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Inhibitory effects of polyphenols toward HCV from the mangrove plant *Excoecaria agallocha* L.

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ABSTRACT

Four new polyphenols namely excoecariphenols A–D (**1–4**) were isolated from the Chinese mangrove plant *Excoecaria agallocha* L. together with 23 known phenolic compounds. The structures of new compounds were elucidated on the basis of extensive spectroscopic analyses including IR, MS, NMR, and CD data. Excoecariphenols A and B presented as the unusual flavane-based 1-thioglycosides. Part of the isolated polyphenols were tested against hepatitis C NS3-4A protease and HCV RNA in huh 7.5 cells. Excoecariphenol D, corilagin, geraniin, and chebulagic acid showed potential inhibition toward HCV NS3-4A protease with IC_{50} values in a range of 3.45–9.03 μM, while excoecariphenol D and corilagin inhibited HCV RNA in huh 7.5 cells significantly. A primary structure–activity relationship (SAR) is discussed.

Hepatitis C virus (HCV) infection caused a serious health problem, and distributed worldwide with 3–4 million new cases reported each year.[1–3] Chronic HCV infection affects 170 million people at risk of developing liver cirrhosis and hepatocellular carcinoma.[4,5] Unfortunately, there is no HCV vaccine, and current limited therapies are often poorly tolerated and frequently ineffective. The standard therapy for chronic hepatitis C patients in hospital is a combination of pegylated interferon (IFN)-α and ribavirin, which inhibit viral indirectly due to no specific target upon HCV protein or RNA element.[6] In addition, this therapy is associated with considerable adverse effects, including depression, fatigue, and 'flu-like' symptoms caused by IFN-α, and hemolytic anemia by ribavirin.[7] Thus, there is an urgent medical need for orally available, small-molecule, direct anti-HCV drugs to treat hepatitis C patients with more effective and fewer side effects. HCV encodes four viral enzymes in its nonstructural protein region including autoprotease NS2-3, serine protease NS3-4A, helicase NS3, and RNA-dependent RNA polymerase NS5B.[8–10] Among them, NS3-4A serine protease is responsible for the proteolytic cleavage at four junctions of the HCV polyprotein precursors, and has been subjected to intense efforts on the discovery of potent, selective inhibitors as potential new therapies for the hepatitis C patients. Significant progress has been made in recent years to identify potent small-molecule inhibitors against the HCV protease, such as clinical proof-of-concept for HCV NS3-4A protease inhibitors BILN 2061 and VX-950,[11,12] indicating that viral proteases, such as the HCV NS3-4A protease, could be excellent targets for a structure-based drug design approach. Discovery of a potent, small-molecule, and orally available drug candidate from natural products would be an enormously challenging task.

The mangrove plant *Excoecaria agallocha* L. (Euphorbiaceae)[13] is a herb medicine traditionally used for the treatment of ulcers, rheumatism, leprosy, and paralysis in the coast regions of South China.[14–17] Its leaves and latex were used as a dart poison and fish poison in some Asian countries, while the latex was used as a purgative and abortifacient. A bioassay guiding fractionation revealed the BuOH extract of *E. agallocha* possessing inhibitory activity against HCV NS3-4A protease. Chromatographic separation of this fraction resulted in the isolation of 27 polyphenols, including four new compounds namely excoecariphenols A–D (**1–4**). This Letter intends to report the structural elucidation of the new compounds and anti-HCV effects of polyphenols.

Excoecariphenol A (**1**) has a molecular formula of $C_{21}H_{24}O_{11}S$ as determined by HRESIMS (*m/z* 507.0941 $[M+Na]^+$, calcd 507.0937), indicating 10° of unsaturation and containing a sulfur atom. The IR absorptions at 3420 and 1640 cm^{-1} in association with ^{13}C NMR data ascertained the presence of hydroxy and aromatic groups. The ^1H NMR spectrum exhibited four aromatic protons for a singlet at δ_H 6.13 (1H, br s, H-6) and an ABX spin system at δ_H 6.81 (1H, d, J = 9.0 Hz, H-5′), 6.77 (1H, br d, J = 9.0 Hz, H-6′) and 6.91 (1H, br s, H-2′), while COSY correlated oxymethine δ_H

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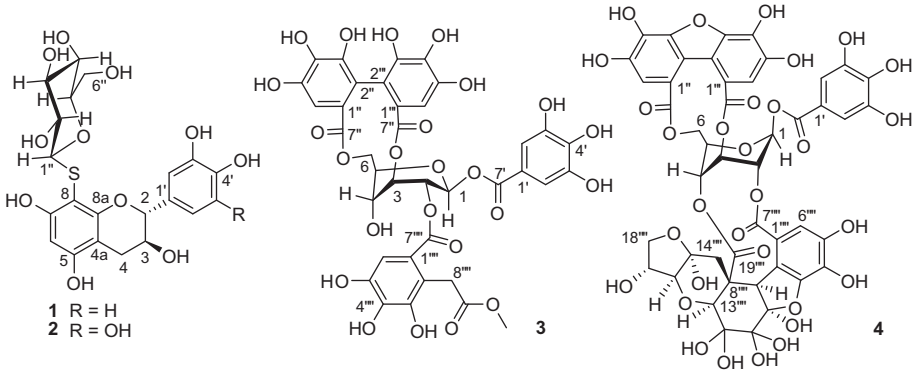
4.13 (1H, ddd, J = 5.0, 7.5, 8.0 Hz, H-3) to an oxymethine (δ_H 4.68, d, J = 7.5 Hz, H-2) and the methylene protons at δ_H 2.56 (1H, dd, J = 5.0, 16.3 Hz, H-4α) and 2.89 (1H, dd, J = 8.0, 16.3 Hz, H-4β) to establish a 1,2-dioxypropyl unit. In addition, the proton signals for a sugar moiety including the hydroxymethylene at δ_H 3.55 (1H, dd, J = 6.2, 12.3 Hz, Ha-6″) and 3.79 (1H, dd, J = 1.8, 12.3 Hz, Hb-6″) and an anomeric proton at δ_H 4.22 (1H, d, J = 9.4 Hz, H-1″) were connected by COSY correlations, while their corresponding carbons were assigned by HMQC. The ^{13}C NMR and DEPT spectra displayed 21 carbon resonances including twelve aromatic carbons for two aromatic rings, two methylenes and seven oxymethines. Analysis of HMBC relationships allowed to establish a nucleus of 5,8,3′,4′-tetrahydroxyfavan-3-ol and a glucosyl unit in the molecule, closely related to catechin-8-*C*-β-D-glucopyranoside.[18] However, the quaternary carbon C-8 of **1** shifted extremely to upfield at δ_C 93.2, and the anomeric carbon of sugar presented at δ_C 89.6 which shifted to downfield with 13.3 ppm more than that of *C*-glucosides.[18] These findings declared **1** to be a 1-thioglycoside. The HMBC interaction between H-1″ and C-8 further confirmed the glucosyl unit to linked to C-8 through a thioether bond. Thus, the structure of **1** was determined as catechin-8-*S*-glucopyranoside. The coupling constant of $J_{H\text{-}2/H\text{-}3}$ (7.5 Hz) was indicative of a *trans* axial-orientation of H-2 and H-3. Based on the revised Snatzke's helicity rule for the assessment of absolute configuration of flavan-3-ols,[19,20] the negative CE_{280} (−3.42) nm (^1L_b transition) and the positive CE_{240} (+4.02) nm (^1L_a transition) in the CD spectrum of **1** was in accordance with 2*R*. Therefore, C-3 was assumed to be *S* configuration. The β-configuration of the glucosyl unit was evident from the coupling constant ($J_{H\text{-}1''/H\text{-}2''}$ = 7.7 Hz). Since the structure of co-existing (+)-catechin-3-*O*-β-D-glucopyranoside was established to be identical to the synthesized product in respect to the physical and chemical properties, the configuration of glucose unit in **1** and also in **2–4** was biogenetically assumed to be in D-form.

Excoecariphenol C (**3**) has a molecular formula of $C_{37}H_{30}O_{24}$, as determined by HRESIMS (m/z 881.1022 $[M+Na]^-$, calcd 881.1019), containing 23° of unsaturation. The ^{13}C NMR and DEPT displayed a total of 37 carbon resonances, including 24 aromatic carbons for four phenyl rings. Based on 2D NMR data analysis, this molecule was consisted of four building blocks. The duplicated aromatic protons at δ_H 6.98 (2H, s) together with their HMBC interactions were characteristic of a galloyl group (unit A). Unit B was assigned to a hexahydroxydiphenic acidic (HHDP) moiety, based on the presence of 12 aromatic carbons whose NMR data were compatible to those of macabarterin,[21] in association with the HMBC relationships from H-3″ (δ_H 6.48, s) and H-3‴ (δ_H 6.68) to C-1″ (δ_C 116.4), C-1‴ (δ_C 116.5), C-5″ (δ_C 135.9), C-5‴ (δ_C 136.3), C-4″ (δ_C 144.4), C-4‴ (δ_C 144.2), and two acetyl carbons at δ_C 167.7 (C-7″) and 166.7 (C-7‴), respectively. The COSY correlations connected the protons of a glucosyl moiety, whose protonated carbons were assigned by HMQC. The NMR data (Supplementary data) of the glucosyl unit exhibited the signals for H-1 (δ_H 6.46, d, J = 7.5 Hz), H-2 (δ_H 5.17, br d, J = 7.5 Hz), H-3 (δ_H 4.64 br s), H-4 (δ_H 4.30 br s), H-5 (δ_H 4.49, dd, J = 7.0, 8.0 Hz), and H_2-6 (δ_H 3.98, 4.38, dd), which featured a skew-boat conformation, the same as the reported data for phyllanemblinin B.[22] The final moiety (unit C) was established as a galloyl-2‴″-methylacetate, which was evident from the presence an aromatic singlet (δ_H 7.01, s), a methoxy group (δ_H 3.46, s), and the geminal protons at δ_H 3.85 (1H, d, J = 17.0 Hz) and 3.92 (1H, d, J = 17.0 Hz), in combination with their HMBC relationships. The connectivity of each moiety was achieved by the HMBC interactions from H-2′ and H-1 to C-7′, H-2 and H-6‴″ to C-7‴″, H_2-6 and H-6″ to C-7″, and H-3 and H-6‴ to C-7‴, respectively, indicating the formation of ester bonds of HHDP to C-3 and C-6, galloyl group to C-1, and the esterification of galloyl-2‴″-methylacetate with the hydroxy group at C-2 (Fig. 1).

The molecular formula ($C_{46}H_{36}O_{31}$) of excoecariphenol D (**4**) was determined on the basis of HRESIMS data (m/z 1083.1171



The NMR data of excoecariphenol B (**2**) were closely resembled those of **1**, except for the presence of two overlapped aromatic protons at δ_H 6.46 (2H, s) in ring B instead of an ABX spin system, suggesting ring B of **2** to be symmetrical substitution and C-5′ to be positioned by a hydroxy group. This assignment was supported by the molecular formula $C_{21}H_{24}O_{12}S$ of **2** showing one oxygen atom more than that of **1**. The stereogenic centers of **2** were assigned to be the same as that of **1** based on the similar CD data and the biogenetic consideration.

$[M-H]^-$, calcd 1083.1168), indicating 29° of unsaturation. The ^{13}C NMR and DEPT spectra displayed 24 aromatic signals for four aromatic rings and five ester carbonyl carbons (δ_C 164.6, 165.3, 165.9, 168.0, 170.8), which were attributed to a galloyl group (unit A), a dibenzofurandicarboxyl group (unit B), and a penta-substituted aromatic ring (unit C). These assignments were inferred from the comparison of NMR data with the structurally similar ellagitannins such as phyllanemblinin A[22] and macabarterin,[21] together with the HMBC data of aromatic protons. A glucosyl moiety was

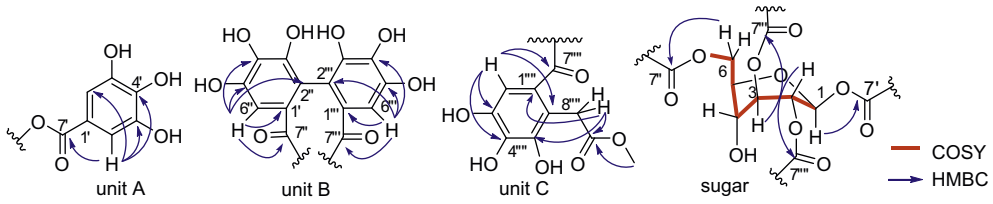


Figure 1. Key HMBC and COSY relationships of **3**.

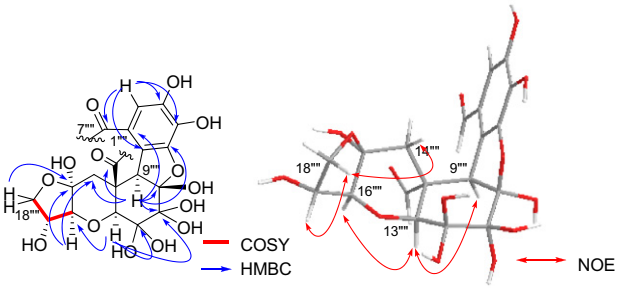


Figure 2. Key COSY, HMBC, and NOESY interactions of unit C in **4**.

recognized by the consecutive COSY cross-peaks from H-1 (δ_H 6.24, d, J = 3.5 Hz) to H_2-6 (δ_H 4.17, 4.55). It is noted that the J values of the glucosyl protons presented as $J_{H\text{-}1/H\text{-}2}$ = 3.5 Hz, $J_{H\text{-}2/H\text{-}3}$ = 0 Hz, $J_{H\text{-}3/H\text{-}4}$ = 3.8 Hz, and $J_{H\text{-}4/H\text{-}5}$ = 0 Hz, indicating the equatorial orientation of H-1 to H-4 when the glucosyl ring adopt a chair-form. In regard to unit C, the uncommon moiety is consist of an aromatic ring, two ester carbonyl carbons (C-7‴ and C-19‴), five aliphatic quaternary carbons, four methines, and two methylenes. A penta-substituted phenyl ring was recognized by the presence of an aromatic singlet at δ_H 7.18 (s, H-6‴). The HMBC relationships of H-6‴ with C-1‴ (δ_C 111.4), C-2‴ (δ_C 117.7), C-4‴ (δ_C 145.4), C-5‴ (δ_C 138.6) and C-7‴ (δ_C 165.3) indicated C-1‴ to be positioned by an ester carbonyl carbon. A methine proton at δ_H 4.50 (s, H-9‴) correlated to the carbon at δ_C 51.6 (C-9‴) in HMQC, and it showed key HMBC interactions (Fig. 2) with aromatic carbons C-1‴, C-2‴, and C-3‴ (δ_C 145.2), two acetal carbons at δ_C 98.7 (C-10‴) and 97.8 (C-11‴), a methylene at δ_C 32.2 (C-14‴), a quaternary carbon at δ_C 52.3 (C-8‴), and a carbonyl carbon at δ_C 170.8 (C-19‴), indicating the presence of a dihydrobenzofuran ring. In addition, the correlations of methine proton H-13‴ (δ_H 4.79, s) to C-9‴, C-14‴, C-19‴, C-8‴, an acetal carbon C-12‴ (δ_C 98.5), and C-11‴ conducted to establish a 4,5,6-triacetalcyclohexane ring, which fused to the dihydrobenzofuran ring at the positions of C-9‴ and C-10‴. In addition, an ester carbonyl group was located at C-8‴. Subsequently, the HMBC cross-peaks from H_2-14‴ (δ_H 1.32, 2.48) to C-15‴ (δ_C 108.8, s) and C-16‴ (δ_C 81.9, d), and from H-16‴ to C-13‴ revealed a perhydropyran ring fusing to the cyclohexane ring at the positions of C-8‴ and C-13‴. The remaining protons included oxymethylene H_2-18‴ (δ_H 3.69, 4.09) and two oxymethines H-16‴ (δ_H 3.90, br s) and H-17‴ (δ_H 3.94), and their corresponding carbons were assigned by HMQC. The COSY cross-peaks from H-17‴ to H_2-18‴ and H-16‴ together with the HMBC relationship between H_2-18‴ and C-15‴ led to the formation of a tetrahydrofuran ring, which was fused to perhydropyran ring. The chemical shift of C-15‴ was in accordance with an acetal carbon. Therefore, the structure of unit C was established to be identical to that of macabarterin. The connectivity of units A–C to glucosyl unit was accomplished by the interpretation of HMBC interactions from glucosyl protons to ester carbonyl carbons (Fig. 2). The relative configurations of the stereogenic centers in unit C were assigned by the NOE relationship and J value. The observation of NOE cross-peak between H_b-14‴ (δ_H 2.48) and H_b-18‴ (δ_H 3.69) ascertained a *cis*-fusion of tetrahydrofuran ring and perhydropyran ring. Additional NOE interactions from H-13‴ to H-16‴ and H-9‴ indicated the same face of these protons, while H-13‴ and H-9‴ should be axial-oriented. Thus, a *cis*-fusion of cyclohexane ring with dihydrobenzofuran ring was supposed. If H-16‴ was arbitrarily assigned to α-face, the $J_{H\text{-}16'''/H\text{-}17'''}$ value (0 Hz) reflected their dihedral angle to be approached to 90°, indicating β-face of H-16‴. These assignments indicated the acyl group of **4** to be the same as that of macabarterin.[21]

Basis on 1D and 2D NMR, and MS spectroscopic data and comparison of spectroscopic data with the reported data in literature, the structures of 23 known phenolic compounds were identified. These included ten flavonoids (2*R*,3*S*)-gallocatechin,[23] (+)-catechin,[24] (+)-catechin-3-*O*-β-D-glucopyranoside,[25] (+)-catechin-7-*O*-β-D-glucopyranoside,[26] (+)-catechin-3-*O*-α-L-rhamnose,[27] quercetin-3-*O*-β-glucopyranoside, myricetin-3-*O*-(6-*O*-galloyl)-β-glucopyranoside,[28] quercetin-3-*O*-(6-*O*-galloyl)-β-glucopyranoside, quercetin-3-*O*-β-galactopyranoside, quercetin-3-*O*-(6-*O*-galloyl)-β-gallcopyranoside.[29] Nine tannins were identical to 1,3,4,6-tetra-*O*-galloyl-β-D-glucose,[30] 1,2,6-tri-*O*-galloyl-β-D-glucose,[31] 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose,[32] corilagin,[33] tercatain,[34] 1,2-di-*O*-galloyl-3,6-(*R*)-HHDP-β-D-glucose,[35] furosin,[36] geraniin,[37] chebulagic acid,[38] ellagic acid 4-*O*-xylopyranoside,[39] gallic acid, 3,4-dihydroxybenzoic acid, and 3,4,5-trihydroxybenzoic acid ethyl ester.[40]

Apart from minor phenols, 22 isolated phenolic compounds were evaluated for the inhibition against HCV NS3-4A serine protease by FRET method.[41] Four compounds including **4**, corilagin, geraniin, and chebulagic acid showed potential inhibition with IC_{50} values in a range of 3.45–9.03 μM. Subsequently, the four active compounds were evaluated for anti-HCV activity in the HCV RNA replicon assay in huh 7.5 cells using real-time qRT-PCR method. As shown in Table 1, **4** and corilagin possessed significant inhi-

Table 1
Inhibitory activities of polyphenols toward HCV NS3-4A protease and HCV DNA in huh 7.5 cells

	NS3-4A protease IC_{50} (μM)	Huh 7.5 cells (MTT) CC_{50} (μM)	Huh 7.5 cells (qRT-PCR) EC_{50} (μM)	SI
Corilagin	3.45	96.65±4.41	13.59 ± 1.90	7.1
4	6.93	56.25 ± 0.55	12.61 ± 1.43	4.5
Geraniin	8.91	63.40 ± 0.76	33.19 ± 8.20	1.9
Chebulagic acid	9.03	104.91 ± 3.86	22.25 ± 8.70	4.7
BILN2061	1.54			

Note: inhibiting HCV RNA replication activity by 50% (EC_{50}), decreasing cell viability by 50% (CC_{50}), and the selective index (SI).

bition against HCV RNA replication in huh 7.5 cells, whereas geraniin, and chebulagic acid showed moderate inhibition. Thus, HCV NS3-4A serine protease is regarded to be a possible pathway for the polyphenols to inhibit HCV.

Primary discussion of structure–activity relationship revealed that flavonoids and falvanes, as well as the glucosides linked by different numbers of galloyl group, are weakly reacted with HCV NS3-4A serine protease (Supplementary data).

In summary, natural products provided a rich bank to assay for anti-HCV promising compounds for progressing molecules into the clinic and to improve therapies. The HCV nonstructural protein 3/4A (NS3/4A) serine protease represents an attractive target as it is essential for viral polyprotein replication. Recently, the NS3-4A protease inhibitors telaprevir and boceprevir for the treatment of chronic hepatitis C are marketed.[42] Up to date, only three plant-derived tannins have been found as the HCV invasion inhibitors.[43] This work firstly reported polyphenols to inhibit HCV virus replication mediated by NS3-4A serine protease, and these compounds maybe possible to be developed as anti-HCV lead compounds.

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Supplementary data

Supplementary data (experimental section, Tables of ^{1}H and ^{13}C NMR data, spectroscopic copies (IR, UV, MS, ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC, NOESY, and CD) of new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.109.

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