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Previously undisclosed bioactive sterols from corbiculid bivalve clam *Villorita cyprinoides* with anti-inflammatory and antioxidant potentials

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ABSTRACT

The estuarine Corbiculid bivalve black clam, *Villorita cyprinoides* collected from the Southwestern coastline regions of Arabian Sea are significant resources of nutritional and bioactive pluralities. The purification of ethyl acetate:methanol (EtOAc:MeOH) extract of *V. cyprinoides* characterized a previously undisclosed specialized abeo-pregnane-type sterol derivative 19 (10 → 5) abeo-20-methyl-pregn-10-en-3β-yl-hex-(3′*E*)-enoate (**1**) along with two cholestenols (22*E*),(24^1*E*)-24^1,24^2-dihomocholesta-5,22,24^1-trien-3β-ol (**2**) and (22*E*)-24^1-homocholesta-5,22-dien-(3β,24$^1\beta$)-diol (**3**). These compounds were characterized by comprehensive spectroscopic investigations. The anti-inflammatory (anti-cyclooxygenase-1, 2/5-lipoxidase) activities of **1** were considerably higher (IC_{50} < 1.10 mg/mL) than **2–3** (IC_{50} > 1.10 mg/mL). These studied compounds registered greater selectivity indices (~1.03) against cyclooxygenase-2 than cyclooxygenase-1. The antioxidant property of abeo-pregnane-type sterol as determined by *in vitro* 2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) quenching potential was significantly greater (IC_{50} 0.94 mg/mL) than those of substituted dihomocholesta-trien-ol (**2**) and homocholesta-dien-diol (**3**) (IC_{50} > 1.00 mg/mL). Structure-activity relationship studies demonstrated that bioactive potentials of the titled compounds were linearly related to their electronic factors along with optimum hydrophobic factors. In addition, molecular docking studies were performed in the active sites of COX-2 and their binding energies and docking scores were well correlated with *in vitro* anti-COX-2 potentials.

1. Introduction

Marine bivalve mollusks of the genus *Villorita* (family: Corbiculidae), known as black clams, are soft-shelled and filter-feeding organisms, which were reported for their antioxidative and anti-inflammatory bioactive potentials [1]. Even though, they are abundant and easily available seafood items, only few publications were available about their bioactive and pharmaceutical pluralities [2]. The isolation and characterization of secondary metabolites responsible for the bioactive potentials from *Villorita* spp are limited that attracted our group to work on Corbiculid *V. cyprinoides*, one of the prominent members of this group of bivalve black clam. The secondary metabolites identified from bivalve mollusks were regarded as pharmacophore leads against inflammatory disorders and oxidative damages [3,4].

Sterols are bioactive secondary metabolites and major constituents in mollusks, such as bivalves, gastropods, cephalopods etc. [5–8]. The oxygenated [9] and polyhydroxylated sterols [10] with bioactive potentials were previously reported. The isolation and characterization of pregnane-type steroids were described in the earlier reports [11,12]. As a part of our investigation towards the isolation of bioactives from marine bivalve mollusks, the crude ethyl acetate:methanol (EtOAc:MeOH) concentrate of *V. cyprinoides* (family Corbiculidae), collected from the Arabian Sea, were screened for its bio-potentials. Herein, we have reported the identification and characterization of previously undisclosed sterol derivatives possessing 19 (10 → 5) *abeo*-20-methyl-pregn-10-en-3β-yl-hex-(3′*E*)-enoate, dihomocholesta-5,22,24^1-trien-ol and homocholesta-5,22-dien-(3,24^1)-diol frameworks. The structures of titled compounds were assigned with the help of mass analyses along with comprehensive nuclear magnetic resonance (NMR) including DEPT (distortionless enhancement by polarization transfer), ^{1}H–^{1}H COSY (homonuclear correlation spectroscopy), HSQC (heteronuclear single-quantum correlation), HMBC (heteronuclear multiple bond correlation), and nuclear overhauser effect (NOE) spectroscopy experiments. The compounds were assessed for their anti-inflammatory {cyclooxygenases-1, 2 (COX-1, 2)/5-lipoxidase (5-LOX) inhibitory properties} and antioxidative {2,2-diphenyl-1-picrylhydrazyl (DPPH)/2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^+$) scavenging activities} properties through various *in vitro* models. Various

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molecular descriptor variables of titled compounds utilized towards structure-bioactivity correlation analyses. The modes of COX-2 enzyme inhibitions by the tilted compounds were determined by the molecular docking models.

2. Materials and methods

2.1. General procedures

The UV spectra were acquired on UV–VIS spectrometer (Varian Cary 50, USA) and Perkin-Elmer Series 2000 FTIR spectrophotometer recorded FTIR (Fourier transform infrared) spectra (scan range of 4000 and 400 cm^{-1}). The NMR spectroscopic analyses (one and two dimensional) were documented on Bruker Avance DPX 500 (500 MHz) in $CDCl_3$ with tetramethylsilane (TMS). The gas-chromatographic mass-spectroscopic (GC–MS) experiments were executed by EI (electronic impact) ionization method (GC–MS Perkin-Elmer Clarus-680), and components were fractionated with non-polar capillary column (Elite-5 bonded phase, 50 m X 0.22 mm i.d. X 0.25 μm film thicknesses). The ESIMS spectra was recorded on a liquid chromatography-mass spectrometry (Applied Biosystems QTrap 2000, Applied Biosystems, Darmstadt, Germany) system in positive and negative modes with turbo ion spray voltage, curtain gas, turbo temperature and nebulizer gas of −4500 V, 30 psi, 500 °C and 50 psi (positive mode), respectively at a flow rate of 1.5 mL/min. All the chemicals/reagents were used in the present study of analytical or spectroscopic solvent grade purchased from Sigma-Aldrich (Missouri, USA) and E-Merck (Darmstadt, Germany). Silica gel (60–120 mesh, E-Merck, Germany; 230–400 mesh, Biotage, Sweden) have used for various column chromatography techniques and precoated plates of GF_{254} were utilized for thin layer chromatographic separation (TLC). The analytical high pressure liquid chromatograph (HPLC) instrument (Shimadzu Corporation, Nakagyo-ku, Japan) connected to RP-C_{18} (bonded reverse-phase; Phenomenex, Torrance, USA; Luna 250 X 4.6 mm, 5 μm) fitted with a binary gradient pump (Shimadzu LC-20AD) column and photodiode array detector (SPD-M20A, Kyoto, Japan) was used to document the purity of isolated compounds. The melting point apparatus, VMP-DS, Veego (Mumbai, India) was used to calculate melting points of studied compounds. The polarimeter, AP-300, ATAGO (Japan), was used to record the angle of rotation [7].

2.2. Sample collection, pre-treatment and extraction

Villorita cyprinoides (10 kg) were freshly collected from the Vembanad Lake (9°35′ N and 76°25′ E), situated along the southwest coast of Arabian Sea and a voucher with specimen number ICAR/CRP-HF/AC 374 was deposited in repository of Indian Council of Agricultural Research funded Consortium Research Platform on Health Food. The edible meat (6 kg) taken from fresh shell-on samples were grinded and freeze dried by lyophilization (Martin Christ alpha 1–4 LD Plus freeze-drier, Germany). The lyophilized powder (1100 g, yield 18.3%) was homogenized in EtOAc:MeOH (1:1, v/v, 750 mL X 3, 40 °C) by sonication (8 h) under the inert atmosphere of N_2. The solvent portion was filtered through anhydrous Na_2SO_4, before being evaporation *in vacuo* by rotary vacuum evaporator (50 °C; Heidolf, Germany) to yield brown colored crude extract of *V. cyprinoides* (50.0 g, yield on dry weight basis 4.54%) [1].

2.3. Isolation and spectroscopic analyses

The crude extract of *V. cyprinoides* (42.0 g) was partitioned by repeated column chromatography. The column was primarily eluted with 100% *n*-hexane followed by dichloromethane (DCM, 100%), ethyl acetate (EtOAc, 100%) and methanol (MeOH, 100%) to obtain a total of 4 column fractions, such as VC_1, VC_2, VC_3 and VC_4, respectively. The bioactivities of the fractions were checked, and based on the results two fractions (VC_2 and VC_3) were selected for further purifications as it exhibited reasonably greater antioxidant and anti-inflammatory activities (IC_{50} 0.93–1.10 mg/mL) compared to other fractions (> 1.15 mg/mL for VC_1 and VC_4) (Table S1). The fraction, VC_2 (13 g) obtained by eluting with 100% DCM was partitioned by vacuum liquid chromatography on glass column (450 mm × 30 mm) packed with silica (230–400 mesh) using *n*-hexane/EtOAc/MeOH to acquire 25 fractions of 25 mL each, which reduced to 9 fractions ($VC_{2\text{-}1}$–$VC_{2\text{-}9}$) after TLC (*n*-hexane:EtOAc, 9:1, v/v) analysis. The fraction, $VC_{2\text{-}4}$ (1.30 g) eluted at 10% EtOAc:*n*-hexane was found to be mixture, which further purified to homogeneity by preparative TLC to yield compound **2** (195 mg, *n*-hexane:EtOAc, 8:2, v/v). Another fraction, $VC_{2\text{-}5}$ (12% EtOAc:*n*-hexane) was found to be mixture, and therefore, sub-fractionated on preparative TLC to yield compound **3** (134 mg, *n*-hexane:EtOAc, 8:2, v/v). The solvents were evaporated from the isolate, and the TLC analysis over silica gel (GF_{254}, 15 mm; *n*-hexane:EtOAc, 95:5, v/v) registered its purity.

Another active fraction, VC_3 (12 g) was eluted at 100% EtOAc, on further purification using *n*-hexane/EtOAc/MeOH on flash chromatography (Biotage AB SP1-B1A, 230–400 mesh, 12 g; Biotage AB, Uppsala, Sweden) on silica gel column at collection UV wavelength of 264 nm. The solvent polarity was initiated from 100% *n*-hexane followed by EtOAc and MeOH to afford 85 portions (12 mL each) and pooled to 7 fractions ($VC_{3\text{-}1}$–$VC_{3\text{-}7}$). The active fraction $VC_{3\text{-}6}$ (2.6 g) was chromatographically fractionated by PTLC (15% EtOAc:*n*-hexane) to achieve three sub-fractions, $VC_{3\text{-}6\text{-}1}$–$VC_{3\text{-}6\text{-}3}$. The fractions, $VC_{3\text{-}6\text{-}1}$ (840 mg) was purified by repetitive RP-C_{18} HPLC (MeOH/MeCN, 6:4 v/v X 3) to afford compound **1** (143 mg). The compound was checked for its purity using TLC {*n*-hexane:EtOAc (90:10, v/v)} and RP-HPLC (MeOH/MeCN, 8:2).

2.3.1. 19 (10 → 5) Abeo-20-methyl-pregn-10-en-3β-yl-hex-(3′E)-enoate (1)

White solid; m.p. 138.2 °C (decom.); $[\alpha]_D^{26}$ −20.5° ($CHCl_3$, c0.011); UV (MeOH) λ_{max}(log ε 3.61): 222 nm; TLC (Si gel GF_{254} 15 mm; EtOAc/*n*-hexane, 10:90, v/v) R_f: 0.42; R_t (RP-C_{18} HPLC, MeOH:MeCN, 8:2, v/v): 3.18 min.; IR (cm^{-1}) (stretching ν, bending δ, rocking ρ): 2923, 2855 (C−Hν), 1719 (C=Oν), 1457 (C−Hδ), 1375 (C−Hρ), 1167 (C−Cν), 1050 (C−Oν), 973 (=C−Hδ), 722, 655 (C−Hδ). ^1H ($CDCl_3$, 500 MHz, ppm), ^{13}C ($CDCl_3$, 125 MHz, ppm), COSY and HMBC data were given in Table 1. HRESIMS (High-resolution electro-spray ionization mass spectrometry): found *m/z* 413.3426 $[M+1]^+$, cal. for $C_{28}H_{45}O_2$ 413.3420 (Δ 1.5 ppm).

2.3.2. (22E),(24¹E)-24¹,24²-Dihomocholesta-5,22,24¹-trien-3β-ol (2)

White solid; m.p. 140.4 °C (decom.); $[\alpha]_D^{26}$ −17.3° ($CHCl_3$, c0.016); UV (MeOH) λ_{max}(log ε 3.32): 220 nm; TLC (Si gel GF_{254} 15 mm; EtOAc/*n*-hexane, 20:80, v/v) R_f: 0.58; R_t (RP-C_{18} HPLC, MeOH:MeCN, 3:2, v/v): 8.99 min.; IR (cm^{-1}): 3366 (br O−Hν), 2934, 2894 (C−Hν), 1663 (C=Cν), 1459 (C−Hδ), 1329 (C−Hρ), 1242, 1133 (C−Cν), 1044 (C−Oν), 963, 836, 801 (=C−Hδ), 737 (C−Hδ). ^1H ($CDCl_3$, 500 MHz, ppm), ^{13}C ($CDCl_3$, 125 MHz, ppm), COSY and HMBC data were given in Table 1. HRESIMS: found *m/z* 410.3555 $[M]^+$, cal. for $C_{29}H_{46}O$ 410.3549 (Δ 1.5 ppm).

2.3.3. (22E)-24¹-Homocholesta-5,22-dien-(3β,24¹β)-diol (3)

White solid; m.p. 139.1 °C (decom.); $[\alpha]_D^{26}$ −18.3° ($CHCl_3$, c0.014); UV (MeOH) λ_{max}(log ε): 221 (3.45); TLC (Si gel GF_{254} 15 mm; EtOAc/*n*-hexane 5:95, v/v) R_f: 0.54; R_t (RP-C_{18} HPLC, MeOH:MeCN 3:2, v/v): 6.00 min.; IR (cm^{-1}): 3366 (br O−Hν), 2934, 2865 (C−Hν), 1653 (C=Cν), 1459 (C−Hδ), 1327 (C−Hρ), 1242, 1191 (C−Cν), 1107 (C−Oν), 928, 881, 801 (=C−Hδ), 737, 625 (C−Hδ). ^1H ($CDCl_3$, 500 MHz, ppm), ^{13}C ($CDCl_3$, 125 MHz, ppm), COSY and HMBC data were given in Table 1. HRESIMS: found *m/z* 414.3504 $[M]^+$, cal. for $C_{28}H_{46}O_2$ 414.3498 (Δ 1.4 ppm).