Hydroxytyrosol Dimers from Medicinal Insect Blaps Rynchopetera and the in Vitro Cytotoxic Activity

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Abstract
The edible Blaps rynchopetera Fairmaire is widely used for its various medicinal effects. From its ethyl acetate fraction, three new hydroxytyrosol dimers, rynchopeterine H (1), rynchopeterine I (2) and trans-2-(3,4-dihydroxy-phenyl)-3-hydroxy-7-(2-hydroxyethyl)-1,4-benzdioxane (3), together with four known similar dimers were obtained by chromatography of silica gel and Sephadex LH-20. Their structures were identified by HRESIMS, 1D and 2D NMR spectra analysis. Compounds 1-4 were obtained as a mixture, and cytotoxicity screening for HepG2, Caco-2, U251, AGS, B16 and Bel-7402 cell lines showed that the mixture of compounds 1-4 exhibited significantly selective cytotoxicity and good inhibitory activity on the proliferation of mouse melanoma cells (B16) with an IC50 value of 27.37 μg mL−1.

Keywords
blaps rynchopetera, insects, hydroxytyrosol dimers, cytotoxic activity, rynchopeterine H, rynchopeterine I

Introduction
Like plants, many animals, including insects, have unique nutrients and pharmacological activities and can be used as food or medicine. Medicinal insects have been utilized by humans for a long time, and they played an important role in the prevention and cure of various diseases by providing antibacterial, anti-inflammatory, antitumor effects.2,2 Besides, they could also enhance immunity and act as sedative and analgesic agents.3 Investigations have found that alkaloids, steroids, fatty acids, aromatic species, and glycosides are the main components of medical insects.4-6 Blaps rynchopetera Fairmaire, a member of the family Tenebrionidae has been used in traditional Yi medicine in Yunnan Province as a regular treatment for tumors, gastritis, fever, cough, whitlow, and rheumatoid arthritis.7 Its main chemical components are polyphenols,8 amino acids,9 cyclic dipeptides10 fatty acids,11 saccharides12 and so on, these compounds exhibit extensive pharmacological activities, including antitumor, analgesic, anti-inflammatory, bacteriostasis, and anti-oxidation activities.13,14 Previous researchs show that the defensive secretion of B. rynchopetera has cytotoxicity against a variety of tumor cell lines.15 Moreover, phenolic components show significant antioxidant activity and antitumor activity.10,14

Hydroxytyrosol (HT) is a kind of polyphenolic compounds which mainly exist in the fruits and branches of olive oil, and acts as a natural antioxidants.16 It is reported that HT has various physiological effects, including lipid-lowering,17 anti-inflammatory,18 and antitumor,19 these effects are usually related to their antioxidant capacity. The HT monomer and a number of dimer derivatives have previously been isolated from the B. rynchopetera,14,20 further chemical constituents provided three new HT dimers, rynchopeterine H (1), rynchopeterine I (2) and trans-2-(3,4-dihydroxy-phenyl)-3-hydroxy-7-(2-hydroxyethyl)-1,4-benzdioxane (3), and another

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four dimers, trans-2-(3,4-dihydroxy-phenyl)-3-hydroxy-6-(2-hydroxyethyl)-1,4-benzodioxane (4), trans-2-(3,4-dihydroxy-phenyl)-3-acylamino-7-(N-acetyl-2-aminoethyl)-1,4-benzodioxane (5), trans-2-(3,4-dihydroxyphenyl)-3-hydroxy-7-(2-hydroxyethyl)-1,4-benzodioxane (6), and trans-2-(3,4-dihydroxyphenyl)-3-acylamino-6-(2-hydroxyethyl)-1,4-benzodioxane (7) (Figure 1). While the hydroxytyrosol dimer, mixture 1-4 had good proliferation inhibitory activity on B16 with an IC50 of 27.37 μg mL−1, and the highest inhibition rate reached 82.0%.

Results

Structural Determination of Compounds 1-7

Compounds 1-4: yellow liquid, HRESIMS: [M - H2O + Na]+ at m/z 327.0840 (calcd for C16H16O6, 327.0845) (compounds 1 and 2); [M + Na]+ at m/z 327.0840 (calcd 327.0845), [2M + Na]+ at m/z 631.1762 (calcd for C34H32O12, 631.1791) (compounds 3 and 4). 1H NMR shows multiple aromatic hydrogens in the mid-field and three groups of mutually coupled hydrogen signals in the midfield: δ 5.33, 4.68 (each 1H, d, J = 5.7 Hz); δ 5.56, 4.90 (each 1H, d, J = 4.0 Hz); δ 3.75, 2.74 (4H, t, J = 7.0 Hz).13C NMR shows four very close tertiary carbon signals at δ 93.2, 93.3, 91.3, 91.2, and δ 78.2, 78.1 and 77.0, 76.9, while two secondary carbon signals at δ 63.1, 38.6 are about double height. The above information indicated that there may be four compounds with very similar structure. There are 20 tertiary carbon signals from 113 to 122 ppm, of with four overlapped peaks, 8 quaternary carbon signals at about 128 ppm and 133 ppm with two overlapped peaks, and 16 quaternary carbon signals with four overlapped peaks from 139 to 145 ppm, which suggested that each compound probably has two groups of 3,4-dihydroxy substituted benzene rings with a side chain of hydroxylated alkyl. According to previous research14 3,4-dihydroxy substituted is the characteristic of polyphenol metabolic product from B. rynchopetra.

Based on 1H-1H COSY and HMBC correlation signal analysis, the main structure is 2-(3,4-dihydroxy phenyl) ethoxy group (unit a) and 2-(3,4-dihydroxy phenyl)-2-hydroxy hemiacetal group (unit b). δ 5.33 (H-3) and δ 4.68 (H-2) correlate with C-4a and C-8a in 2-(3,4-dihydroxyphenyl) ethanol, respectively. Figures 2 (A and B) and 3 (compounds 3 and 4), suggesting that the 2 and 3 sites hydroxyl groups of unit b and the 4a and 8a hydroxyl groups of unit a form 1,4-dioxane. Among the four different structures, only the chemical shifts of C-4a, C-8a, which linked two parts or formed dioxane are quite different, while the other signals are basically identical. The main differences of the four structures are the configurations of the two tertiary carbons of the dioxane and the position of C-6/7 (different ring-forming positions). According the HMBC correlated signals (Figure 3), compounds 3 and 4 is formed by the ether bond of C-2 and C-8a, C-3 and C-4a to form dioxane. The signals of δ 5.33 and δ 4.68 with a coupling constant of 5.7 Hz, suggest that H-2 and H-3 have trans configuration.

| No. | δ1 (J in Hz) | δC | δ2 (J in Hz) | δC |
|-----|--------------|-----|--------------|-----|
| 1   | 128.4 s      |      | 128.4 s      |     |
| 2   | 7.16 (1H, d, 1.8) | 114.9 d | 7.15 (1H, d, 1.8) | 114.9 d |
| 3   | 144.7 s      |      | 144.7 s      |     |
| 4   | 145.1 s      |      | 145.1 s      |     |
| 5   | 6.86 (1H, d, 8.0) | 114.7 d | 6.86 (1H, d, 8.0) | 114.7 d |
| 6   | 6.94 (1H, d, 8.0, 1.8) | 119.2 d | 6.93 (1H, d, 8.0, 1.8) | 119.2 d |
| 7   | 4.91 (1H, brd, 4.0) | 77.0 d | 4.90 (1H, brd, 4.0) | 76.9 d |
| 8   | 5.55 (1H, d, 4.0) | 91.2 d | 5.56 (1H, d, 4.0) | 91.3 d |
| 1'  | 143.7 s      |      | 143.7 s      |     |
| 2'  | 139.3 s      |      | 140.7 s      |     |
| 3'  | 6.79 (1H, brd, 8.0) | 117.1 d | 6.82 (1H, d, 1.8) | 116.4 d |
| 4'  | 6.75 (1H, brd, 8.0) | 122.0 d | 132.9 s      |     |
| 5'  | 132.7 s      |      | 117.8 d      |     |
| 6'  | 6.82 (1H, d, 1.8) | 116.2 d | 6.75 (1H, brd, 8.0) | 121.8 d |
| 7'  | 2.74 (2H, t, 7.0) | 38.6 t | 2.74 (2H, t, 7.0) | 38.6 t |
| 8'  | 3.75 (2H, t, 7.0) | 63.1 t | 3.75 (2H, t, 7.0) | 63.1 t |

The structures are respectively: trans-2-(3,4-dihydroxy-phenyl)-3-hydroxy-7-(2-hydroxyethyl)-1,4-benzodioxane (3), trans-2-(3,4-dihydroxy-phenyl)-3-hydroxy-6-(2-hydroxyethyl)-1,4-benzodioxane (4).21,22

For another two compounds, only δ 5.56 (H-8) is correlated with C-1’, and δ 4.90 (H-7) is uncorrelated with C-2’. Figure 2(C), Figure 3 (compounds 1 and 2), suggesting that only C-8 is dehydrated to form an ether with one of the two phenolic hydroxyl groups of the other phenyl ring, compound 1 and 2 is etherified by C-8 hydroxyl group and C-1’ hydroxyl group. Generally, an open chain structure(C-7,8 sites) should

Table 2. NMR Spectroscopic Data of Compounds 3-4. 1H, 400 MHz; 13C, 100 MHz in CD3COCD3.

| No. | δ1 (J in Hz) | δC | δ2 (J in Hz) | δC |
|-----|--------------|-----|--------------|-----|
| 1   | 2.48 (1H, d, 5.7) | 78.2 d | 2.46 (1H, d, 5.7) | 78.1 d |
| 2   | 5.33 (1H, d, 5.7) | 93.2 d | 5.31 (1H, d, 5.7) | 93.3 d |
| 3   | 6.79 (1H, brd, 8.0) | 116.9 d | 6.82 (1H, d, 1.8) | 117.3 d |
| 4   | 6.75 (1H, brd, 8.0) | 121.9 d | 6.75 (1H, brd, 8.0) | 121.9 d |
| 5   | 129.0 s       |      | 129.0 s      |     |
| 6   | 6.82 (1H, d, 1.8) | 116.6 d | 6.79 (1H, brd, 8.0) | 117.1 d |
| 7   | 145.3 s       |      | 145.3 s       |     |
| 8   | 144.9 s       |      | 144.9 s       |     |
| 5'  | 6.86 (1H, d, 8.0) | 115.0 d | 6.86 (1H, d, 8.0) | 115.0 d |
| 6'  | 6.82 (1H, dd, 8.0, 1.8) | 119.3 d | 6.82 (1H, dd, 8.0, 1.8) | 119.3 d |
| 1'' | 2.74 (2H, t, 7.0) | 38.6 t | 2.74 (2H, t, 7.0) | 38.6 t |
| 2'' | 3.75 (2H, t, 7.0) | 63.1 t | 3.75 (2H, t, 7.0) | 63.1 t |
| 4a  | 140.4 s       |      | 141.9 s       |     |
| 8a  | 142.9 s       |      | 141.5 s       |     |
Figure 1. Structure of compounds 1-7.

have an coupling constant of about 7 Hz between H-7 and H-8, the fact is that the coupling constant is smaller (4.0 Hz). We speculate that the compounds form intramolecular hydrogen bonds, Figure 4, which not only made the hemiacetal structure stable, but also made H-7 and H-8 in an ortho-cross configuration with a dihedral angle about 60°, and showed a small coupling.23 The structures are confirmed as 1-[2-hydroxyl-5-(2- hydroxyethyl)-phenoxy]-2-(3,4-dihydroxy-benzenyl)-1,2-diol (I), 1-[2-hydroxyl-4-(2-hydroxyethyl)-phenoxy]-2-(3,4- dihydroxy-benzenyl)-1,2-diol (2). 1 and 2 are new compounds, and named as rynchopeterine H (1) and rynchopeterine I (2). The HRESIMS can’t detect molecular ion peaks, which may be because the molecular ion peak is easy to lose water, and the [M - H2O+Na]+ ion peak is similar as compounds 3 and 4. The 1H and 13C NMR signals were assigned through HMQC and HMBC (Tables 1 and 2).

Compound 5: yellow, amorphous powder. HRESIMS: [M + H]+ at m/z 387.1556 (calcd 387.1556), [M + Na]+ at 409.1386 (calcd for C20H22N2O6, 409.1376). 1H NMR (CD2COCD3, 100 MHz): δ 7.0 Hz, H-2), 3.69 (2H, t, J = 6.8 Hz, H-3), 4.76 (1H, d, J = 7.0 Hz, H-2), 3.69 (2H, t, J = 7.0 Hz, H-2′), 2.69 (2H, t, J = 6.8 Hz, H-1′), 1.81 (3H, s, H-2″′); 13C NMR (CD2COCD3, 100 MHz): δ 169.2 (C-1""), 145.7 (C-3""), 145.0 (C-4""), 142.9 (C-8a), 140.8 (C-4a), 133.0 (C-7), 128.1 (C-1′), 122.2 (C-6), 119.6 (C-6′), 117.2 (C-8), 116.6 (C-6), 115.0 (C-5′), 114.7 (C-2′), 77.0 (C-2′), 76.8 (C-3), 63.0 (C-2′′), 38.6 (C-1′′′), 22.0 (C-2′′′). The structure was confirmed by HRESIMS, 1D and 2D NMR. Compared with compound 5, except for the reduction of one acetamide group, only the side chain ethyl signals, H-1′′′ [δ 2.69/2.64 (2H, m) and δ 3.69 (2H, t, J = 6.8 Hz)], H-2′′′ [δ 3.50/3.33 (2H, m) and δ 3.69 (2H, t, J = 7.0 Hz)], C-1′′′ [δ 35.0 and 38.6], C-2′′′ [δ 40.7 and 63.0] were significantly different, revealing that C-6/7 was linked an ethoxyl group, compound 6 is identified as trans-2-(3,4-dihydroxyphenyl)-3-acetamidino-7-(N-acetyl-2-aminoethyl)-1,4-benzodioxane.

Compound 6: yellow, amorphous powder. HRESIMS: [M + Na]+ at m/z 368.1158 (calcd for C18H19NO6, 368.1110). 1H NMR (CD2COCD3, 400 MHz): δ 8.09 (2H, brs, OH-3′, 4′), 6.92 (1H, brs, H-2′), 6.79/6.75 (3H, m, H-5, 8′, 6′), 6.73/6.69 (2H, m, H-5, 6), 5.75 (1H, dd, J = 9.7, 7.0, H-3), 4.76 (1H, d, J = 4.0 Hz, H-2), 3.69 (2H, t, J = 7.0 Hz, H-2′), 2.69 (2H, t, J = 6.8 Hz, H-1′), 1.81 (3H, s, H-2″′); 13C NMR (CD2COCD3, 100 MHz): δ 169.2 (C-1""), 145.7 (C-3""), 145.0 (C-4""), 142.9 (C-8a), 140.8 (C-4a), 133.0 (C-7), 128.1 (C-1′), 122.2 (C-6), 119.6 (C-6′), 117.2 (C-8), 116.6 (C-6), 115.0 (C-5′), 114.7 (C-2′), 77.0 (C-2′), 76.8 (C-3), 63.0 (C-2′′), 38.6 (C-1′′′), 22.0 (C-2′′′). The structure was confirmed by HRESIMS, 1D and 2D NMR. Compared with compound 6, except for the reduction of one acetamide group, only the side chain ethyl signals, H-1′′′ [δ 2.69/2.64 (2H, m) and δ 3.69 (2H, t, J = 6.8 Hz)], H-2′′′ [δ 3.50/3.33 (2H, m) and δ 3.69 (2H, t, J = 7.0 Hz)], C-1′′′ [δ 35.0 and 38.6], C-2′′′ [δ 40.7 and 63.0] were significantly different, revealing that C-6/7 was linked an ethoxyl group, compound 6 is identified as trans-2-(3,4-dihydroxyphenyl)-3-acetamidino-7-(N-acetyl-2-aminoethyl)-1,4-benzodioxane.
Figure 2. Partial correlation signals in HMBC of compounds 1-4.

Figure 3. Key COSY (−) and HMBC (−−−) correlations of compounds 1-4.
122.0 (C-7), 119.6 (C-6′), 117.3 (C-5), 116.4 (C-8), 115.0 (C-5′), 114.7 (C-2′), 76.9 (C-2, 3), 63.0 (C-2″), 38.6 (C-1″), 22.0 (C-2‴). The structure and signal assignments were confirmed by data analysing of HRESIMS, 1D and 2D NMR. The data are very close to those of compound 6, only the carbon signals of second benzene ring are slightly different, presumably the substitution of hydroxyethyl is different, and compound 7 was identified as trans-2-(3,4-dihydroxyphenyl)-3-acetylamino-6-(2-hydroxyethyl)-1,4-benzodioxane.

Cytotoxic Activity of Compounds
The cytotoxicity of mixture 1-4 and compounds 5-7 against cancerous cell lines, B16, U251, Caco-2, AGS, Bel-7402 and HepG2 were tested in vitro using a modified MTT method. The result showed that only mixture 1-4 exhibited anti-tumor activity against B16, with the highest inhibitory rate as 82.0%, and an IC_{50} value of 27.37 μg·mL\(^{-1}\) (Figure 5), while the positive control cisplatin (DDP) generated IC_{50} values of 1.82 μg·mL\(^{-1}\). Compounds 5-7 were inactive against the above tested cancer cell lines.

Discussion
Studies have shown that HT dimers could bring beneficial biological outcomes in neurodegenerative diseases, nervous system diseases, cardiovascular diseases, metabolic syndrome; these effects are often related to its anti-inflammatory activities and antioxidant capacities. Besides, the anticancer properties of HT dimers were confirmed in vitro on different cell lines, predominantly breast, thyroid, liver and digestive cancer cell lines. B. rynchopetera has been used in many areas of Yunnan Province in China for the treatment of tumors; the phenolics are responsible for its therapeutic effect. Xiao Hua14 isolated a variety of antioxidant phenolics from the ethyl acetate fraction of B. rynchopetera, and HT dimers are the main phenolics and exhibited significant antioxidant activities, rynchopeterines B and rynchopeterines C inhibited the proliferation of tumor cell Caco-2 with IC_{50} values of 119.7 μg·mL\(^{-1}\) and 158.7 μg·mL\(^{-1}\). Xu Fa isolated the anti-inflammatory active ingredient HT from the B. rynchopetera, increase cell apoptosis by inhibiting tumor growth and enhancing immune surveillance against cancer cells.18

In this report, seven hydroxytyrosol dimers were isolated from the EtOAc fraction of B. rynchopetera. The cytotoxicity of mixture 1-4 and compounds 5-7 was evaluated on all aforementioned human and mouse cancer cell lines. The mixture of compounds 1-4 exhibits significant selective cytotoxic activity and good proliferation inhibitory activity against mouse melanoma cells (B16) at low concentrations, while compounds 5-7 had no antiproliferative activity for testing cell lines. According to the structural characteristics of testing compounds, it may be necessary for antiproliferative activity that the 1,4-benzodioxane part of the structural was opened and the active constituent could be 1 and 2 which with a open loop structure.

Conclusions
In summary, three new and four known hydroxytyrosol dimers were isolated and identified from B. rynchopetera. The mixture of compounds 1-4 could selectively inhibit the proliferation of B16 cells in vitro. The further biological activity and the mechanism
of HT and the possible molecule substances in *B. rynchopetera* were necessary for deeply researched.

**Materials and Methods**

**General Procedures**

NMR spectra were performed on a Bruker AV-400 spectrometer (Karlsruhe, Germany) with TMS as an internal reference. Mass spectra analyses were measured on an Agilent G3250AA LC/MSD TOF spectrometer. Cooling water circulation device (EYELA, Tokyo, Japan). Sephadex LH-20 (GE Healthcare, USA) were used for column chromatography.

The specimen were collected from Dali, Yunnan Province, China. Compared with the voucher specimen (No. 2008071001), it was identified as *Blaps rynchopetera* Fairmaire by Prof. Guo-Dong Ren of Hebei University and preserved in Special Medicinal Insect Development National Engineering Research Center of Dali University.

Cell lines of human HepG2 hepatocellular carcinoma, human Caco-2 colorectal adenocarcinoma, human U251 glioma, human AGS gastric adenocarcinoma, mouse melanoma cells B16 and human Bel-7402 hepatocellular carcinoma cell lines, were all purchased from Shanghai Cell Institute, Chinese Academy of Sciences, China. Cisplatin was purchased from Qilu Pharmaceutical Co., Ltd (A1A1004007, Shandong, China).

**Extraction and Isolation**

*B. rynchopetera* adult body was extracted with 95% EtOH for three times. The crude extract were obtained by decompressing the solvent and then fractionated with Petroleum ether, EtOAc and butyl alcohol successively to obtain three soluble parts. The EtOAc fraction (295 g) was separated into eleven fractions (Fr.A-Fr.K) by gradient elution of petroleum ether and EtOAc on silica gel (10:1 to 0:1). Fr.F was further purified by eluting silica gel with CHCl3-CH3OH to afford compounds 1-4 (12 mg). Fr.G was isolated by Sephadex LH-20 and silica gel to obtain compound 5 (5 mg), 6 (3 mg) and 7 (4 mg).

**Cytotoxic Activity Assay**

Antiproliferative activity was detected by modified MTT reduction assay on B16, U251, Caco-2, AGS, Bel-7402 and HepG2. The mixture 1-4, compounds 5-7 and cisplatin (positive sample) were respectively dissolved in phosphate buffer saline (PBS) to gain a stock solution of 50 mg·mL−1, and stored at −20 °C, light protected, for further use. The exponentially growing cells were trypsinized, 10 μL cell suspension was absorbed, mixed with an equal volume of Trypan blue, and the cell concentration was adjusted to 1×10^5 cells/mL by cell counting board. Seeded into 96-well plates and placed in a CO₂ incubator for culture for 24 h, then treated with the sample and cisplatin for 48 h at a final concentrations of 1, 3, 10, 30 and 100 μg·mL⁻¹. 15 μL/well MTT was used to replace the medium, and 150 μL/well triple fluid was added after 4 h incubation. After 16 h, the optical density (OD) of each well was measured at 490 nm wavelength. The inhibition rate (1 %) was counted as follows: I% = (ODcontrol - ODsample)/(ODcontrol - ODblank)×100%, the control group refers to the PBS solution adding MTT and triple fluid without adding positive drugs and tested drugs, cells receiving no treatment were used as blank control throughout the experiments. SPSS 21.0 statistical analysis software was used to calculate IC₅₀ value.

**Acknowledgments**

We are appreciated for the NMR testing from the Analysis and Testing Center of Dali University and the technical support from National-Local Joint Engineering Research Center of Entomoeutics, Dali University.

**Author Contributions**

Zhong-Tao Ding and Huai Xiao conceived and designed the experiments; Xiu-Qin Pang, Xiu-Mei Wu, Yan-Ming Huang, Jing-Lei Xu, Yue Li, and Heng Liu performed the experiments; Qi Wang and Di Meng analyzed the data; Xiu-Qin Pang, Huai Xiao and Xiu-Mei Wu wrote the manuscript; and Zhong-Tao Ding polished it. Zhong-Tao Ding and Huai Xiao received a research grant.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China, the Special Program of Science and Technology of Yunnan Province, Special Fund Project for the Development of TCM Decoction Pieces Industry of Yunnan Province (grant number 81860742, 81960755, 82160822, 202002AA100007, 2019-YG-067).

**Ethical Approval**

Not applicable, because this article does not contain any studies with human or animal subjects.

**Informed Consent**

Not applicable, because this article does not contain any studies with human or animal subjects.

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**Supplemental Material**

Supplemental material for this article is available online.

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**Trial Registration**

Not applicable, because this article does not contain any clinical trials.
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