'), 3.51 (1H, m, H-3'), 3.45 (1H, m, H-4'), 3.68 (1H, td, J = 7.8, 1.9 Hz, H-5'), 4.45 (1H, dd, J = 11.8, 6.8 Hz, H-6'a), 4.55 (1H, dd, J = 11.8, 2.1 Hz, H-6'b), 7.07 (2H, s, H-2'', H-6''). $^{13}$C-NMR (CD$_3$OD, 100 MHz) δ (ppm): 206.2 (C-1), 33.5 (C-2), 30.3 (C-3), 61.1 (C-4), 148.6 (C-5), 129.1 (C-6), 119.4 (C-7), 159.5 (C-8), 116.1 (C-9), 135.1 (C-10), 104.5 (C-1'), 75.3 (C-2'), 77.9 (C-3'), 71.7 (C-4'), 75.8 (C-5'), 64.6 (C-6'), 121.0 (C-1''), 110.4 (C-2''), 146.9 (C-3''), 140.4 (C-4''), 146.9 (C-5''), 110.3 (C-6''), 168.3 (C-7'').

(4S)-4-Hydroxy-a-tetralone-4-O-ß-D-(6'-O-4''-hydroxybenzoyl) glucopyranoside (25). Yellow powder, mp 128–132 °C; $[a]_D^23$ = -28.2 (c 0.50, MeOH); UV$\lambda_{max} = 262$ nm; CD (MeOH) $\lambda_{max} (\Delta\varepsilon)$: 236 nm (−12.6),
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258 nm (+1.23). IR (KBr) ν max 3396, 1728, 1260, 1180 cm⁻¹; ¹H-NMR and ¹³C-NMR data see Table 1; HR-ESI-MS (positive): m/z 445.0262 [M + H]⁺ (calcld for C₂₃H₂₅O₉, 445.0267).

(4S)-4,5-Dihydroxy-α-tetralone-4-O-β-D-(6'-O-4''-hydroxybenzoyl) glucopyranoside (26). yellow powder, mp 132–135 °C; [α] D 30.2 (c 0.52, MeOH); UV λ max = 274 nm; CD (MeOH) λ max (Δε): 234 nm (~8.60), 261 nm (+2.21). IR (KBr) ν max 3420, 2960, 1726, 1255, 1135 cm⁻¹; ¹H-NMR and ¹³C-NMR data see Table 1; HR-ESI-MS (positive): m/z 483.4276 [M + Na]⁺ (calcld for C₂₃H₂₄NaO₁₀, 483.4279).

(4S)-4,5,8-Thihydroxy-α-tetralone-5-O-β-D-(6'-O-4''-hydroxybenzoyl) glucopyranoside (27). yellow powder, mp 145–148 °C; [α] D 31.4 (c 0.47, MeOH); UV λ max = 264 nm; CD (MeOH) λ max (Δε): 241 nm (~9.46), 266 nm (+0.93), 271 nm (~0.55), 292 nm (~0.35). IR (KBr) ν max 3400, 2994, 1725, 1056 cm⁻¹; HR-ESI-MS (positive): m/z 477.1058 [M + H]⁺ (calcld for C₂₃H₂₅O₁₁, 477.1054).

3.5. Acid Hydrolysis and Sugar Analysis

Compounds 18, 25–27 (1.5 mg) were refluxed with 1.0 mol/L HCl (5 mL, dioxane/H₂O, v/v) for 7 h. After filtration, the acid aqueous layer was neutralized with 5% NaOH and desalted with Sephadex LH-20 to obtain the sugar residue (0.8 mg). The residues were dissolved in pyridine (5 mL) and 1-(trimethylsilyl)-imidazole (0.5 mL) at 60 °C for 10 min. The reaction mixtures were dried with a stream of N₂, the residues were partitioned between CHCl₃ and H₂O. The organic layers were analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of injector and detector were maintained at 200 °C. A temperature gradient system was used for the oven; the initial temperature remained at 100 °C for 1 min and then was raised to 180 °C at the rate of 5 °C/min. Peaks of the hydrolysate of 18 and 25–27 were respectively detected at 14.72 min, 14.72 min, 14.73 min, 14.72 min. The final result was to compare the retention time of authentic sample of D-glucose (Sigma-Aldrich, St. Louis, MO, USA) treated in the same manner with 1-(trimethylsilyl)-imidazole in pyridine which was detected at 14.72 min. Thus, it was concluded that all the sugar moieties of 18 and 25–27 are D-glucose.

3.6. Cytotoxicity Assays

3.6.1. Cell Culture

The cytotoxicity of compounds 1–27 was performed against human liver carcinoma cells (HepG-2) by MTT assay [25,26]. HepG-2 cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco-BRL). The cells were incubated in 5% CO₂ humidified at 37 °C for growth.

3.6.2. Measurement of Cell Proliferation by MTT Assay

HepG-2 cells in logarithmic growth phase were seeded in a 96-well microtiter plates and kept overnight for attachment. Twenty-seven compounds and positive control (cisplatin), dissolved in dimethyl sulfoxide, were diluted to various concentrations with Dulbecco’s Modified Eagle Medium (DMEM) from 200 to 0.5 μM for 24 h. The optical density (OD) was measured at 570 nm using a
Multiscan microplate reader. All experiments were performed in triplicate. Data were expressed as the concentration required for inhibiting growth of HepG-2 by 50% (IC50).

4. Conclusions

Twenty-seven naphthoquinones and their derivatives, including four new naphthalenyl glucosides and twenty-three known compounds, have been isolated with the aim of exploring the relationship between cytotoxicity and structures. The results indicated that in naphthoquinones with 2,3-unsaturated moieties, the position of the substituents was at the aryl ring portion or the quinone ring portion of naphthoquinone played an important role in the cytotoxic activity. Moreover, the type of substituents also had an effect on the activity. And in general, when these compounds were substituted with the phenolic hydroxyl group, they had stronger activity against the HepG-2 cells. Naphthoquinone glycosides had no activity or weaker activity. So far, we are not able to definitely confirm that the type of saccharide is an essential factor for cytotoxic activity, since compounds obtained are all substituted with glucose. These results will provide experimental bases for further structural modifications to yield better active derivatives.

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Author Contributions

Yuanyuan Zhou, Bingyou Yang and Haixue Kuang came up with the conception of this experiment. Yuanyuan Zhou and Zhaoxi Liu wrote the manuscript. Yuxin Liu and Yanqiu Jiang carried out the experiments and analyzed the spectral data. Xiaoli Wang conducted the cytotoxicity assays against HepG-2 and deduced the preliminary structure-activity relationships for these naphthoquinones from green walnut husks of Juglans mandshurica Maxim. All authors had read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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*Sample Availability:* Samples of the compounds 1–27 are available from the authors.

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