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Specialized oxygenated heterocyclics from *Villorita cyprinoides* with cyclooxygenase-2 and 5-lipoxygenase inhibitory properties

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ABSTRACT

Villorita cyprinoides is traditional seafood in the coastal regions of Arabian Sea. Bioactivity-guided purification of ethyl acetate:methanol extract of *V. cyprinoides* resulted in the identification of two O-spirocyclic ether derivatives (**1–2**) along with one O-heterocyclic irregular meroterpenoid (**3**). The structures and their relative stereochemistries were elucidated through comprehensive spectroscopic experiments. These specialized metabolites were found to exhibit potential antioxidative (IC_{50} < 0.70 mg/mL) and anti-inflammatory activities against pro-inflammatory inducible 5-lipoxygenase (anti-5-LOX IC_{50} ≤ 0.80 mg/mL) and cyclooxygenase-2 (anti-COX-2 IC_{50} < 0.75 mg/mL) enzymes. Molecular docking simulations were used to describe the interactions of the isolated compounds (ligands) with COX-2 and 5-LOX inflammation model. The permissible hydrophobic-hydrophilic balance and lesser steric bulk of spirocyclic ether derivative (compound **2**), along with greater number of hydrogen bonding interactions in the active sites of COX-2 and 5-LOX manifested towards its greater bioactivities compared to other compounds isolated from *V. cyprinoides*.

1. Introduction

The marine organisms are rich sources of structurally different biofunctional specialized metabolites with potential therapeutic properties and their significance as novel bioactive components are a rapidly emerging subject (Fung, Hamid, & Lu, 2013; Lin-Rui, Qing, Zhen-Xing, Yu-Chao, & Lin, 2012). About 90% of the bivalve clam aquaculture at the coastal regions of Arabian Sea reported to comprise one of the main productive marine bivalve fisheries sectors of the Southeast Asia. Notably, these species were considered as undervalued by-catch fishery resources in various parts of the world (Murray & Burt, 2001). The bivalve mollusks were reported as low-valued sources of protein, polyunsaturated fatty acids, minerals along with different bioactive metabolites with antioxidant, anti-inflammatory and anti-diabetic potentials (Joy & Chakraborty, 2017a; Joy, Chakraborty, & Pananghat, 2016; Luan, Wang, Wu, Jin, & Ji, 2011). Therefore, the bioactive secondary metabolites could be considered as popular pharmacophores against inflammations and oxidative stress.

The mollusks were reported for various secondary metabolites classified under different classes, such as spirocyclics, oxygenated heterocyclics, terpenoids, pyranoids and polyketides with promising biological functions (Blunt, Copp, Keyzers, Munro, & Prinsep, 2016; Joy & Chakraborty, 2017b). The compounds with O-heterocyclic-spiro and bicyclic (fused bicyclic lactones) functionalities were found to be significant bioactive agents among various classes of organic compounds (Zheng, Tice, & Singh, 2014). Spiro compounds enclosed two rings (identical or different rings), which have been shared with one atom (the quaternary spiroatom). Conformation of ligands (compounds) has been rigidified by the introduction of ring framework, whereas the cyclic derivatives appeared to experience a reduced conformational stress while binding to a target site (enzymes or proteins). A search for newer compounds with O-heterocyclic-spiro or fused systems as principle bioactive domains along with lower lipophilic (log P_{ow} < 5) factors and greater electronegative functionalities in the pharmacophore templates appeared to be vital for bioactive potentials and bioavailability. Spirocyclic and bicyclic analogues reported to be present in various natural products (Blunt et al., 2016; Jin et al., 2016). The spirocyclic ether compounds possessing unrearranged monocyclofarnesane skeleton were found in mollusk, *Aplysia dactylomela* (Schmitz, McDonald, & Vanderahl, 1978).

The black clam, *Villorita cyprinoides* Gray (1825) is a common seafood item among the coastal populations of South-western coast of India (Joy & Chakraborty, 2017a). As a part of investigation of bioactive secondary metabolites from commercially significant bivalve

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clams, the ethyl acetate:methanol (EtOAc:MeOH) extract of *V. cyprinoides* was fractionated by repetitive chromatographic methods. Herein, we report the identification and characterization of two spirocyclic ether derivatives (**1–2**) and one O-heterocyclic irregular meroterpenoid (**3**) from this species, for the first time. These oxygenated heterocyclics were characterized using various spectroscopic methods, such as Fourier transform infrared (FTIR), mass and nuclear magnetic resonance (NMR) experiments. *In vitro* inhibitory properties of the oxygenated heterocyclic analogues isolated in the present study towards pro-inflammatory cyclooxygenases, 5-lipoxygenase and free radical inhibition activities by 2,2-diphenyl-1-picrylhydrazyl along with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays determined their anti-inflammatory and antioxidant potentials, respectively. Structure-activity correlations of the titled compounds were analyzed by various physico-chemical attributes (hydrophobic/electronic/steric parameters). The radical scavenging mechanism of oxygenated heterocyclic analogues (**1–3**) in DPPH radical model was proposed to correlate their antioxidant potentials. The modes of inhibition of pro-inflammatory (COX-2 and 5-LOX) enzymes by these compounds were determined by molecular docking simulations.

2. Materials and methods

2.1. Sample collection, pre-treatment and extraction

The samples of *V. cyprinoides* (10 kg) were freshly collected from Vembanad Lake, located along the south-western coastline of Arabian Sea (9°35′ N and 76°25′ E) and a voucher with specimen number ICAR/CRP-HF/AC-374 was deposited in repository of Indian Council of Agricultural Research Consortium Research Platform on Health Food. The edible meat (6 kg) from fresh shell-on samples were grinded to homogeneity before being lyophilized (Martin Christ alpha 1–4 LD Plus, Germany). Lyophilized powder (1100 g, yield 18.3%) was homogenized in EtOAc:MeOH (1:1, v/v, 750 mL × 3) through sonication (8 h) under the N_2 atmosphere. The solvent portion was filtered through anhydrous Na_2SO_4 followed by evaporation *in vacuo* by rotary vacuum evaporator (55 °C; Heidolf, Germany) to yield a brown colored crude extract of *V. cyprinoides* (50.0 g, yield 4.54% on dry basis).

2.2. Isolation and spectroscopic analysis

The extract of *V. cyprinoides* (45.0 g) was repeatedly partitioned by exhaustive column chromatography. The extract was slurried through silica (60–120 mesh, 4.2 g) and packed into a column (1000 mm × 40 mm) filled with silica gel (60–120 mesh, 50 g). 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 these fractions were checked and two fractions (VC_2 and VC_3) were selected for further purifications due to their greater antioxidant and anti-inflammatory activities (IC_{50} 0.93–1.10 mg/mL) compared to other fractions (IC_{50} > 1.15 mg/mL for VC_1 and VC_4).

The fraction, VC_2 (8 g; 17.78%) was fractionated by vacuum liquid chromatography on a column (450 mm × 30 mm) filled with silica (230–400 mesh) using *n*-hexane/EtOAc/MeOH to yield 25 fractions (25 mL each), which were reduced to 9 groups ($VC_{2\text{-}1}$-$VC_{2\text{-}9}$) after TLC (9:1, v/v, *n*-hexane:EtOAc) analysis. The fraction, $VC_{2\text{-}6}$ (15% EtOAc:*n*-hexane; 1323 mg; 2.94%) registered greater bioactive properties compared to other fractions, and therefore, was selected for further fractionation. The fraction, $VC_{2\text{-}6}$ was flash chromatographed (Biotage AB SP1-B1A, 25 + M, 230–400 mesh; Biotage AB, Uppsala, Sweden) at collection UV wavelength of 250 nm with *n*-hexane/EtOAc/MeOH solvent system to afford 52 fractions (8 mL each) before being pooled to six fractions ($VC_{2\text{-}6\text{-}1}$-$VC_{2\text{-}6\text{-}6}$). The bioactive fraction, $VC_{2\text{-}6\text{-}2}$ (205 mg; 0.46%) on repeated column chromatography followed by PTLC (4% EtOAc:*n*-hexane) purifications afforded **3** (79 mg; 0.18%) and its purity was checked by TLC (9:1, *n*-hexane:EtOAc).

The fraction, VC_3 (11 g; 24.44%) eluted at 100% EtOAc was fractionated with *n*-hexane/EtOAc/MeOH on flash chromatography (230–400 mesh, 25 + M) using a silica gel column $\lambda_{collection}$ 264 nm to furnish a total of 85 fractions (12 mL each). The latter were pooled to 6 sub-fractions ($VC_{3\text{-}1}$-$VC_{3\text{-}6}$) after TLC experiment (*n*-hexane:EtOAc, 9:1, v/v). The active sub-fraction, $VC_{3\text{-}6}$ (1540 mg) was separated by flash (230–400 mesh, 12 + M) chromatography on silica column at 252 nm ($\lambda_{collection}$) with *n*-hexane/EtOAc/MeOH solvent system to yield three sub-fractions ($VC_{3\text{-}6\text{-}1}$-$VC_{3\text{-}6\text{-}3}$). The active fraction, $VC_{3\text{-}6\text{-}1}$ (546 mg), on PTLC using 2% EtOAc:*n*-hexane solvent system resulted compound **1** (147 mg) and its homogeneity was determined by TLC analyses {*n*-hexane:EtOAc (90:10, v/v)}. Another fraction, $VC_{3\text{-}6\text{-}2}$ (489 mg) on PTLC (5% EtOAc:*n*-hexane) fractionation yielded **2** (96 mg) that was found to be homogenous after TLC {*n*-hexane:EtOAc (90:10, v/v)}.

2.2.1. 16-Hydroxyhexyl-(2-ethyl-2,6-dimethyl-1-oxaspiro[4.5]dec-3,8-dien)-10-propanoate (1)

Yellow oily; UV (MeOH; UV–VIS spectrophotometer, Varian Cary 50, Creek, USA) λ_{max} (log ε 2.55): 231 nm; TLC (Silica GF_{254} 15 mm; 10:90, v/v, EtOAc/*n*-hexane) R_f: 0.63; R_t (HPLC, Shimadzu Corporation, Nakagyo-ku, Japan; RP-C_{18} Luna 250 × 4.6 mm, 5 μm; Phenomenex, USA, 2:3, v/v, MeOH:MeCN): 5.33; IR (cm^{-1}; FTIR Perkin-Elmer Series 2000): 3411 (br, O—Hν), 2924 (C—Hν), 1731 (C═Oν), 1460 (C—Hδ), 1376 (C—Hρ), 1167 (C—Cν), 1045 (C—Oν), 972 (═C—Hδ), 721 (C═Hδ). ^{1}H, ^{13}C, ^{1}H—^{1}H COSY and HMBC (Table 1; Bruker Avance DPX 500 NMR spectrometer, 500 MHz). HRESIMS (High resolution electrospray ionization mass spectrometry; Applied Biosystems QTrap 2000, Applied Biosystems, Darmstadt, Germany): found *m/z* 364.2620 $[M]^+$, cal. for $C_{22}H_{36}O_4$ 364.2614 (Δ 1.6 ppm).

2.2.2. (E)-18-Ethyl-17,19-dihydroxyhept-14-enyl-(2-ethyl-2,6-dimethyl-1-oxaspiro[4.5]dec-3,8-dien)-10-acetate (2)

Yellow oily; UV (MeOH) λ_{max} (log ε 2.17): 236 nm; TLC (Silica GF_{254} 15 mm; 10:90, v/v, EtOAc/*n*-hexane) R_f: 0.51; R_t (RP-C_{18} HPLC, 2:3, v/v, MeOH:MeCN): 3.28; IR (cm^{-1}): 3389 (br, O—Hν), 2923 (C—Hν), 1728 (C═Oν), 1459 (C—Hδ), 1375 (C—Hρ), 1042 (C—Oν), 971 (═C—Hδ), 730 (C—Hδ). ^{1}H, ^{13}C, ^{1}H—^{1}H COSY and HMBC (Table 1). HRESIMS: found *m/z* 406.2725 $[M]^+$, cal. for $C_{24}H_{38}O_5$ 406.2719 (Δ 1.5 ppm).

2.2.3. 8-(1,3,3a,4,5,7a-Hexahydro-1-(hydroxymethyl)-3-oxoisobenzofuran-4-yl)ethyl-pentanoate (3)

Dark brown oily; UV (MeOH) λ_{max} (log ε 2.76): 235 nm; TLC (Silica GF_{254} 15 mm; 1:9, v/v, EtOAc/*n*-hexane) R_f: 0.81; R_t (RP-C_{18} HPLC, 2:3, v/v, MeOH:MeCN): 3.03; IR (cm^{-1}): 2984, 2935 (C—Hν), 1737 (C═Oν), 1446 (C—Hδ), 1372, (C—Hρ), 1233, 1043 (C—Oν), 917 (═C—Hδ), 786 (C—Hδ). ^{1}H, ^{13}C, ^{1}H—^{1}H COSY and HMBC (Table 1). HRESIMS *m/z*: 296.1630 $[M]^+$, cal. for $C_{16}H_{24}O_5$ 296.1624 (Δ 2.0 ppm).

2.3. Antioxidative and anti-inflammatory assays

2.3.1. Antioxidative assays

The antioxidative activities were determined by *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH)(Chew, Lim, Omar, & Khoo, 2008) and $ABTS^+$ {2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)} (Joy et al., 2016) radical quenching experiments. The absorbance was recorded against a reagent blank (MeOH) at 517 nm. The $ABTS^+$ reagent was prepared by mixing $ABTS^+$ (7 mM) and potassium persulfate (2.45 mM). The appropriately diluted $ABTS^+$ (5 mL) solution was mixed with 0.1 mL of test samples and the absorbances were recorded at 734 nm.