doi.org/10.1002/cbdv.202401388 Research Article CHEMISTRY & BIODIVERSITY

www.cb.wiley.com

Bioactive Alkaloids from Nepalese *Corydalis chaerophylla* D.C. Acting on the Regulation of PCSK9 and LDL-R *In Vitro*

Binita Maharjan,[a] Ilaria Rossi,[b] Stefania Sut,[b] Timila Shrestha,[a] Lok Kumar Shrestha,[c, d] Jonathan P. Hill,[c] Katsuhiko Ariga,[c, e] Veronica Benetazzo,[b] Maria Pia Adorni,[f] Bianca Papotti,[g] Shyam Sharan Shrestha,[h] Ram Lal Swagat Shrestha,*[a] Nicola Ferri,[i, j] and Stefano Dall'Acqua*[b]

Four new alkaloids Chaeronepaline-A (**1**), Chaeronepaline-B (**2**), Chaeronepaline-C (**3**), and Chaeronepaline-D (**4**) were isolated from *Corydalis chaerophylla* D.C. collected from Nepal and their structures were elucidated by spectroscopic data, 1D, 2D NMR and mass spectrometry. The structures were established as 3,12- Dimethoxy-5,6-dihydroisoquinolino [2,1-b] isoquinolin- 7-ium- 2, 9- diol (**1**), 7-Methyl-2,3:11,12-bis(methylenedioxy)-7,13a-secoberbin-13–14-epoxide (**2**), 7- methyl-5, 6, 7, 8-tetrahydro- 8H-spiro-9,14-dihydroxy-11,12-methylenedioxy-indane-isoquinoline (**3**) and 7- methyl-5, 6, 7, 8- tetrahydro- 8H-spiro-9,14-dihydroxy-11,12-methylenedioxy-indane-isoquinoline-N-oxide (**4**). The new alkaloids were tested in human hepatoma cell line to assess their ability to modulate the expression of low-density lipoprotein receptor (LDL–R), of proprotein convertase subtilisin/kexin 9 (PCSK9) and to affect cellular cholesterol biosynthesis with the aim to evaluate their potential hypocholesterolemic effect. Results indicated that compounds **2** and **3** upregulate the LDLR, and inhibited the cholesterol biosynthesis with compound **2**, which also reduced the secretion of PCSK9 by Huh7 cells. These *in vitro* data indicated a potential hypocholesterolemic effect of compound **2** that requires further *in vivo* validation.

1. Introduction

The genus *Corydalis*, comprising over 470 species in Eurasia and North America, is indigenous to the temperate Northern Hemisphere. Among them, fifty-seven species can be found in Nepal. These plants, characterised by their vibrant and attractive flowers, are classified as both annual and perennial herbaceous plants. They produce significant number of isoquinoline alkaloids,[1–4] which exhibit diverse biological properties.[4,5] Traditionally, some *Corydalis* species have been widely employed in China, Korea, Japan, and other Eastern Asian nations to treat gastric and duodenal ulcers, dysmenorrhoea, rheumatism and cardiac arrhythmia disease.[2–4,6] The phytochemical analysis of *Corydalis* plant extracts resulted in the isolation of more than 100 isoquinoline alkaloids from this genus.[4] Some of these alkaloids have been studied as possible treatment of serious diseases such as cancer, Alzheimer's disease, and microbial infections. *Corydalis* alkaloids present good drug-like properties demonstrated by the numerous reported biological activities.[4] Various alkaloids derived from *Corydalis* have been studied for their *in vitro* metabolic effects,[7] for hepatoprotective properties,[8] as urease activity inhibitors,[9] as leishmanicidal,[10] analgesic,[11] apoptosis inducers,[12] as cytotoxic agents on human cancerous cell lines[13] and as cholesterol controlling agents.[14]

Corydalis chaerophylla D.C. is a glabrous herb found in high-altitude areas of Nepal, India, and Pakistan. It survives in wet, shadowy conditions at elevations ranging from 2400–4800 a.l.s.

[a] *B. Maharjan, T. Shrestha, R. L. S. Shrestha*
Department of Chemistry, Amrit Campus, Tribhuvan University, Kathmandu 44600, Nepal
E-mail: swagatstha@gmail.com

[b] *I. Rossi, S. Sut, V. Benetazzo, S. Dall'Acqua*
Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy
E-mail: stefano.dallacqua@unipd.it

[c] *L. K. Shrestha, J. P. Hill, K. Ariga*
Research Center for Materials Nano Architectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

[d] *L. K. Shrestha*
Department of Materials Science, Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573 Japan

[e] *K. Ariga*
Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8561, Japan

[f] *M. P. Adorni*
Department of Medicine and Surgery, University of Parma, Parma, Italy

[g] *B. Papotti*
Department of Food and Drug, University of Parma, Parma, Italy

[h] *S. S. Shrestha*
Himalayan Research and Development Center Nepal, Kathmandu, Nepal

[i] *N. Ferri*
Department of Medicine, University of Padova, Padova, Italy

[j] *N. Ferri*
Veneto Institute of Molecular Medicine (VIMM), Padova, Italy

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202401388

C. chaerophylla was used as traditional medicine in Nepal for peptic ulcers in doses of about four teaspoons, three times per day. The administration of a dosage of around six teaspoons, three times per day, of the root juice is recommended for the treatment of dyspepsia. This therapeutic approach involves blending the root juice with an equal amount of the root juice derived from *Cyathula capitates* Moq. (N. Kuro).[15] Previous investigations reported the isolation from *C. chaerophylla* collected in Nepal, of a new alkaloid called chaerophylline, as well as (−)-corypalmine, berberine, (−)-isocorypalmine, (−)-corydalmine, and (+)-bicuculline.[16] In a recent paper our group also considered Nepalese *C. chaerophylla* showing the presence of fifteen different alkaloids, with N–Me-tetrahydropalmatine, bicuculline, protopine, hunnemanine and jatrorrhizine as most abundant derivatives.[17]

In a previous study, our research group considered a series of alkaloids isolated from plants as potential bioactive compounds targeted on the key enzyme involved in cholesterol metabolism, namely the low-density lipoprotein receptor (LDL–R) and the proprotein convertase subtilisin/kexin 9 (PCSK9). In that work, we observed significant induction of LDLR, similar to the hydroxy-methyl glutaryl Coenzyme A (HMG-CoA) inhibitor, simvastatin, berberine, californidine and govaniadine. Californidine and berberine reduced the expression of PCSK9, while govaniadine, similar to simvastatin, induced the PCSK9 expression. Additionally, it was shown that all of the tested compounds reduced the total cholesterol level in the hepatocytes.[14]

Focusing on the discovery of novel bioactivities associated with alkaloids, with a particular emphasis on the biodiversity of the Nepalese plant kingdom, starting from our previous investigation on alkaloid content of this plant[17] we considered *C. chaerophylla*, as potential source of bioactive compounds being the Nepalese population of this plant, up to now, only in limited part investigated.

2. Result and Discussion

2.1. Structural Elucidation of New Isolated Compounds 1–4

The compound (**1**) was analysed by mass spectrometry and showed molecular ion $[M+H]^+$ at *m/z* 324, and fragmentation showed the loss of methyl group leading to ion at *m/z* 309. From the HR-MS data, the molecular formula was calculated as $C_{19}H_{18}NO_4$.

The ^{1}H-NMR showed the presence of four singlets at δ 9.30, 8.03, 7.39 and 6.93, each integrating for one proton. Furthermore, two singlets integrating three protons each were observed at δ 3.94 and 3.90, suggesting the presence of two methoxy groups. Two ortho coupling doublets (*J* = *8.0*) were observed at δ 7.58 and 6.94, supporting the presence of an aromatic ring, and two triplets at δ 4.63 and 3.16, showing aliphatic coupling (*J* = *6.2*), each integrating for two protons were observed. The HSQC-DEPT spectrum allowed to observe all the non-quaternary positions and revealed that the compound possesses six aromatic CH, two aliphatic CH_2, one benzylic (δ_H 3.16-δ_C 27.3; C-5) and one N-linked (δ_H 4.64-δ_C 54.2; C-6). Also, the two methoxyl positions were confirmed. The complete structure of the compound was obtained by combining the data of HMBC, COSY and NOESY spectra, structure and main observed diagnostic NMR correlations are reported in Figures 1 and 2. The singlet at δ_H 7.39 (H-1) showed HMBC correlation with C-4a (δ_C 126.2), C-3 (δ_C 149.3) and C-14 (δ_C 134.6), while the other at δ_H 6.93 (H-4) showed HMBC with C-2 (δ_C 146.6) supporting the presence of an electron attractive group, C-14a (δ_C 120.2) and C-5 (δ_C 27.3). The methoxyl group at δ_H 3.94 presents HMBC correlation with C-3, confirming the presence of 3-methoxyl substitution. The HMBC observed from H-5 (δ_H 3.16) with C-4 (δ_C 110.0), C-14a, and C-4a support the direct linkage between positions 4a and 5. The COSY coupling from H-5 to H-6 and the diagnostic HMBC correlation from H-6 with C-4a and C-14 allowed to establish the presence of a 3,4-dihydroquinoline moiety. Further diagnostic HMBC from H-6 is observed with C-8 (δ_C145.9). From the H-8 (δ_H 9.30), HMBC correlations are observed with C-14 confirming the presence of the six-member ring of 3,4-dihydroisoquinoline, C-12a (δ_C 132.4) and C-9 (δ_C 162.0) this latter supporting the presence of a hydroxyl group. The H-13 present long range HMBC correlations with C-8a (δ_C 120.1) and C-12 (δ_C 149.7) this latter correlation is in common with the singlet at δ_H 3.90, supporting the presence of a 12-linked methoxy group. Two ortho coupling doublets were finally assigned to positions H-10 and H-11, and they present long range correlations with C-8a, C-12 and C-9 and C-12a, respectively. NOESY correlation confirmed the methoxylation position shown by the cross peak between H-16 and H-4 and H-15 with H-13 H-11. All the obtained data allowed to establish for the compound **1** the structure of 3,12-Dimethoxy-

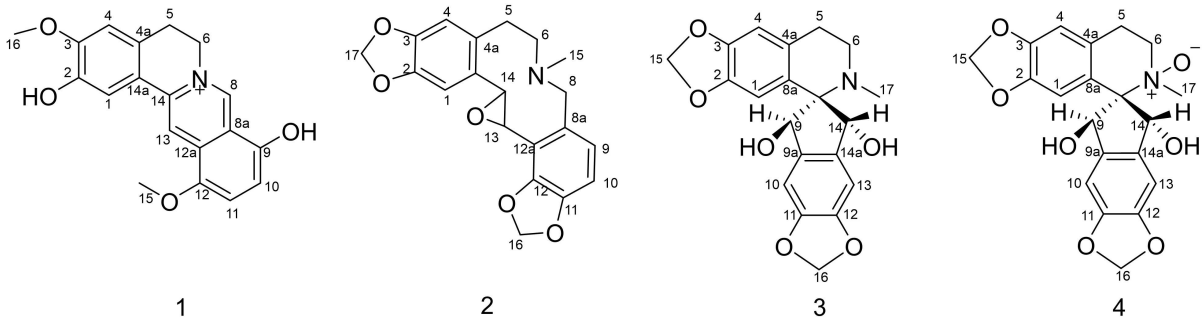

Figure 1. Structures of compounds 1–4.

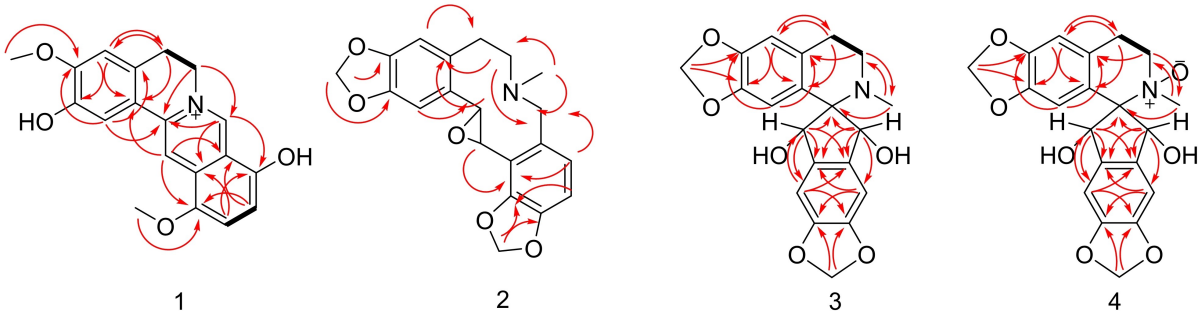

Figure 2. Key HMBC(red arrows), ^{1}H-^{1}H COSY (bold) correlation of compounds **1**–**4**.

5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-2,9-diol and named chaeronepaline-A. As per our knowledge, this is the first report of the isolation of this compound from natural sources.

The compound (**2**) presented molecular ion $[M+H]^+$ at *m/z* 354 and the HR-MS data allowed to obtain the molecular formula of $C_{20}H_{20}NO_5$. The ^{1}H-NMR spectrum showed four singlets in the deshielded region, two at δ_H 7.14, 6.79, integrating for one proton each and two ortho coupled doublets ($J=7.0$) at δ_H 6.86 and 6.81 integrating one proton each. The signals support the presence of two aromatic rings, the spectrum in the aromatic is similar to the one published for protopine.[19] Singlets at δ_H 6.02, 6.03 integrating two protons each indicate the presence of two methylenedioxy groups.

In the aliphatic region, broad signals at δ_H 4.48 (2H), 3.86 (1H), 3.85 (1H), 3.77 (1H), 3.51 (2H) and 3.22 (2H) suggested the presence of aliphatic N-linked methylene and methyne bearing electron attractive group. Broad signals ascribable to methylene have been described for protopine suggesting that the compound (**2**) may be belonging to this family of alkaloids.[19,20] Another signal suggesting the structure of protopine-type alkaloid is the singlet integrating for three protons at δ_H 2.91, ascribed to a N-linked methyl group. Compound appear quite unstable and further purification lead to alteration of pattern of signals in the ^{1}H-NMR so we decided to perform the structure elucidation on the partially purified compound. The HSQC-DEPT revealed the presence of four aromatic CH at δ_H 7.14-δ_C 115.9 (C-1), δ_H 6.79-δ_C 108.9 (C-4), δ_H 6.86-δ_C 122.9 (C-9), δ_H 6.81-δ_C 108.6 (C-10), two methylene dioxy groups at δ_H 6.00-δ_C 102.0 (C-17), δ_H 6.00-δ_C 102.0 (C-16). In the aliphatic region, two partially overlapped CH were observed at δ_H3.85- δ_C 56.5 (C-13 and C-14), due to their chemical shifts they were assigned as benzylic CH bearing an epoxide group. Other significant signals were one benzylic CH_2 at δ_H 3.25-δ_C 24.6 (C-5) and two N-linked CH_2 at δ_H 4.48-δ_C 53.7 (C-6) one at 3.77–3.51 broad signals δ_C 55.0 (C-8). N-linked methyl group was observed at δ_H 2.91-δ_C 41.3 (C-15). The structure was elucidated combining the data obtained from HSQC-DEPT, HMBC, COSY and NOESY. The HMBC observed from H-1 with carbon resonances at δ_C 148.8 (C-3), 124.5 (C-4a) and 56.5 (C-14) as well as the diagnostic long range correlation observed from H-4 with carbon at δ_C 148.7 (C-2), 127.8 (C-14a). NOESY correlation was observed from H-4 with δ_H 3.25 (H-5) an from H-1 with δ_H 3.86–3.85 (H-14 and H-13). Furthermore, the long range correlation observed from H-5 with C-4 and C-14a as well as from H-14 with C-4a suggest that the compound present protopine type moiety. NOESY correlations were also observed from H-6 with H-5 and N-Methyl group as well as from H-8 with N-Methyl group. Diagnostic HMBC correlation was observed from H-13 with C-12 (δ_C 148.1) confirming the epoxide position in 13–14. Furthermore, the long range correlations from H-9 with C-8, C-11, C-12a allowed to establish the position of the second methylenedioxy group. Complete structure assignments are reported in Tables 1 and 2. The structure of the compound was assigned to derivative of protopine bearing the 13–14-epoxy group instead of the keto function in position 14. Compound can be nominated as 7-Methyl-2,3 : 11,12-bis(methylenedioxy)-7,13a-secoberbin-13–14-epoxide and we here propose the name chaeronepaline-B. The MS^n fragmentation support the proposed assignment in fact we can observe in MS^2 the intense fragment at *m/z* 188 and 149. The compound presents optical activity and the CD spectrum revealed a negative effect at 205 and 215 nm, but with the isolated amount was not possible to assign absolute configuration.

The MS of the compound (**3**) presented molecular ion at *m/z* $[M+H]^+$ 370. The molecular formula deduced from the HR-MS data was $C_{20}H_{20}NO_6$. The H-NMR revealed the presence of four singlets at δ_H 6.89, 6.87, 6.68 and 6.30, the first integrating for two protons and the other three integrating for one proton. Further signals are detected as singlets at δ_H 6.00 and 5.81, both integrating for two protons. Finally, two more singlets, integrating one proton each, are observed at δ_H 5.53 and 5.34. in the aliphatic region, three multiplets were detected at δ_H 3.68, 3.62 and 2.97, the first two integrating one proton each and the third integrating for two protons. One singlet suggesting the presence of one N-linked methyl group was detected at δ_H 2.84 (3H). Complete structure assignment was obtained by combining HSQC-DEPT, HMBC, COSY and NOESY data. One tetrahydro isoquinoline moiety was supported by the two CH observed at δ_H 6.30-δ_C 109.1 (C-1) and δ_H 6.68-δ_C 108.2 (C-4) and by the relevant long range HMBC correlations observed from H-1 with δ_C 128.3 (C-4a), δ_C 147.4 (C-3) and a quaternary carbon resonance at δ_C 81.6 (C-8) as well as by the one observed from H-4 with δ_C 145.3 (C-2), δ_C 120.0 (C-8a) and δ_C 21.5 (C-5). The other correlations allowing the identification of the dehydro pyperidine ring were observed from H-6 with C-4a, C-8 and the δ_C 37.9 (C-17), this latter assigned to the N-linked methyl group.

The singlets at δ_H 5.53 and 5.34 were assigned to oxygen-bearing CH due to the shift of their carbons that, were δ_C 76.3 and 77.8, respectively. From these signals assigned to positions H-14 and H-9 diagnostic HMBC were observed with C-8 and C-8a, supporting the direct linkage of these CH with the tetrahydroquinoline moiety. The chemical shift of the quaternary position C-8 suggests the presence of a spiro derivatives.[21] The protons H-14 and H-9 present a COSY correlation, suggesting a "w" type long range coupling. From the H-9 and H-14, identical and numerous long range HMBC correlations can be observed and are depicted in the Figure 2. In particular, long range correlations were observed from the H-9 and C-14 and from H-14 and C-9, supporting the linkage of the two CH to one quaternary carbon (C-8). Furthermore, from H-14, two bond correlations are observed with C-8 and C-14a. Three bond correlations were observed with C-8a, C-9a and C-13, but also four bond correlations were observed with C-10 and C-12, and five bond correlations were observed with C-11. The same correlations were also observed from H-9. These HMBC correlations suggest the presence of a planar and rigid portion of the compound. Due to the presence of the H-9 and H-14 as well as the singlets ascribed to H-10 and H-13 and the methylenedioxy H-16, the second portion of the molecule was assigned to an indane derivative bearing a methylene dioxy substituent and sharing one carbon of the pentacyclic hydrocarburic cycle with the isoquinoline moiety. NOESY correlations allowed to establish relative stereochemistry, and we imposed the alpha configuration to H-14. The H-14 showed NOESY with signal at δ_H 3.62 that on the basis of our hypothesis can be ascribed to H-6α, while H-9 presents NOESY correlation with proton at δ_H 3.68 that consequently can be ascribed to H-6β. Both H-9 and H-14 show NOESY correlation with the N-methyl group.

The structure of the compound is a spiroindane isoquinoline characterised by the presence of two methylenedioxy substituents, one for each aromatic ring. The structure is formed by a tetrahydro isoquinoline moiety fused with an oxygenated indane, and the basic carbon skeleton is like ochrobirine,[22] but with a different position of the methylenedioxy substituent in the indane portion. The compound (**3**) is characterized as 7-methyl-5, 6, 7, 8- tetrahydro- 8H-spiro-9,14-dihydroxy-11,12-methylenedioxy-indane-isoquinoline and named chaeronepaline-C.

The compound present optical activity (+38), and the CD spectrum showed two negative cotton effects, one at 215 nm and one at 295, while a positive effect was recorded at 270 nm. The literature[23] assigned absolute configuration for ochrobirine since opposite cotton effects in CD spectrum recorded in methanol. On the basis of our data, we can assume that compound **3** presented opposite absolute configuration at the carbons 6, 9 and 14 of ochrobirine. Complete assignment of absolute stereochemistry could be confirmed only after asymmetric synthesis.

The MS of compound (**4**) presented molecular ion at $[M+H]^+$ 386, with relevant fragments at *m/z* 370, suggesting that it contained a further oxygen atom compared to the previous derivative. The molecular formula deduced from the HR-MS data was $C_{20}H_{20}NO_7$. H-NMR presented similarity to compound **3** with an almost superimposable spectrum related to the indane portion and small differences in the chemical shift of position 1 and 4 and 9 and 14. In contrast, significant differences were observed in the chemical shift of the N-methyl that appeared deshielded with δ_H 2.97. The analysis of 2D spectra allowed to establish the same spiroindane isoquinoline moiety and compound was characterised as the N-Oxide derivative of **3** as 7-methyl-5, 6, 7, 8- tetrahydro- 8H-spiro-9,14-dihydroxy-11,12-methylenedioxy-indane-isoquinoline-N-oxide and named chaeronepaline-D. Compound **4** presented the same behaviour as 3 at circular dichroism and presented optical rotation activity, as for the compound 3 we imposed H-14 as alpha. NOESY correlations are observed from H-14 with H-6α, while from H-9 with H-6 β and from H-9 and H-14 with the N-Methyl group. On the basis of the literature, we assume that compound **4** presented opposite absolute configuration at the carbons 6, 9 and 14 compared to ochrobirine. In any case, the complete assignment of absolute stereochemistry could be confirmed only after asymmetric synthesis Figure 1.

2.2. LC–MS^n of the Isolated Alkaloids

The behaviour of the new alkaloids was evaluated using liquid chromatography with multiple stage mass spectrometry (LC–MS^n) in positive ion mode, allowing the observation of relevant ion species useful for structural elucidation and for the development of further analytical methods.

Compound **1** presented a charged nitrogen in the structure; in the spectrum, it was observed as $[M]^+$ ions at *m/z* 324 in positive ion mode. When CID was performed in an ion trap, the $[M]^+$ ion produced the prominent product at *m/z* 309, due to the loss of methyl radical (See supplementary material S36). This product ion was further subjected to MS^3 analysis leading to the ion at *m/z* 294 corresponding to the loss of a further methyl radical, confirming the presence of two methoxyl groups in the structure. Ion at *m/z* 294 was subjected to MS^4, which afforded the product ion at *m/z* 266, corresponding to a loss of 28 Da.

Compound **2** presented peculiar behaviour in mass spectrometry. When ionised in positive mode, it presented ion $[M+H]^+$ at *m/z* 354. When CID was performed, ion at *m/z* 354 produced ions at *m/z* 336(2a), 206 (2b), 188 (2c) and 149 (2d) *m/z* corresponding to sequential loss of 18, 148, 166, 206 Da (Figure S37). The ion *m/z* 188 and 149 are diagnostic for protopine-type alkaloids.[24]

Compound **3** presented ion $[M+H]^+$ at *m/z* 370 in positive ion mode. When CID was performed, ion at *m/z* 370 produced ions at *m/z* 352, corresponding to a loss of 18 Da (Figure S38). In MS^3, ion at *m/z* 352 afforded the product ion at *m/z* 190 corresponding to the formation of methylenedioxy 1,2-dehydro methylisoquinoline ion. This latter was subjected to CID fragmentation leading to the formation of 131, 149 and 175, corresponding to 1,2-dehydroisoquinoline, a species that originated due to nitrogen loss (uneven ion) and a methylenedioxy 1,2 dehydro isoquinoline.

Compound **4** presented ion $[M+H]^+$ at *m/z* 386 in positive ion mode, with a difference of 16 Da compared to compound **3**, corresponding to the N oxide derivative. When CID was performed, the $[M+H]^+$ ion produced the prominent product at *m/z* 368, which was formed by the loss of 18 Da. In MS3, ion at *m/z* 368 afforded the product ion at *m/z* 206, corresponding to the formation of methylenedioxy 1,2-dehydromethylisoquinoline N oxide ion. This latter undergo to CID fragmentation leading the formation of 191 corresponding to methylenedioxy 1,2 dehydro isoquinoline N oxide ion, which was formed by the loss of methyl radical (Figure S39).

The observed behaviour in mass spectrometry allowed to identify these compounds with the observation of diagnostic fragments and can be a useful strategy for the further study of the presence of such derivatives also in other *Corydalis* species.

2.3. Bioactivity of the Isolated Compounds

These four compounds were tested for their possible action on main cholesterol players, such as LDL–R and PCSK9. We first determined the cytotoxicity activity in human hepatocyte-derived cancer cell line Huh7 after a concentration-dependent exposure to the compounds for 72 hours in a cultured medium with 10% fetal bovine serum (FBS). The SRB assay revealed that only compounds **2** and **3** elicited a significant cytotoxic action at 50 μM and 100 μM, respectively (Figure 3).

We next performed a series of experiments aimed at evaluating the effect of the four alkaloids on LDL–R and PCSK9 intracellular expression by Western blot analysis of total protein extracts. For these experiments Huh7 cells were incubated for 72 hours with 100 μM of compound **4**, 50 μM of compound **1** and **3**, and 25 μM of compound **2**. The HMG-CoA reductase inhibitor simvastatin was utilized as positive control, as well as berberine, an isoquinoline alkaloid, with well-known inhibitory effect on PCSK9 transcription. As expected, simvastatin significantly induced the expression of both LDLR and PCSK9 by 8.3 ± 2.4 and 4.9 ± 2.3 fold, respectively, while berberine showed a minor effect on the LDL–R and a strong inhibition on PCSK9 (−75%) (Figure 4). Compound **2** and **3** determined a significant increase of the LDL–R expression by 3.0 ± 1.2 fold and 2.4 ± 0.4 fold, respectively (Figure 4). On the contrary compounds **1** and **4** did not show any significant effect. Compound **3** appeared to induce the expression of PCSK9, although the difference was not statistically significant. Taken together, only compounds **2**

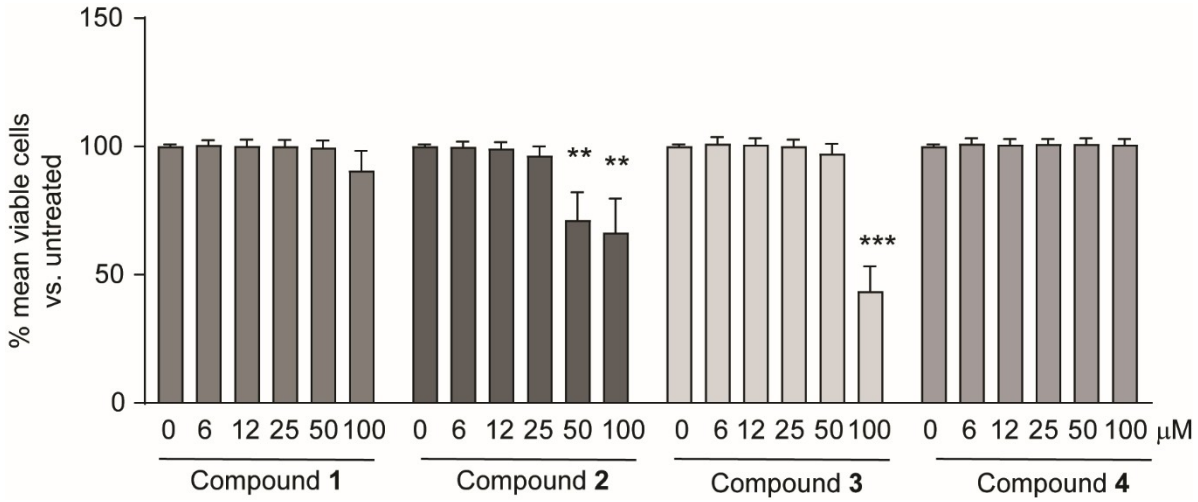

Figure 3. Cytotoxicity assay of alkaloids from Nepalese *C. chaerophylla* D.C. The cytotoxic effect was determined by SRB assay after 72 hours incubation of Huh7 with 6.25, 12.5, 25, 50 or 100 μM of compounds. Data are expressed as mean ± SD of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ *vs* untreated control by Student's t-test.

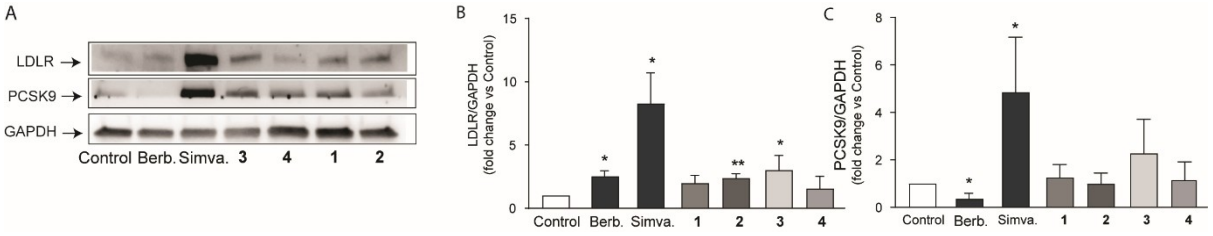

Figure 4. Effect of alkaloids from Nepalese *C. chaerophylla* D.C. on LDL–R and PCSK9 expression in Huh7 cell line. Cells were incubated with simvastatin 5 μM or compound **4** (100 μM), compound **1** and **3** (both at 50 μM), and compound **2** (25 μM) for 72 h. A) Representative western blotting analysis for the expression of LDL–R and PCSK9 upon treatments. GAPDH was used as loading control; B) Densitometric analysis of LDL–R/GAPDH ratio; C) Densitometric analysis of PCSK9/GAPDH ratio. Data are presented as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control by Student's T-test. Simva: simvastatin.

and **3** showed a positive effect on LDL–R expression suggesting a possible hypocholesterolemic activity.

To further investigate the potential hypocholesterolemic effect of these new alkaloids, we determined their action on the secretion of PCSK9 from Huh7 cell line. Berberine was utilised as positive control and, as previously described,[25] reduced PCSK9 extracellular levels by 40.5% (Figure 5). All the compounds showed, to a different extent, a reduction in PCSK9 secretion, with compounds **1** and **2** that had the most potent and effective activity.

These data indicate that compounds **2** and **3** may have some potential hypocholesterolemic effect by determining an upregulation of the LDL–R (Figure 4), however compound **2** seems to act differently from **3** with an additive inhibitory action on the secretion of PCSK9 (Figure 5). Thus, compound **2** seems to have a similar mechanism of action of berberine that was shown to inhibit PCSK9 transcription, and, as a consequence, to lower its intracellular and extracellular (secretion) levels.[26]

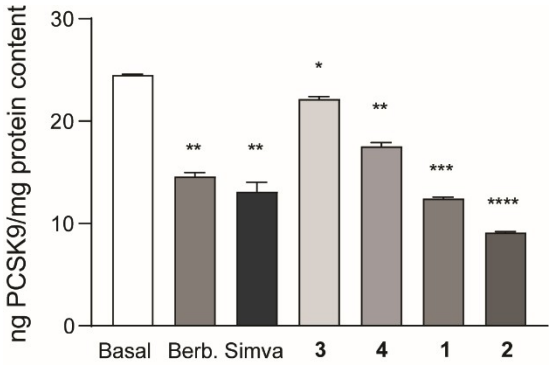

Figure 5. Effect of alkaloids from Nepalese *C. chaerophylla* D.C. on PCSK9 secreted by Huh7 cell line. Cells were incubated with berberine 10 μM, simvastatin 5 μM or compound **4** (10 μM), compound **1** and **3** (both at 5 μM), and compound **2** (2.5 μM) for 72 h. At the end of the incubation, the conditioned media were collected, and PCSK9 levels were determined by ELISA assay. Data are presented as mean ± SD of three independent experiments. *$p < 0.05$ vs control by Student's T-test. Berb: berberine.

We then determined their effect of cholesterol biosynthesis in hepatoma HepG2 cell line. As expected, simvastatin and berberine significantly reduced cholesterol biosynthesis (Figure 6). Although to a lower extent than simvastatin and berberine, all the alkaloids partially inhibited cholesterol biosynthesis, with compounds **2** that showed the most potent activity (Figure 6).

These data indicated that these alkaloids, especially **2**, showed a similar mechanism of action than berberine, by both reducing the expression of PCSK9 and cholesterol biosynthesis, effects that determined a significant increase of the LDL–R expression. Further consideration can be done considering the chemical structure, in fact compound **2** is a protopine derivative that differs from afromentioned alkaloid due to the presence, in position 13–14 of epoxy group, while in protopine the position 14 is occupied by a keto group and position 13 is a CH_2. This difference strongly influence the bioactivity of the compound in fact in our previous paper we observed that 50 μM protopine strongly downregulated both the LDLR and PCSK9 in the same cellular model.[14]

Finally, we tested the activity of *C. chaerophylla* D.C. derived compounds on lipid accumulation in Huh7 cell line. As shown in Figure 7, after the treatments with the compounds, there was no significant variation in the quantity of neutral lipids, specifically triglycerides and cholesterol esters, except after incubation with compounds **2** and **4**. In this instance, a significant reduction in lipid accumulation was observed with **2** resembling the effect of simvastatin rather than berberine while **4** seems to have a more berberine-like lipid accumulation. Although compound **2** has previously exhibited a berberine-like mechanism of action by reducing PCSK9 secretion and cholesterol biosynthesis with an induction of LDL–R expression, this assay revealed a protective effect on liver lipid accumulation. It is, thus, tempting to speculate that there could be a potential additional effect on the triglyceride synthesis pathway.

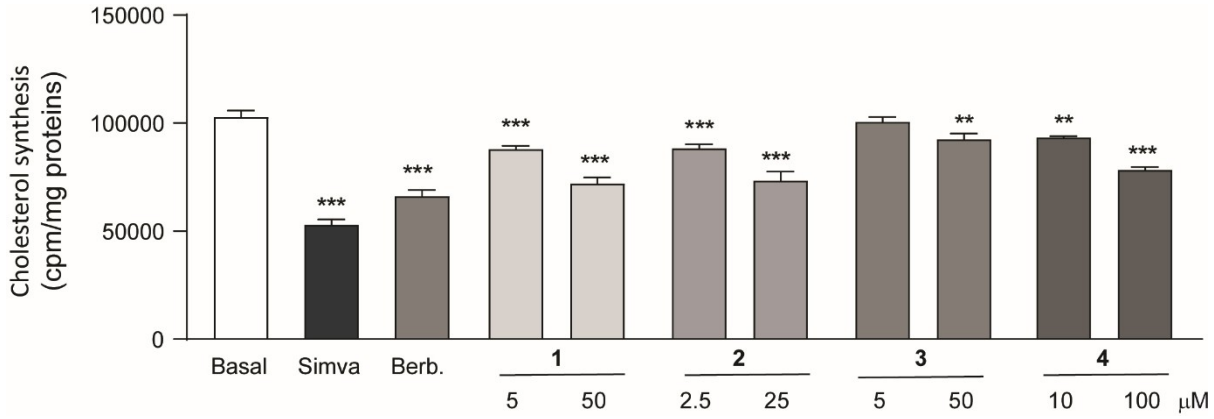

Figure 6. Effect of alkaloids from Nepalese *C. chaerophylla* D.C. on cholesterol biosynthesis in HepG2 cell line. Cells were incubated with simvastatin 5 μM, berberine 20 μM, or compounds at the indicated concentrations for 48 h. At the end of the incubation, cells were incubated with ^{14}C-acetate in MEM containing 0.4% FCS. After 24 h the ^{14}C-acetate incorporation into cholesterol was determined. Data are presented as mean ± SD of three independent determinations. **$p < 0.01$; ***$p < 0.001$ vs control by Student's T-test. Simva: simvastatin; Berb: berberine.

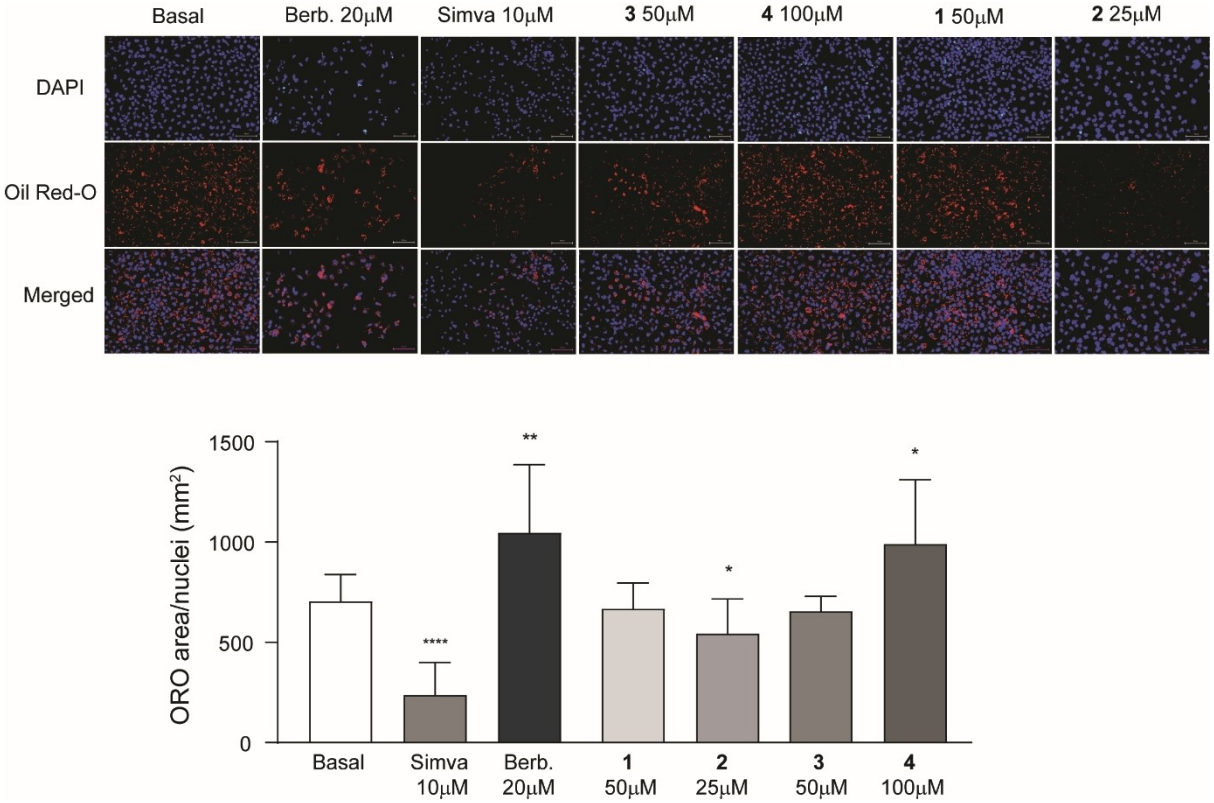

Figure 7. Accumulation of neutral lipids after *C. chaerophylla* D.C. treatments on Huh7 cell line. Cells were incubated with the positive controls and the compounds at the indicated concentrations for 72 h. At the end of the incubation, neutral lipids were stained with Oil Red-O. Data are presented as mean ± SD of three independent determinations. The upper panel showed representative images of Oil Red O staining (n = 6) *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ vs control by Student's T-test. Simva: simvastatin; Berb: berberine.

3. Conclusions

The Nepalese *C. chaerophylla* D.C. was subjected to detailed phytochemical analysis. Four new alkaloids were isolated, and their structures were established using spectroscopic techniques. Compounds **1** is a protoberberine type compound with two methoxyl functions in position 3 and 12 and with two hydroxyl groups in position 2 and 9. The compound **2** is a protopine derivative with epoxy group. Compounds **3** is a spiroindane benzoquinoline and compound **4** is its N-oxide derivative. The new compounds were assayed on key proteins involved in cholesterol metabolism and **2** expressed the most promising activity by inhibiting cholesterol biosynthesis, intracellular lipid accumulation, and PCSK9 secretion with a significant increase in LDL–R expression. However, the hypocholesterolemic effect of compound **2** still needs to be determined in *in vivo* experimental models.

4. Experimental Section

4.1. General Experimental Procedure

All NMR spectra were recorded on Bruker 400 MHz spectrometers operating at 400.11 MHz for ^{1}H and 100 MHz for ^{13}C. ^{1}H-NMR, ^{13}CNMR, COSY, HMBC, HSQC and NOESY experiments were acquired using standard Bruker pulses sequences optimising values of p1, d1 and mixing times. Mass spectrometry and fragmentations were obtained on a Varian 500MS Ion trap, while the HR-ESI-MS spectra were obtained from Waters Xevo G2 QTof. IR spectra were recorded on a Perkin Elmer FTIR spectrometer. Jasco digital 2000 polarimeter was used for the measurement of optical rotation power, while Jasco J-2000 circular dichroism was used for CD measurements.

Column chromatography was performed on silica-gel 40 mesh. Precoated silica-gel 60 F254 (0.25 mm) plates were used for TLC. The purity of isolated compounds was checked by HPLC using an Agilent 1260 system equipped with a diode array and using an Agilent C18 XDB column (3×150 mm, 3.5 μm) eluting in isocratic mode acetonitrile/water 70/30.

4.2. Plant Material

The complete botanical specimen of *Corydalis chaerophylla* D.C. was procured from Phulchowki, Lalitpur, Nepal, at an elevation ranging from 2400–2700 metres. The identification of the specimen was conducted, and a voucher specimen (901) was deposited by Mr. Ganga Datt Bhatt, a Research Officer at the National Herbarium and Plant Laboratories located in Godawari, Lalitpur, Nepal.

4.3. Extraction and Isolation

The air dried powdered whole plant (10 kg) of *C. chaerophylla* was cold percolated with hexane (20 L). The hexane fraction was separated, and the plant residue was extracted with methanol using 15 L of solvent. The methanol extract was concentrated on the rotary evaporator under a vacuum, and 900 g methanol extract

was obtained. The methanol extract was stirred with a 7% citric acid and filtered. The filtrate was neutralized with NH_3 solution and extracted with chloroform. The chloroform extract (120 g) was subjected to column chromatography over a silica gel column (70 cm×10 cm) by using an ethyl acetate/hexane (0.5/95.5) solvent system. Then elution was obtained increasing the percentage of ethyl acetate/hexane (5/95) and (10/90). Fractions were checked using TLC and based on the detected spots three main fractions were obtained pooling the collected fractions namely fraction A (16 g), B (22 g) and C (24 g). After eluent system was changed using chloroform/methanol (90/10), and two fractions, D (9 g) and E (32 g), were obtained pooling the collected fractions. From fraction E significant spots were observed in TLC and was. subjected to silica gel column chromatography (20 cm×5 cm) using a dichloromethane (DCM)/methanol (95/5) solvent system with a few drops of NH_3 solution with increasing polarity. A total of 11 sets of fractions (E1-E11) were collected, which yielded four new compounds. Compound (**1**) (60 mg) from E9 fraction and compound (**2**) (30 mg) from E6 fraction yielded from the solvent system DCM/Methanol with a few drops of NH_3 solution in the ratio of 8:2. Similarly, compound (**3**) (600 mg) from E3 fraction and compound (**4**) (500 mg) from E4 fraction were yielded from 9:1 DCM/Methanol with few drops of NH_3 solution.

Chaeronepaline-A

3,12-Dimethoxy-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-2,9-diol (**1**) $C_{19}H_{18}NO_4$, dark red powder. $[\alpha]^D$ + 15.2(c 0.050, MeOH); UV max 228 274 350 444 nm; IR (KBr) νmax 3372 2922 1609 1513 1454 1277 1236 1214 cm^{-1};^{1}H-NMR (400 MHz, MeOD) and ^{13}C-NMR (100 MHz, $CDCl_3$) data, see Tables 1 and 2; HR-ESI-MS *m/z* 324.1238 $[M]^+$, calcd for $C_{19}H_{18}NO_4^+$,324.1236.

Chaeronepaline-B

7-Methyl-2,3:11,12-bis(methylenedioxy)-7,13a-secoberbin-13-14-epoxide (**2**) $C_{20}H_{20}NO_5$, white amorphous solid. $[\alpha]^D$−5.1 (c 0.050, MeOH); UV max 240 288 nm; IR (KBr) νmax 3416 2907 1612 1472 1362 1236 1037 cm^{-1} ;^{1}H-NMR (400 MHz, MeOD) and ^{13}C-NMR (100 MHz, $CDCl_3$) data, see Tables 1 and 2; HR-ESI-MS *m/z* 354.0980 $[M+H-2H]^+$, calcd for $C_{20}H_{20}NO_5$, 354.0977.

Chaeronepaline-C

7-methyl-5, 6, 7, 8- tetrahydro- 8H-spiro-9,14-dihydroxy-11,12-methylenedioxy-indane-isoquinoline (**3**) $C_{20}H_{20}NO_6$, yellow powder. $[\alpha]^D$ + 38.17 (c 0.050, MeOH); UV max 228 242 288 nm; IR (KBr) νmax 3416 2892 1620 1476 1391 1236 1037 cm^{-1};^{1}H-NMR (400 MHz, MeOD) and ^{13}C-NMR (100 MHz, $CDCl_3$) data, see Tables 1 and 2; HR-ESI-MS *m/z* 370.1289 $[M+H]^+$, calcd for $C_{20}H_{20}NO_6$, 370.1290.

Chaeronepaline-D

7-methyl-5, 6, 7, 8- tetrahydro- 8H-spiro-9,14-dihydroxy-11,12-methylenedioxy-indane-isoquinoline-N-oxide (**4**) $C_{20}H_{20}NO_7$, yellow powder. $[\alpha]^D$ + 58.7 (c 0.050, MeOH); UV max 224 242 290 nm; IR (KBr) νmax 3409 2922 2649 1620 1480 1380 1236 1037 cm^{-1};^{1}H-NMR (400 MHz, MeOD) and ^{13}C-NMR (100 MHz, $CDCl_3$) data, see Tables 1 and 2; HR-ESI-MS *m/z* 386.1240 $[M+H]^+$, calcd for $C_{20}H_{20}NO_7^+$, 386.1239.

Table 1. ^{13}C NMR Data for compounds **1**–**4** (δ in ppm, 100 MHz, in $MeOD_4$), *overlapped signals.

Position	1	2	3	4
1	111.1 CH	115.9 CH	109.1 CH	108.8 CH
2	146.6 C	148.7 C	145.3 C	144.9 C
3	149.3 C	148.8 C	147.4 C	147.4 C
4	110.0 CH	108.8 CH	108.2 CH	107.7 CH
4a	126.2 C	124.5 C	128.3 C	127.9 C
5	27.3 CH_2	24.6 CH_2	21.5 CH_2	21.5 CH_2
6	54.2 CH_2	53.7 CH_2	47.2 CH_2	47.0 CH_2
7	–	–	–	–
8	145.9 CH	55.0 CH_2	81.6 C	82.4 C
8a	120.1 C	127.8 C	120.0 C	118.1 C
9	162.0 C	122.9 CH	77.8 CH	77.4 CH
9a	–	–	120.3 C	120.8 C
10	107.9 CH	108.6 CH	108.7 CH	108.8 CH
11	123.3 CH	148.2 C	149.1 C	148.9 C
12	149.7 C	148.1 C	143.5 C	142.8 C
12a	132.4 C	124.0 C	–	–
13	117.9 CH	56.5 CH*	115.8 CH	115.1 CH
14	134.6 C	56.5 CH*	76.3 CH	75.9 CH
14a	120.2 C	127.8 C	135.9 C	135.1 C
15	55.6 CH_3	41.3 CH_3	100.7 CH_2	101.4 CH_2
16	55.2 CH_3	102.0 CH_2	101.7 CH_2	102.4 CH_2
17	–	102.0 CH_2	37.9 CH_3	36.9 CH_3

Table 2. ^{1}H-NMR Data for compounds **1–4** (δ in ppm, 400 MHz, in $MeOD_4$).

Position	1	2	3	4
1	7.39, s, 1H	7.14, s, 1H	6.30, s, 1H	6.30, s, 1H
2	–	–	–	–
3	–	–	–	–
4	6.93, s, 1H	6.79, s, 1H	6.68, s, 1H	6.71, s, 1H
4a	–	–	–	–
5	3.16, t, 2H, *J* = *6.2*	3.25, m, 2H	2.97, m, 2H	3.08, m, 2H
6	4.64, t, 2H, *J* = *6.2*	4.48, m, 2H	3.62 3.68, m, 2H	3.80 3.74, m, 2H
7	–	–	–	–
8	9.30, s, 1H	3.77 3.51, m, 2H	–	–
8a	–	–	–	–
9	–	6.86, d, 1H, *J* = *7.0*	5.34, s, 1H	5.39, s, 1H
9a	–	–	–	–
10	6.94, d, 1H, *J* = *8.0*	6.81, d, 1H, *J* = *7.0*	6.87, s, 1H	6.91, s, 1H
11	7.58, d, 1H, *J* = *8.0*	–	–	–
12	–	–	–	–
12a	–	–	–	–
13	8.03, s, 1H	3.85 m*	6.89, s, 1H	6.90, s, 1H
14	–	3.86 m*	5.53, s, 1H	5.58, s, 1H
14a	–	–	–	–
15	3.90, s, 3H, OCH_3	2.91, s, 3H	5.81, s, 2H	5.85, s, 2H
16	3.94, s, 3H, OCH_3	6.00, s, 2H	6.00, s, 2H	6.02, s, 2H
17	–	6.00, s, 2H	2.84, s, 3H	2.97, s, 3H

4.4. Reagents Used for *In Vitro* Experiments

Eagle's minimum essential medium (MEM), trypsin- EDTA, penicillin, streptomycin, sodium pyruvate, L-glutamine, nonessential amino acid solution and fetal bovine serum (FBS) were purchased from EuroClone (Milan, Italy) as well as all plastic supplies. *C. chaerophylla* compounds were diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to a final concentration of 0.08 μM. Simvastatin (Merck, Sharp, and Dohme Research Laboratories, Kenilworth, NJ, USA) was dissolved to a stock concentration of 50 mM in 0.1 M NaOH, and the pH was adjusted to 7.2 according to the manufacturer's instructions. The solution was then sterilised by filtration. Berberine chloride (cod. B3251, Sigma-Aldrich) was dissolved to a final concentration of 80 mM in DMSO.

4.5. Cell Cultures

Human hepatic cancer cells (Huh7 and HepG2) were cultured in MEM supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine 200 mM, 1% sodium pyruvate 100×, 1% nonessential amino acids 100×, and 1% penicillin/streptomycin solution (10,000 U/mL and 10 mg/mL, respectively), at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, cells were incubated with the indicated final concentrations in MEM/10% FBS. The final concentration of solvent (DMSO) did not exceed 0.5% v/v, and the same amount was added to all of the experimental points in each assay.

4.6. Cell Viability Assay

Cells were seeded in MEM/10% FBS in a 96-well tray at a cellular density of 8000 cells/well. The day after, treatments were added (four experimental points for each compound: 50 μM, 25 μM, 12.5 μM and 6.25 μM) for 72 h, after which the cell viability was evaluated by the sulforhodamine B assay (SRB) according to a previously published protocol.[18]

4.7. Western Blot Analysis

Huh7 cells were seeded in MEM/10% FBS in 6-well trays at the cellular density of 300,000 cells/well. The day after, the medium was replaced with the compounds at the indicated concentrations in DMEM/10% FBS. After 72 h of incubation, intracellular protein content was extracted in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 1% Nonidet-P40, containing 1% v/v of protease and phosphatase inhibitor cocktails). Protein samples (25 μg) and a molecular mass marker (Bio-Rad, Hercules, CA, USA) were separated using 4–20% SDS-PAGE (Bio-Rad) under denaturing and reducing conditions. The protein samples were then transferred to a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad), and nonspecific binding sites were blocked with a 5% non-fat dried milk tris-buffered tween 20 (TBS–T20) solution, with agitation for 60 min at room temperature. The blots were incubated overnight at 4 °C with a diluted solution (5% non-fat dried milk) of anti-LDLR (rabbit polyclonal antibody, GeneTex GTX132860; dilution 1:1000), anti-PCSK9 (rabbit polyclonal antibody, GeneTex GTX129859; dilution 1:1000), anti-GAPDH (rabbit polyclonal antibody, GeneTex GTX100118; dilution 1:10000). The membranes

were washed with TBS–T20 and exposed for 90 min at room temperature to a diluted solution (5% non-fat dried milk) of the secondary antibodies (peroxidase-conjugate goat anti-rabbit, Jackson ImmunoResearch, dilution 1:5000, cod. 111–036-045). Immunoreactive bands were detected by exposing the membranes to Clarity™ Western Enhanced Chemi Luminescence (ECL) chemiluminescent substrates (Bio-Rad) for 5 min, and images were acquired with an Uvitec Alliance Q9 (Uvitec, Cambridge, UK). The densitometric readings were evaluated using Image Lab™ software (Bio-Rad).

4.8. Cholesterol Biosynthesis Assay

HepG2 cells were seeded in MEM supplemented with 10% FBS in 12-well trays with a density of 1×10^6 cells/well. After 24 hours, cells were treated with the compounds at the indicated concentrations dissolved in DMEM supplemented with 0.2% (w/v) Bovine Serum Albumin (BSA; Merck, Darmstadt, Germany) for 48 hours. Subsequently, cells were incubated for an additional 24 hours with MEM supplemented with 2 μCi/mL $^{[2-14C]}$ acetate (Perkin-Elmer, MA, USA), 0.4% (v/v) FBS, 0.5% v/v of a solution of sodium acetate [8 mg/ml] together with the compounds, that were thus incubated for a total of 72 hours. Cell monolayers were lysed with 0.1 M NaOH overnight at 4 °C; 10^5 cpm/sample of $^{[1,2-3H]}$ cholesterol (Perkin-Elmer, MA, USA) were then added as an internal reference to each cell lysate and saponification was performed at 60 °C for 1 hour in alcoholic KOH. Lipid extraction was carried out using low-boiling point Petroleum Ether; thin-layer chromatography (TLC) was performed using petroleum ether 40°–60 °C/diethyl ether/acetic acid (70:30:1) as mobile phase to separate cholesterol from the other cellular sterols and to allow the quantification of radioactivity derived from the incorporation of $^{[2-14C]}$ acetate through liquid scintillation counting. Cellular cholesterol biosynthesis was expressed as cpm per milligram of protein for each cell lysate, measured through the bicinconic acid (BCA) assay (Thermofisher, MA, USA) following the manufacturer's instructions (PMID: 36293049).

4.9. Neutral Lipid Staining with Oil Red-O

To perform Oil Red-O staining, Huh7 cells were seeded 50,000 cells/well in 24-well plates with sterile microscope cover glasses 10 mm Ø (VWR international). After 24 hours, the medium was replaced by fresh MEM/10% FBS containing the described treatments. 72 hours later, cells were rinsed with PBS and fixed in 2% formaldehyde for 10 min and washed with PBS without calcium and magnesium. After a short rinse in 20% isopropanol, Oil Red-O (Sigma-Aldrich, Cod. O0625) solution was added. Oil Red-O stock solution is 0.5% w/v in isopropanol, diluted in distilled water 6:4 and filtered twice with 0.220 μm PVDF and PES filters prior to use. The staining is left for 20 minutes, then rinsed with 20% isopropanol and tap water respectively, for 1–2 min. Nuclei were stained with DAPI solution in PBS (Sigma-Aldrich, cod. D9542), then rinsed in tap water and twice in distilled water.

The slides or the coverslips were mounted with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, Cod. F4680). Images were obtained with Leica DMRE mounting Leica camera with Leica 541 517 HC zoom and Leica Application Suite X Software. Oil Red O-stained areas were quantified using ImageJ (v.1.52 h, NIH) and normalised with nuclei count.

4.10. Statistical Analysis

Data are expressed as mean ± standard deviation. Differences between the two groups were analysed via Student's t-test analysis (GraphPad, San Diego, CA, USA). P-values lower than 0.05 were considered statistically significant.

Author Contributions

Conceptualisation: B.M., R.L.S., S.S., S.D.A., N.F.; Data curation: B.M., I.R., V.B., S.S., N.F.; Formal analysis: B.M., R.L.S., I.R., V.B., S.S., S.D.A., B.P., M.P.A.; Funding acquisition: R.L.S., N.F., S.D.A.; Investigation and methodology: I.R., V.B., S.S. ; Project administration: R.L.S., N.F., S.D.A.; Supervision: S.D.A, N.F., R.L.S.; Visualisation: B.M., I.R., V.B., S.S.S.; Roles/Writing - original draft: S.S., B.M., R.L.S., S.D.A; Writing - review & editing: B.M., S.S.S., S.S., S.D.A., B.P., M.P.A., I.R., R.L.S., L.K.S., J.P.H., K.A.

Acknowledgements

B. Maharjan acknowledges University Grants Commission (UGC), Sanothimi, Bhaktapur, Nepal, for awarding research grants under PhD Fellowship and Research Support (PhD-75/76-S&T-6) Open Access publishing facilitated by Università degli Studi di Padova, as part of the Wiley - CRUI-CARE agreement.

Conflict of Interests

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available on request.

Keywords: Isoquinoline alkaloids · *Corydalis chaerophylla* D.C. · PCSK9 · LDL–R

[1] W. Majak, Y. Bai, M. H. Benn, *Biochem. Syst. Ecol.* **2003**, *31*, 649–651.
[2] H. L. Li, W. D. Zhang, R. H. Liu, C. Zhang, T. Han, X. W. Wang, X. L. Wang, J. B. Zhu, C. L. Chen, *J. Chromatogr. B* **2006**, *831*, 140–146.
[3] Z. Z. Ma, W. Xu, N. H. Jensen, B. L. Roth, L. Y. Liu-Chen, D. Y. W. Lee, *Molecules* **2008**, *13*, 2303–2312.
[4] M. Iranshahy, R. J. Quinn, M. Iranshahi, *RSC Adv.* **2014**, *4*, 15900–15913.
[5] S. Bhambhani, K. R. Kondhare, A. P. Giri, *Molecules* **2021**, *26*, DOI: 10.3390/molecules26113374.
[6] P. Wangchuk, P. A. Keller, S. G. Pyne, T. Sastraruji, M. Taweechotipatr, R. Rattanajak, A. Tonsomboon, S. Kamchonwongpaisan, *Nat. Prod. Commun.* **2012**, *7*, 575–580.
[7] L. M. M. Marques, D. R. Callejon, L. G. Pinto, M. L. de Campos, A. R. M. de Oliveira, R. Vessecchi, A. Adhikari, R. L. S. Shrestha, R. G. Peccinini, N. P. Lopes, *J. Pharm. Biomed. Anal.* **2016**, *131*, 464–472.
[8] A. Jahan, S. Shams, S. Ali, S. Samrana, A. Ali, A. Adhikari, M. Sajid, A. Ali, H. Ali, *ACS Omega* **2021**, *6*, 2462–2472.
[9] R. L. Shrestha, A. Adhikari, B. P. Marasini, R. N. Jha, M. I. Choudhary, *Phytochem. Lett.* **2013**, *6*, 228–231.
[10] D. R. Callejon, T. B. Riul, L. G. P. Feitosa, T. Guaratini, D. B. Silva, A. Adhikari, R. L. S. Shrestha, L. M. M. Marques, M. D. Baruffi, J. L. C. Lopes, N. P. Lopes, *Molecules* **2014**, *19*, 5692–5703.
[11] N. Muhammad, R. L. Shrestha, A. Adhikari, A. Wadood, H. Khan, A. Z. Khan, F. Maione, N. Mascolo, V. De Feo, *Nat. Prod. Res.* **2015**, *29*, 430–437.

[12] N. Sivakumaran, S. R. Samarakoon, A. Adhikari, M. K. Ediriweera, K. H. Tennekoon, N. Malavige, I. Thabrew, R. L. S. Shrestha, *Biomed Res. Int.* **2018**, *2018*, DOI: 10.1155/2018/3171348.
[13] L. M. M. Marques, M. Behrens, S. Kalinina, U. Rottkord, A. Adhikari, R. L. S. Shrestha, H. U. Humpf, N. P. Lopes, *Rev. Bras. Farmacogn.* **2020**, *30*, 374–380.
[14] B. Maharjan, D. T. Payne, I. Ferrarese, M. G. Lupo, L. K. Shrestha, J. P. Hill, K. Ariga, I. Rossi, S. S. Shrestha, G. Panighel, R. L. S. Shrestha, S. Sut, N. Ferri, S. Dall'Acqua, *Bioorg. Chem.* **2022**, *121*, 105686.
[15] N. P. Manandhar, *Contrib. Nepal. Stud. J.* **1993**, *20*, 184–196.
[16] R. N. Jha, M. B. Pandey, A. K. Singh, S. Singh, V. P. Singh, *Nat. Prod. Res.* **2009**, *23*, 250–255.
[17] B. Maharjan, L. K. Shrestha, J. P. Hill, K. Ariga, S. S. Shrestha, S. Sut, R. L. S. Shrestha, S. Dall'Acqua, *Chem. Biodivers.* **2023**, *20*, DOI: 10.1002/cbdv.202301209.
[18] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. Mcmahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, *J. Natl. Cancer Inst.* **1990**, *82*, DOI: 10.1093/jnci/82.13.1107.
[19] C. Simoes-Pires, K. Hostettmann, A. Haouala, M. Cuendet, J. Falquet, B. Graz, P. Christen, *Int. J. Parasitol. Drugs Drug Resist.* **2014**, *4*, 338–346.
[20] F. A. L. Anet, M. A. Brown, *Tetrahedron Lett.* **1967**, *48*, 4881–4884.
[21] M. E. Popova, A. N. Boeva, L. Dolejs, *Planta Med.* **1980**, *40*, 156–160.
[22] R. H. F. Manske, R. G. A. Rodrigo, D. B. Maclean, D. E. F. Gracey, J. K. Saunders, *Can. J. Chem.* **1969**, *47*, 3589–3592.
[23] M. Shamma, J. L. Moniot, R. H. F. Manske, W. K. Chan, K. Nakanishi, *J. Chem. Soc. Chem. Commun.* **1972**, *6*, 310–311.
[24] H. J. Shim, J. Y. Lee, B. Kim, J. Hong, *Mass Spectrom. Lett.* **2013**, *4*, 79–82.
[25] M. G. Lupo, C. Macchi, S. Marchianò, R. Cristofani, M. F. Greco, S. Dall'Acqua, H. Chen, C. R. Sirtori, A. Corsini, M. Ruscica, N. Ferri, *Nutr. Metab. Cardiovasc. Dis.* **2019**, *29*, DOI: 10.1016/j.numecd.2019.06.001.
[26] J. Cameron, T. Ranheim, M. A. Kulseth, T. P. Leren, K. E. Berge, *Atherosclerosis* **2008**, *201*, 266–273.

Manuscript received: June 4, 2024
Accepted manuscript online: July 27, 2024
Version of record online: October 28, 2024