New sterols with anti-inflammatory potentials against cyclooxygenase-2 and 5-lipoxygenase from *Paphia malabarica*

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**ABSTRACT**

Marine bivalves occupy a leading share in the total edible molluscs at the coastline regions of south-eastern Asia, and are found to possess significant nutritional and biological potential. Various *in vitro* evaluation (antioxidant and anti-inflammatory) guided purification of ethyl acetate–methanol (EtOAc–MeOH) extract of bivalve clam, *Paphia malabarica* characterised two new sterol derivatives as 23-*gem*-dimethylcholesta-5-en-3β-ol (1) and (22E)-241,242-methyldihomocholest-5,22-dien-3β-ol (2) collected from the south-west coast of Arabian Sea. Their structures were unambiguously assigned on the basis of 1D, 2D NMR spectroscopy and mass spectrometry. The antioxidant and anti-inflammatory activities of 2 as determined by DPPH/ABTS*⁺* radical scavenging and anticyclooxygenase-2/5-lipoxygenase assays were significantly greater (IC₅₀ < 1 mg/mL) than 1 (IC₅₀ > 1 mg/mL). Structure–activity relationship analysis revealed that the bioactivities of these compounds were directly proportional to the electronic and lipophilic parameters. This is the first report of the occurrence and characterisation of 23-*gem*-dimethyl-3β-hydroxy-Δ⁵-cholestane nucleus and C-30 dihomosterol from marine organisms.

**KEYWORDS**

Bivalve clam; *Paphia malabarica*; 23-*gem*-dimethylcholesta-5-en-3β-ol; (22E)-241,242-methyldihomocholest-5,22-dien-3β-ol; anti-inflammatory activity

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1. Introduction

The bivalves are low-cost sources of protein, amino acids, essential minerals, vitamins and low saturated fat (Joy et al. 2016). The importance of clam aquaculture has competitively increased against finfish fisheries with concerns as an alternative to relatively expensive food supplements, nutraceuticals and synthetic drugs available in the pharmaceutical stores (Chakraborthy et al. 2016). Considering the underutilisation of these species, exploring bioactive compounds and development of any biologically useful products has duel benefits as health products and their commercial farming in coastal habitats. The bioactive secondary metabolites produced by marine bivalve molluscs were identified as popular pharmacophores against inflammations (Chakraborthy et al. 2016; Joy & Chakraborthy 2016) and various oxidative stress-induced diseases (Ouyang 2006; Chakraborthy et al. 2014). Cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) have been implicated to be induced by various inflammatory stimuli, such as cytokines, prostaglandins (such as PGE$_2$, PGF$_2$$\alpha$), leukotrienes (such as LTB$_4$) under inflammatory responses (Mitchell et al. 1993) and the antioxidants were reported to suppress COX-2, 5-LOX enzymes, thus inhibit the release of inflammatory prostaglandins or cytokines (D’Orazio et al. 2012). The (24$R$)-24-propylcholesterol was isolated from *Hiptage benghalensis*, a plant species reported for suppression of PGE$_2$ inflammatory effects as well as anti-iNOS/anti-COX-2 potentials (Hsu et al. 2015).

Sterols are known as the bioactive lipid metabolites, and were found as major constituents in marine invertebrates, such as sponges, corals, bryozoans and molluscs (Goad & Scheuer 1978). More than 200 sterols of 3$\beta$-hydroxy-Δ$^5$ (or saturated) cholestane nucleus and a C$_8$–C$_{10}$ side chain occurring in marine organisms were reported (Sarma et al. 2005). In particular, sponges and molluscs were extensively investigated for different types of steroids than invertebrates of other phyla (Joosse 1978; Sica 1980). Among different groups of steroids, poly-oxygenated tetracyclic nucleus with varying degrees of unsaturation and atypical side chain substitution (D’Auria et al. 1993), steroidal alkaloids, 3$\beta$-cholestane esters, steroid-amino acid conjugates, steroids with spiro A/B ring system (Su et al. 2007) and bicyclo[4.4.1] or bicyclo[4.3.1] A/B steroids were prevalent among the compounds isolated from marine organisms (Amagata et al. 2003; Wang et al. 2015).

As part of our ongoing program towards the isolation of biologically active compounds from marine molluscs, the crude EtOAc–MeOH extract (1:1 v/v) of the bivalve yellow-foot mollusc *Paphia malabarica* (family, Veneridea), collected from Arabian Sea, was screened for antioxidative and anti-inflammatory properties by various *in vitro* assays. The EtOAc–MeOH extract showed promising antioxidative and anti-inflammatory activities in the initial screening as determined by DPPH/ABTS$^+$ radical scavenging and anti-cyclooxygenase-2/5-lipoxgenase assays. We herein describe the $^1$H NMR guided isolation and structure elucidation of two new sterol derivatives, 23-$\text{gem}$-dimethylcholesta-5-en-3$\beta$-ol (1) and (22E)-24$^1,24^2$-methylidihomocholest-5,22-dien-3$\beta$-ol (2) from the EtOAc–MeOH extract (1:1 v/v) of *P. malabarica*. Structure–activity relationship analysis was used to correlate different physicochemical parameters that significantly contribute towards the antioxidative and anti-inflammatory properties of the title compounds.

2. Results and discussion

The freeze-dried powder (1200 g) of the edible flesh separated from *P. malabarica* was exhaustively extracted with EtOAc–MeOH (1:1, v/v) and the crude extract separated on silica
gel as adsorbent column chromatography using a step gradient of 100% n-hexane followed by ethyl acetate and methanol afforded column fractions PM₁–PM₃. The fraction, PM₂ was selected for bioassay (antioxidant and anti-inflammatory) guided repeated chromatographic fractionation using n-hexane/EtOAc/MeOH, resulted in the isolation of two new sterol derivatives, 23-gem-dimethylcholesta-5-en-3β-ol (1) and (22E)-24₁,24²-methylidihomocholest-5,22-dien-3β-ol (2).

23-gem-dimethylcholesta-5-en-3β-ol (1) is a new cholestane derivative, in which the side chain at C-17 contains a gem dimethyl group at C-23 position (Figure 1(A)). It was isolated as white crystalline solid upon repeated chromatography over silica gel as adsorbent. The molecular ion peak at m/e 414 (HRESIMS m/e 414.3892 [M]+, calcd for C₂₉H₅₀O, 414.3862), which in combination with its ¹H and ¹³C NMR data (Table S1) indicated the elemental

Figure 1. Structures of (A) 23-gem-dimethylcholesta-5-en-3β-ol (1) and (B) (22E)-24₁,24²-methylidihomocholest-5,22-dien-3β-ol (2).

Table 1. *In vitro* bioactivities (antioxidant and anti-inflammatory) of the pure compounds (1 and 2) from *P. malabarica*.

| Bioactivities           | 1                 | 2                 |
|------------------------|-------------------|-------------------|
| **Antioxidant activity** |                   |                   |
| DPPH scavenging activity | 1.01⁺ ± 0.02      | 0.81⁰ ± 0.09      |
| ABTS⁺ scavenging activity | 1.12⁺ ± 0.06      | 0.98⁰ ± 0.02      |
| **Anti-inflammatory activity** |            |                   |
| COX-2 inhibition activity | 1.15⁺ ± 0.03      | 0.92⁺ ± 0.08      |
| 5-LOX inhibition activity | 1.02⁺ ± 0.07      | 0.96⁺ ± 0.01      |

*The bioactivities were expressed as IC₅₀ values (mg/mL). The samples were analysed in triplicate (n = 3) and expressed as mean ± standard deviation. Means followed by the different superscripts (p–q) within the same row indicate significant differences (P < 0.05).
composition of $C_{29}H_{50}O$ as 23-gem-dimethylcholesta-5-en-3β-ol (1) with 5° of unsaturation containing one double bond and four-ring systems. Previous works on $^1H$ and $^{13}C$ NMR spectroscopy established the characteristic chemical shifts of various types of protons and carbons of steroids; and based on these data steroid shift assignments were made for the present study. The absence of the characteristic aromatic proton signals in the $^1H$ NMR spectrum confirmed that the four cyclic rings were not of aromatic origin. The $^1H$ NMR spectrum contains several overlapping second-order multiplets with the type ABCDEF. The olefinic signals appeared at $\delta$ 140.76 and 121.72 in the $^{13}C$ NMR spectrum indicating a double bond. The relative downfield shift at $\delta_C$ 140.76 referred to quaternary carbon adjacent to a double bond in the cyclic ring. The highly deshielded $^1H$ signal at $\Delta^2$ (H-6, $\delta$ 5.35, dd), which was found to be a double doublet due to the adjacent $\delta_H$ 1.98 and 1.52 protons attached to carbon (C-7, $\delta$ 31.91) with coupling constant of 5.24 and 3.36 Hz. The presence of an olefinic group in the carbocyclic ring was identified by comparison with cholestane analogues as detailed in a previous literature (Reich et al. 1969). An earlier study of steroid derivatives explained the olefinic proton resonances in the region $\delta$ 5.0–5.6 (br, $J = 5$ Hz) due to the $>C=CH–CH_2$ skeleton (Goad & Akihisa 1997). The proton at H-3 ($\delta$ 3.50) attached to tertiary carbon ($\delta$ 71.82) bearing the hydroxyl group (–OH) appeared as a quintet at the downfield position of the $^1H$ NMR spectrum. The downfield shift of carbinol carbon at C-3 was the result of a greater electron-withdrawing power of the hydroxyl group. It is of note that for the axial cyclohexanols, there is a $\delta$ 1 downfield shift of C-3, probably because of smaller 1, 3-interaction of the –OH group with the H-3 relative to the hydroxyl group. Further specific deuteration of the hydroxyl proton was used for identifying the C-3 carbon. The signal for deuterated –OH proton essentially disappeared. The spectroscopic analysis of $^1H$ NMR, $^{13}C$ NMR along with $^1H$–$^1H$ COSY, HSQC and HMBC relations allowed the elucidation of a cholestane network with $\delta$ 5.35 (H-6) as double bond ($\delta_C$ 121.72) and $\delta$ 3.50 (H-3) hydroxyl ($\delta_C$ 71.8), which was consistent with the literature study (Tian et al. 2011). The $^1H$–$^1H$ COSY correlations $\delta$ 1.85, 1.08 (assigned as H-1)/$\delta$ 1.50, 1.85 (H-2)/$\delta$ 3.50 (H-3)/$\delta$ 2.25, 2.29 (H-4) and $\delta$ 5.35 (H-6)/$\delta$ 1.98, 1.52 (H-7)/$\delta$ 0.91 (H-8) supported the presence of adjacent protons from H-1 to H-4 and H-6 to H-8, respectively. The HMBC correlations from H-4 ($\delta$ 2.25/2.29) to $\delta$ 31.66 (attributed to C-2), $\delta$ 71.82 (C-3), $\delta$ 140.76 (C-5), $\delta$ 121.72 (C-6), $\delta$ 36.52 (C-10) and those from H-6 ($\delta$ 5.35) to $\delta$ 42.31 (C-4), $\delta$ 31.91 (C-7), $\delta$ 36.52 (C-10) supported the bicyclic framework. The three high-field methine (–CH–) protons, $\delta$ 0.95, 1.10 and 1.02 were assigned to carbons at C-9, C-14 and C-17 positions, respectively. The two quaternary carbons (C-10 and C-13) were attributed to characteristic chemical shift and signal pattern of steroids (Reich et al. 1969). The $^1H$–$^1H$ COSY relations, such as $\delta$ 0.95 (H-9)/$\delta$ 1.46 (H-11)/$\delta$ 2.01, 1.15 (H-12) and $\delta$ 1.10 (H-14)/$\delta$ 1.56, 1.06 (H-15)/$\delta$ 1.83 (H-16)/$\delta$ 1.02 (H-17) established the presence of ring C (hexacyclic) and D (cyclopentane), respectively. The HMBCs from H-11 ($\delta$ 1.46) to C-13 ($\delta$ 42.52); H-12 ($\delta$ 2.01/1.15) to C-13 ($\delta$ 42.50); H-8 ($\delta$ 0.91) to C-14 ($\delta$ 56.11) appropriately supported the attachment of hexacyclic ring, C; whereas the correlations from H-16 ($\delta$ 1.83) to C-13 ($\delta$ 42.52); H-14 ($\delta$ 1.10) to C-16 ($\delta$ 28.33); H-15 ($\delta$ 1.56) to C-16 ($\delta$ 28.33) unambiguously described the attachment of cyclopentane (D) moiety in compound 1 as also supported by previous literature (Díaz-Marrero et al. 2013). The C-4 proton has been flanked on both side by downfield shifting of functional groups (OH at C-3 and C=C at C-5, 6) and the two H-4 protons are pulled downfield to $\delta$ 2.25–2.29 away from the pack of overlapped resonances in the $^1H$ NMR spectrum. The $^{13}C$ NMR signal at the far downfield region ($\delta$ 140.76) was less intense (shorter) than other peak at $\delta$ 121.72, because of slow relaxation, it must be a
quaternary carbon. It is of note that the proximity of protons is the primary means of relaxation of $^{13}$C nuclei, and therefore, the carbons lacking proton relax much more slowly, and give less intense peaks, if the relaxation delay is short ($RD = 1.7$ s). The more substituted carbon (at C-5) shifted more downfield relative to C-6 due to the steric crowding effects. The resonances of the cholestane side chain (C-20 to C-27) can readily be identified by comparison with 2, 6-dimethyloctane as a model compound as detailed in a previous literature (Reich et al. 1969) and confirmed by $^1$H–$^1$H COSY. The $^1$H–$^1$H COSY connections between H-17 ($\delta$ 1.02) and H-20 ($\delta$ 1.35) along with their HSQC values confirmed the attachment of side chain, C-20 at C-17 (Díaz-Marrero et al. 2013). The HMBC relations from H-17 ($\delta$ 1.02) to C-21 ($\delta$ 18.72); H-21 ($\delta$ 0.92) to C-13 ($\delta$ 42.52) and C-20 ($\delta$ 36.27) further supported the presence of side chain attachment to the sterol moiety. The two quaternary carbons, C-10 and C-13 were assigned using a long-range $^1$H–$^{13}$C correlation (HMBC) spectrum. A two bond correlation to the methyl protons in each case yields the assignment of C-10 at $\delta$ 36.52 and C-13 at $\delta$ 42.52. The $^1$H and $^{13}$C connectivity deduced from HSQC and HMBC experiments confirmed the side chain framework. The structure contained four singlets which made the compound different from other reported steroids from molluscs (Santalova et al. 2007). The $^1$H NMR spectrum displayed seven upfield methyl signals at $\delta^H$ 1.01 (s), 0.68 (s), 1.59 (s), 1.25 (s), 0.92 (d), 0.86 (d) and 0.87 (d), which were found to exhibit connectivities with carbons at $\delta$ 19.41, 11.87, 28.01, 29.72, 18.72, 19.31 and 22.82, respectively, based on the HSQC experiments. The numbers of carbon atoms were confirmed as 29 through $^{13}$C NMR and DEPT analysis in which 7 –CH$_3$, 10 –CH$_2$ and 8 –CH groups with a total proton integral of 51.93. The methyl groups, including the two gem-dimethyl groups give rise to tall, sharp peak at the upfield region. The $^1$H, $^{13}$C NMR and HMBC correlations were specified (Table S1). The configurations at the individual carbons were determined using their $J$ values and detailed NOESY experiments (Figure S17A). The relative configuration of the carbons (C-3 and C-8) in 1 were deduced by $^1$H coupling constants, $J_{3a,2a} = J_{3a,2b} = 4.2$ Hz and $J_{8b,9a} = 12.3$ Hz. In NOESY, the proton H-3 ($\delta^H$ 3.50) exhibited correlation with H-6 ($\delta^H$ 5.35)/H$^\alpha$-4 ($\delta^H$ 2.29), and therefore, have been considered as $\alpha$ protons, which in turn indicated the $\beta$-disposition of hydroxyl group at C-3 (Tian et al. 2011; Sun et al. 2013). The methyl groups (H-18 and H-19) of the cholestane derivative were correlated with H$^\beta$-4 ($\delta^H$ 2.25)/H$^\beta$-12 ($\delta^H$ 2.01)/H-8 ($\delta^H$ 0.91)/H-20 ($\delta^H$ 1.35), which apparently suggested their $\beta$-orientation, and these attributions were supported by the literature reports (Calderón et al. 2004; Tian et al. 2011). Based on these interpretations, the compound was characterised as 23-gem-dimethylcholesta-5-en-3$\beta$-ol.

The molecular ion peak at $m/e$ 414 (C$_{29}$H$_{50}$O$^+$, [M]$^+$) appeared to undergo elimination of one molecule of water to yield 23-gem-dimethylcholesta-2,5-diene (1a) ($m/e$ 396). One of the most common types of fragmentation in C-17 substituted steroids is that the loss of side chain leads to an abundant ion at $m/e$ 255 in the corresponding spectra. The side chain elimination (2, 4, 4-trimethylheptane) from the fragment ion at $m/e$ 396 yielded the fragment with $m/e$ 255 (1s), which on subsequent rearrangement yielded the fragments at $m/e$ 163 (1t), 95 (1v), 81 (1w) and 69 (1x). The molecular ion peak at 69 (1x) was found to be the base peak and corresponding to penta-1, 4-diene. The fragment ion at $m/e$ 345 was formed from $m/e$ 399 (23-gem-dimethylcholesta-2-ene), through a Retro-Diels-Alder mechanism. Further decomposition of the ion at $m/e$ 399 was perceived to be accompanied by the loss of the C-18 methyl group and results in an ion at $m/e$ 203. The molecular ion peak at $m/e$ 264 (1y) resulted from the fragmentation of ions through elimination of water molecule and side chain. The olefinic (C=C) and alkyl (C–H) groups IR stretching vibrations were represented.
by the 1664 and 2945 cm\(^{-1}\) absorption bands, respectively. The distinctive absorption at 3427 cm\(^{-1}\) indicated O–H stretching vibration. The FTIR absorption bands at 1374, 1332 (C–H rocking), 1243, 1188 (C–C stretch), 881, 835, 733 cm\(^{-1}\) (=C–H bend) substantiated the structure of substituted cholestane.

(22\(E\))-24\(^1\),24\(^2\)-methylenecholest-5,22-dien-3\(\beta\)-ol (2), a new derivative of the substituted dihomocholestdienol, was isolated as white crystalline solid upon repeated chromatography on silica gel. The ultraviolet absorbance at \(\lambda_{\text{max}}\) (log \(\varepsilon\) 228.9 (1.74) nm have been assigned to be chromophores with olefinic system. Its mass spectrum exhibited a molecular ion peak at \(m/e\) 426 (HRESIMS m/e 426.3892 [M]\(^+\), calcd. for \(C_{30}H_{50}O\), 426.3862), which in combination with its \(^1H\) and \(^{13}C\) NMR data (Table S1) indicated the elemental composition of \(C_{30}H_{50}O\) as (22\(E\))-24\(^1\),24\(^2\)-methylenecholest-5,22-dien-3\(\beta\)-ol (2) with 6\(^°\) of unsaturation containing two double bonds and four ring systems (Figure 1(B)). The NMR signal and peak characteristics of 2 were closely related to 1; the main difference found in the \(^1H\) NMR spectrum was the presence of an additional double bond which was between C-22 and C-23. The \(^{13}C\) NMR spectrum of 2 in combination with DEPT experiments indicated the occurrence of 30 carbon atoms including 1 carbinol carbon at \(\delta\) 71.81 and olefinic carbons at \(\delta\) 140.76, 121.71, 135.83 and 131.72. A total of 6 methyl carbons, 10 methylene and 11 methine groups were recorded. Since no aromatic signals were recorded in the \(^1H\) NMR data, these peaks which appeared downfield above \(\delta\) 130 were assigned to the olefinic carbons in the \(^{13}C\) NMR spectrum. The olefinic signal at \(\delta\) 140.76 appeared at the far downfield region and less intense than other peak at \(\delta\) 121.71, because of slow relaxation, it must be a quaternary carbon. The intense olefinic signals at \(\delta\) 131.72 and 135.83 might be due to the fact that these are less substituted carbons and sterically less crowded. The similarity in chemical shift apparently indicated these as sp\(^2\) hybridised (–CH=CH–) and shifted more upfield relative to the sp hybridised carbon atom. The broad IR absorption band at 3427 cm\(^{-1}\) was due to free –OH stretching vibrations, which has been supported by the \(^1H\) NMR signal at \(\delta\) 3.50. The presence of olefinic protons was supported by the \(^1H\) NMR signals at \(\delta\) 5.35 (1H; dd) with a coupling constant of 5.28 and 3.58 Hz. This supported the presence of E-geometrical isomer of the olefinic proton (assigned to the proton at C-6). The \(^1H\) NMR, \(^{13}C\) NMR and HSQC experiments attributed the parent steroid nucleus with C-3 hydroxyl at \(\delta\) 3.50 ppm (\(\delta\)C 71.81) and C5-C6 double bond at \(\delta\) 5.35 ppm that were similar to those reported in the earlier studies (Wilson et al. 1996). The \(^1H–^1H\) COSY correlations were confirmed between \(\delta\) 1.83, 1.08 (assigned to H-1)/\(\delta\) 1.50, 1.82 (H-2)/\(\delta\) 3.50 (H-3)/\(\delta\) 2.24, 2.28 (H-4); \(\delta\) 5.35 (H-6)/\(\delta\) 1.96, 1.56 (H-7)/\(\delta\) 0.93 (H-8) and \(\delta\) 0.95 (H-9)/\(\delta\) 1.49 (H-11)/\(\delta\) 2.01, 1.15 (H-12) along with their H–C connectivities, which were deduced from the HSQC and HMBC experiments. The \(^{13}C\) NMR signals at C-22 and C-23 (\(\delta\) 135.83 and \(\delta\) 131.72, respectively) in combination with the HSQC experiments (\(\delta\) \(H_1\) 5.18 and \(\delta\) 5.17) assigned the olefinic (–HC=CH–) group at the side chain. The large coupling constants, (\(J_{\text{c}}\)) 12.4 and 16.1 Hz of the olefinic protons at H-22 and H-23, respectively attributed to its trans (E-form) configuration, as supported by the previous studies (Goad & Akihisa 1997). The occurrence of olefinic proton at \(\Delta^{22}\) (\(\delta\) 5.18 (H-22) and \(\delta\) 5.17 (H-23)) was further confirmed by the long-range HMBC correlations from H-21 (0.91) to C-23 (\(\delta\) 131.72) and from H-17 (1.10) to C-22 (\(\delta\) 135.83) along with one bond \(^1H–^{1H}\) COSY relations such as \(\delta\) 1.86 (H-20)/\(\delta\) 5.18 (H-22); \(\delta\) 5.17 (H-23)/\(\delta\) 2.03 (H-24)/\(\delta\) 1.25 (H-24\(^2\))/\(\delta\) 1.35 (H-24\(^3\))/\(\delta\) 0.92 (H-24\(^4\)), \(\delta\) 1.52 (H-25) and \(\delta\) 1.52 (H-25)/\(\delta\) 0.86 (H-26), \(\delta\) 0.87 (H-26) (Tian et al. 2011). The HMBC relations from H-24\(^2\) (\(\delta\) 1.35) to C-27 (\(\delta\) 22.69) and from H-24 (\(\delta\) 2.03) to C-24\(^3\) (\(\delta\) 20.53), C-25 (\(\delta\) 28.02) supported the occurrence of side chain framework attached to the parent
steroid skeleton. Further the long-range HMBC correlations between H-4 (δ 2.24) to C-2 (δ 31.80), C-6 (δ 121.71), C-10 (δ 36.50); and those between H-6 (δ 5.35) to C-10 (δ 36.50); H-7 (δ 1.96) to C-5 (δ 140.76), C-6 (δ 121.71); H-1 (δ 1.83) to C-3 (δ 71.81), C-10 (δ 36.50); H-11 (δ 1.49) to C-14 (δ 56.70) assigned to the tricyclic framework. 1H–1H COSY couplings were apparent between the protons at δ 1.0 (assigned to H-14)/δ 1.57, 1.06 (H-15)/δ 1.85 (H-16)/δ 1.10 (H-17). Further the HMBC correlations between H-14 (δ 1.00) and C-8 (δ 46.05), C-9 (δ 50.14), C-16 (δ 28.2) and the C–H connectivities between H-17 (δ 1.1) and C-22 (δ 56.7), C-14 (δ 56.7) confirmed the side chain framework. Six methyl groups, including the two angular –CH₃ groups (δ 0.91 and 0.68) located at the ring junction of A/B and C/D gave rise to tall, sharp peak at the upfield region. Long-range HMBC correlations between H-19 (δ 0.80) to C-1 (δ 37.20), C-2 (δ 31.80) and C-5 (δ 140.76) also attributed the presence of C-19 and C-18 methyl groups. The relative stereochemistries of chiral centers, particularly at C-3, C-8, C-14, C-17, and C-20, were deduced from the NOESY spectrum and coupling constants (J-values) which were similar to compound 1, suggesting the OH-3, H-8 and H-14 for β-orientation (Figure S17A). NOE couplings were observed between Ha-3/Ha-6 thus indicating that these groups must be on the α-side of the molecule. Therefore, the C-3 hydroxyl group is equatorial and β-oriented (Sun et al. 2013). NOE correlations between Ha-6/Ha-14 indicated that these groups on their α-disposition. The methine proton at C-21 did not exhibit NOE interactions with Me-18 and Me-19, which are at the β-face of the molecule, thereby indicating that H-21 is at the α position. Based on the interpretations, the compound was deduced as (22E)-24¹,24²-methyldihomocholest-5,22-dien-3β-ol.

The molecular ion peak at m/e 426 (C₃₀H₅₀O·+, [M]+) appeared to undergo elimination of one molecule of water and an isopropyl group to yield 24¹,24²-methyldihomocholest-5,22-trien-3β-ol (2h) (m/e 409), which underwent side chain elimination followed by rearrangement at ring D to afford a fragment with m/e 229 (2i). The fragment ion at m/e 357 were formed from m/e 411 (24¹,24²-methyldihomocholest-5,22-dien-3β-ol), through a Retro-Diels-Alder mechanism. Fragmentation of the ion at m/e 366 (2b) was perceived to be accompanied by the loss of a C-6 fragment (assigned to hex-1-ene) resulted in an ion at m/e 285 (2c), which on subsequent rearrangement yielded the fragments at m/e 177 (2d), 135 (2e), and 69 (2f). It is of note that the fragment ion at m/e 69 (C₅H₈·+) appeared as base peak of 2. The IR spectrum revealed broad absorption band at ν_max 3427 cm⁻¹ attributed to hydroxyl and ν_max 1664 cm⁻¹ to olefinic (C=C) functionalities.

The antioxidant and anti-inflammatory activities of 1 as inferred by DPPH/ABTS⁺ radical scavenging and anti-cyclooxygenase-2/5-lipoxygenase assays were significantly lesser (IC₅₀ > 1 mg/mL) than 2 (IC₅₀ < 1 mg/mL) (Table 1). Commercially available antioxidative agent α-tocopherol has been used as standard in the present study. It is of note that no significant differences in the DPPH and ABTS⁺ radical scavenging activities of compound 2 (IC₅₀ < 1 mg/mL) and α-tocopherol (IC₅₀ < 1 mg/mL) were recorded (P < 0.05). Selective inhibition of lipoxygenase (5-LOX) is a favored system to deter inflammatory stimuli (Joy et al. 2016). The commercially available synthetic anti-inflammatory drug ibuprofen has been used to compare the results. Notably, 5-LOX inhibitory activity of ibuprofen (IC₅₀ 0.93 mg/mL) showed no significant difference (P < 0.05) compared to the title compounds (IC₅₀ 0.96–1.02 mg/mL), particularly (22E)-24¹,24²-methyldihomocholest-5,22-dien-3β-ol (2) (IC₅₀ 0.96 mg/mL) isolated from P. malabarica. Structure–activity relationship analysis revealed that the bioactivities of the title compounds purified in this study were directly proportional to the electronic and lipophilic parameters. The electronic factor such as polarizibility (Pl)
was found to significantly contribute towards the antioxidant and anti-inflammatory activities of the compounds 1 and 2. It is of note that the compound 2 was found to possess an additional olefinic group at C22=C23 position, and therefore might contribute towards greater bioactive characteristics. The antioxidant activity of 1 was found to be significantly lesser (IC$_{50}$ 1.01 mg/mL) than 2 (IC$_{50}$ 0.81 mg/mL) apparently due to the absence of an additional double bond in the former, resulting in lesser electron delocalisation. This resulted in higher electronic properties of 2 (PI 53.21 × 10$^{-24}$ cm$^3$) than 1 (PI 51.23 × 10$^{-24}$ cm$^3$). This hypothesis has been supported by studies conducted previously to find that the radical scavenging activities of a compound proportionately increases with the presence of double bonds due to effective electron transfer through the process of electron delocalisation (Cai et al. 2006). Similar reasons might be attributed to the greater anti-inflammatory activity of 2 than that of compound 1. The antioxidant activities of the substituted cholestane derivatives were also found to be directly proportional to their hydrophobic character as determined by octanol–water coefficient (log $P_{ow}$). The greater the value of log $P_{ow}$, the greater the molecular hydrophobicity of the molecules. The hydrophobic character of 1 (log $P_{ow}$ 8.19) is lesser than those of 2 (log $P_{ow}$ 8.23). The lowering of activity of 1 might be explained due to the decrease in its log $P_{ow}$, the membrane permeability properties and the reactivity towards DPPH free radical. It has been hypothesised that the free radical DPPH can interact with the compounds with higher hydrophobic coefficients (greater log $P_{ow}$ value) and therefore, showed positive correlation with the scavenging efficiency towards lipophilic DPPH. Since antioxidants have to diffuse into liposomes to react with lipophilic free radicals (such as peroxides and DPPH as the in vitro model), it is reasonable to expect that a greater hydrophobicity of antioxidants is relevant to their radical scavenging efficiency in liposomes and cells. Based on these results, it can be inferred that hydrophobicity is particularly important factor in determining the antioxidant and anti-inflammatory activities. In particular, the presence of gem-dimethyl groups at C-22 position of the cholestane framework attributed to greater steric restrictions of 1, resulting in lesser target bioactivities than 2.

More significantly, the marine-derived steroids with their diverse structures were found to exhibit interesting therapeutic properties (Goad & Akihisa 1997; Whitson et al. 2009). Anti-inflammatory properties of steroidal compounds isolated from marine invertebrates against pro-inflammatory COX-2 and cytokines were reported in earlier literature for example, the inhibitory effect of diunsaturated C-27 polyhydroxy sterols isolated from marine gastropod, _Trimusculus peruvianus_ (Chao et al. 2008; Su et al. 2008; Díaz-Marrero et al. 2013; Thao et al. 2013). Consequently, the detection and identification of COX-2/5-LOX-specific inhibitors could have a potentially profound impact on the treatment of a number of inflammatory diseases and disorders.

3. Experimental section

3.1. General procedures

All reagents and solvents were of spectroscopic or chromatographic or analytical grade from Merck (Darmstadt, Germany). Fourier-transform infrared (FTIR) spectra (KBr) scanned between 4000 and 400 cm$^{-1}$ (Perkin-Elmer Series 2000 FTIR spectrophotometer). 1D and 2D NMR spectra were analysed on a Bruker Avance DPX 500 (500 MHz) spectrometer in CDCl$_3$ aprotic solvent with TMS (standard). The GC-MS (Perkin-Elmer Clarus 680 GC-MS fitted with
Elite 5 MS non-polar) analyses were performed in electronic impact (EI) ionisation mode in bonded phase capillary column (50 m × 0.22 mm i.d. × 0.25 μm film thicknesses) (Chakraborty et al. 2014). UV spectra were acquired on a Varian Cary 50 UV–vis spectrometer (Varian Cary, USA) (Joy & Chakraborty 2016). The melting point of the compounds were determined using Melting Point Apparatus (VMP-DS, Veego, Mumbai, India) and the angle of rotation of the compounds were recorded on a polarimeter (AP-300, ATAGO, Japan).

3.2. Animal material and extraction

According to Joy and Chakraborty (2016), *P. malabarica* (10 kg) samples were freshly collected from Ashtamudi Lake (8°59′ N and 76°36′ E) situated along the south-west coast of India. A voucher specimen has been deposited in the repository of the project titled ‘Development of nutraceutical supplements from marine molluscs, macroalgae and shrimps’ (grant number ICAR/CRP-HF/2016) under the ICAR Consortium Research Platform funded by the Indian Council of Agricultural Research, New Delhi, India, with a voucher specimen No. ICAR/CRP-HF/AC 368. The edible flesh (6 kg) separated from the cleaned shell-on samples were ground and freeze dried by lyophilisation (Martin Christ alpha 1-4 LD Plus freeze-drier, Germany). The lyophilised powder (1200 g, yield 20.0%) was extracted with EtOAc–MeOH (1:1, v/v, 500 mL × 3) at 40 °C followed by sonication (8 h) under an inert atmosphere of N2. The extracts were filtered over anhydrous Na2SO4 (100 g), before being evaporated in vacuo using a rotary vacuum evaporator (50 °C) (Heidolph, Germany) to dryness to get a dark brown oily viscous residue, which was referred to as the crude extract of *P. malabarica* (55.0 g, yield on dry basis 4.58%).

3.3. Isolation and spectroscopic analysis

The crude extract of *P. malabarica* (45.0 g) was partitioned by repeated column chromatography over silica gel as adsorbent by modified methods of Joy and Chakraborty (2016). The extract was made into slurry with silica gel (4 g, 60–120 mesh) and packed into a column (1000 mm × 40 mm) containing silica gel as adsorbent (60–120 mesh). The column was initially eluted with 100% *n*-hexane followed by ethyl acetate and methanol to obtain different factions. The major column fractions (PM1–PM3) were evaluated for antioxidant and anti-inflammatory activities. The fraction PM2 (6.40 g, yield 11.6%) was subjected to further chromatographic fractionation due to its relatively greater antioxidant and anti-inflammatory activities compared to other two fractions (PM1 and PM3). The fraction PM2 was subjected to vacuum liquid chromatography on a glass column (450 mm × 30 mm) packed with silica (230–400 mesh). The eluent polarity was gradually increased by the addition of EtOAc (*n*-hexane:EtOAc, 99:1 to 60:40, v/v) to furnish 21 fractions of 30 mL each, which were reduced to 8 groups (PM2-1–PM2-8) after TLC analysis (*n*-hexane:EtOAc, 9:1, v/v). The fraction PM2-3 (1.25 g) eluted with *n*-hexane:EtOAc (19:1, v/v) was found to be a mixture, which was flash chromatographed (Biotage AB SP1-B1A, 230–400 mesh, 12 g; Biotage AB, Uppsala, Sweden) on a silica gel column at a collection UV wavelength of 254 nm. The eluent polarity initiated from 100% *n*-hexane followed by EtOAc and MeOH to furnish a total of 55 fractions (9 mL each), which were pooled to 4 fractions (PM2-3-1 through PM2-3-4) based on analytical TLC (*n*-hexane:EtOAc, 9:1, v/v). The fraction PM2-3-1 was further fractionated over preparatory TLC on silica gel GF254 using *n*-hexane:EtOAc (49:1, v/v) afforded compound 1, 23-*gem*-dimethylcholesta-3β-ol (82 mg) as major component. Evaporation of solvents from the fractions
followed by TLC over silica gel GF$_{254}$ (particle size 15 mm) using n-hexane:EtOAc (95:5, v/v) supported the purity. The fraction, PM$_{2-3-2}$ was further fractionated over preparatory TLC on silica gel GF$_{254}$ using n-hexane:EtOAc (46:4, v/v) afforded compound 2, (22E)\textsuperscript{-24\text{I}}, 24\textsuperscript{2}-methyl-dihomocholest-5,22-dien-3\textsuperscript{\beta}-ol (148 mg) as major component. Evaporation of solvents from the fractions followed by TLC over silica gel GF$_{254}$ (particle size 15 mm) using n-hexane:EtOAc (95:5, v/v) supported the purity.

3.3.1. 23\textsuperscript{gem}-dimethylcholesta-5-en-3\textsuperscript{\beta}-ol (1)
White crystalline solid; m.p. 139.4 °C; $[\alpha]_D^{26} = 16.4^\circ$ (CHCl$_3$, $c=0.015$); UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$): 226.4 (1.36); TLC (Si gel GF$_{254}$ 15 mm; EtOAc:n-hexane 5:95, v/v); $R_f$: 0.39, $R_t$ (HPLC, MeOH:ACN, 6:4 v/v): 5.97 min.; IR (KBr, cm$^{-1}$) $\nu_{\text{max}}$: 3427.16 (br O–H $\nu$), 2945.52 (C–H $\nu$), 1664.58 (C=C $\nu$), 1459.26, 1374.62, 1332.94 (C–H $\rho$), 1243.57, 1188.40, 1122.79 (C–C $\nu$), 961.64 (CH=CH) 881.06, 835.96, 806.20, 596.38 (C–H $\delta$); $^1$H NMR (500 MHz, CDCl$_3$) 5.35(dd, $J = 5.13,3.36$ Hz), 3.50(p), 2.29(d), 2.25(d), 2.01(t), 1.98(t), 1.85(m), 1.84(t), 1.83(t), 1.59(s), 1.56(t), 1.52(t), 1.50(m), 1.49(m), 1.46(q), 1.35(m), 1.30(d), 1.25(s), 1.15(t), 1.14(d), 1.10(m), 1.08(t), 1.06(m), 1.02(m), 1.01(s), 0.95(t), 0.92(d), 0.91(m), 0.87(d), 0.86(d), 0.68(s); $^{13}$C NMR (125 MHz, CDCl$_3$) 140.76, 121.72, 71.81, 56.72, 56.11, 50.14, 46.06, 42.52, 42.31, 39.79, 39.61, 37.22, 36.52, 36.27, 36.22, 35.71, 31.91, 31.66, 29.72, 28.33, 28.01, 24.21, 23.83, 22.82, 21.09, 19.41, 19.31, 18.72, 11.87, COSY and HMBC (Table S1); HRESIMS $m/e$ found 414.3892 [M$^+$], calcd for C$_{29}$H$_{50}$O 414.3862.

3.3.2. (22E)-24\textsuperscript{I},24\textsuperscript{2}-methyl-dihomocholest-5,22-dien-3\textsuperscript{\beta}-ol (2)
White crystalline solid; m.p. 140.7 °C; $[\alpha]_D^{26} = -18.6^\circ$ (CHCl$_3$, $c=0.012$); UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$): 228.9 (1.74); TLC (Si gel GF$_{254}$ 15 mm; EtOAc:n-hexane 5:95, v/v); $R_f$: 0.48, $R_t$ (HPLC, MeOH:ACN, 6:4 v/v): 5.332 min.; IR (KBr, cm$^{-1}$) $\nu_{\text{max}}$: 3427.16 (br O–H $\nu$), 2945.52 (C–H $\nu$), 1664.58 (C=C $\nu$), 1459.26, 1374.62, 1332.94 (C–H $\rho$), 1243.57, 1188.40, 1122.79 (C–C $\nu$), 961.64 (CH=CH) 881.06, 835.96, 806.20, 596.38 (C–H $\delta$); $^1$H NMR (500 MHz, CDCl$_3$) 5.35(dd, $J = 5.28,3.58$ Hz), 5.18(dd, $J = 12.4,6.1$ Hz), 5.17(dt, $J = 16.1,7.3$ Hz), 3.50(p), 2.28(d), 2.24(d), 2.03(m), 2.01(t), 1.96(t), 1.86(t), 1.85(m), 1.83(t), 1.82(m), 1.57(m), 1.56(m), 1.52(m), 1.50(m), 1.49(m), 1.35(m), 1.25(m), 1.15(t), 1.10(m), 1.08(t), 1.06(m), 1.01(s), 1.00(m), 0.96(m), 0.93(m), 0.92(d), 0.91(d), 0.87(d), 0.86(d), 0.68(s); $^{13}$C NMR (125 MHz, CDCl$_3$) 140.76, 135.83, 131.71, 121.71, 71.81, 56.70, 56.16, 50.14, 46.05, 42.54, 42.33, 42.32, 41.90, 39.72, 35.72, 37.22, 36.50, 31.91, 31.83, 28.42, 28.22, 28.02, 24.26, 22.69, 21.14, 20.53, 19.89, 19.40, 18.39, 11.68; COSY and HMBC (Table S1); HRESIMS $m/e$ found 426.3892 [M$^+$], calcd for C$_{30}$H$_{50}$O 426.3862.

3.4. **Antioxidative and anti-inflammatory assay**
The antioxidant and anti-inflammatory activities of purified compounds (1–2) isolated from *P. malabarica* were carried out. The antioxidant properties were evaluated by DPPH (Chew et al. 2008) and ABTS$^+$ radical decolourisation assay (Vijayabaskar & Shiyamala 2012; Chaudhary et al. 2015). In vitro anti-inflammatory properties (Chavan et al. 2012) were determined by inhibition of COX-2 (Larsen et al. 1996) and 5-LOX (Baylac & Racine 2003) enzymes. The plot of scavenging and pro-inflammatory enzyme inhibitory activities were recorded, and the results were expressed as IC$_{50}$ (the concentration of samples at which it inhibits/scavenges 50% of enzyme/radical activities and expressed in mg/mL) value. The structure–activity relationship analysis was carried out using different physicochemical parameters of the purified compounds. The structural descriptors were acquired from ChemDraw Ultra
(8.0 database), steric (molar volume, molar refractivity), hydrophobic (log $P_{ow}$: logarithmic value of the octanol–water partition coefficient) and polarisability (electronic descriptor) factors.

3.5. **Statistical analysis**

Statistical Program for Social Sciences 13.0 (SPSS, USA, ver. 13.0) was assessed for calculating significant differences between the means (One way analysis of variance, ANOVA) of triplicates $\pm$ standard deviation of all assays and were represented as $P < 0.05$.

4. **Conclusion**

To the best of our knowledge, 23-**gem**-dimethyl-3β-hydroxy-$\Delta^5$-cholestan nucleus and C-30 dihomosterol (1 and 2) represent the first examples of steroids possessing the 23-**gem**-dimethyl derivative of sterol and C-30 dihomosterol system from a natural source. The C30 dihomocholest-dien-3β-ol from *P. malabrica* has potential bioactive potential as natural antioxidant and anti-inflammatory pharmacophore. The extensive biochemical analyses and bioassay guided purification followed by identification of bioactive secondary metabolites from this species can positively influence the clam agriculture, seafood exports and pharmaceutical fields in the future.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**

Amagata T, Amagata A, Tenney K, Valeriote FA, Lobkovsky E, Clardy J, Crews P. 2003. Unusual C25 steroids produced by a sponge-derived *Pencillium citrinum*. Org Lett. 5:4393–4396.

Baylac S, Racine P. 2003. Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. Int J Aromather. 13:138–142.

Cai YZ, Mei S, Jie X, Luo Q, Corke H. 2006. Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sci. 78:2872–2888.

Calderón GJ, Castellanos L, Duque C, Echigo S, Hara N, Fujimoto Y. 2004. Ophirasterol, a new C31 sterol forms the marine sponge *Topsentia ophiraphidites*. Steroids. 69:93–100.
Chakraborty K, Chakkalakal SJ, Joseph D. 2014. Response of pro-inflammatory prostaglandin contents in anti-inflammatory supplements from green mussel *Perna viridis* L. in a time dependent accelerated shelf-life study. J Funct Foods. 7:527–540.

Chakraborty K, Chakkalakal SJ, Joseph D, Joy M. 2016. Nutritional composition of edible Oysters (*Crassostrea madrasensis* L.) from the South West coast of India. J Aquat Food Prod. doi: 10.1080/10498850.2015.1039682.

Chao CH, Wen ZH, Su JH, Chen IM, Huang HC, Dai CF, Sheu JH. 2008. Further study on anti-inflammatory oxygenated steroids from the octocoral *Dendronephthya griffini*. Steroids. 73:1353–1358.

Chaudhary AK, Ahmad S, Mazumder A. 2015. Isolation, structural elucidation and *in vitro* antioxidant activity of compounds from chloroform extract of *Cedrus deodara* (Roxb.) Loud. Nat Prod Res. 29:268–273.

Chavan MJ, Wakte PS, Shinde DB. 2012. Analgesic and anti-inflammatory activities of the sesquiterpene fraction from *Annona reticulata* L. bark. Nat Prod Res. 26:1515–1518.

Chew yL, Lim YY, Omar M, Khoo KS. 2008. Antioxidant activity of three edible seaweeds from two areas in South East Asia. LWT-Food Sci Tech. 41:1067–1072.

D’Auria MV, Minale L, Riccio R. 1993. Polyoxygenated steroids of marine origin. Chem Rev. 93:1839–1895.

Díaz-Marrero AR, Dorta E, Cueto M, Rovirosa J, San-Martin A, Loyola A, Darias J. 2003. New polyhydroxylated steroids from the marine pulmonate *Trimusculus peruvianus*. Arkivoc. 10:107–117.

D’Orazio N, Gammone AM, Gemello E, De Girolamo M, Cusenza S, Riccioni G. 2012. Marine bioactives: pharmacological properties and potential applications against inflammatory diseases. Mar Drugs. 10:812–833.

Goad LJ, Akihisa T. 1997. Analysis of sterols. London: Blackie Academic and Professional, Chapman & Hall, 2–6 Boundary Row.

Goad LJ, Scheuer PJ. 1978. Marine natural products, vol. 2. New York: Academic Press; p. 76–172.

Hsu C-L, Fang S-C, Huang H-W, Yen G-C. 2015. Anti-inflammatory effects of triterpenes and steroid compounds isolated from the stem bark of *Hiptage benghalensis*. J Funct Foods. 12:420–427.

Joosse J. 1978. Endocrinology of molluscs. Actualites sure les Hormones d’Invertibrates. CNRS. 251:107–123.

Joy M, Chakraborty K. 2016. First report of two new antioxidative meroterpeno 2H-pyranoids from short-necked yellow-foot clam *Paphia malabarica* (family: Veneridae) with bioactivity against pro-inflammatory cyclooxygenases and lipoxygenase. Nat Prod Res. doi:10.1080/14786419.2016.1209670.

Joy M, Chakraborty K, Pananghat V. 2016. Comparative bioactive properties of bivalve clams against different disease molecular targets. J Food Biochem. 40:593–602. doi:10.1111/jfbc.12256.

Larsen LN, Dahl E, Bremer J. 1996. Peroxidative oxidation of leuco-dichloroluorescein by prostaglandin-H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. BBA: Lipid Lipid Met. 1299:47–53.

Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, Vane JR. 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc Natl Acad Sci. U.S.A. 90:11693–11697.

Ouyang MA. 2006. A new adenosyl-alkaloid from *Ostrea rivularis*. Nat Prod Res. 20:79–83.

Reich HJ, Jautelat M, Messe MT, Weigert FJ, Roberts JD. 1969. Nuclear magnetic resonance spectroscopy, Carbon–13 spectra of steroids. J Am Chem Soc. 91:7445–7454.

Santalova EA, Denisenko VA, Chernyshev AV, Gavagnin M., Sanamyan KE. 2007. Ketosteroids from the far-east marine prosobranch mollusk *Onchidiopsis variegate*. Chem Nat Compd. 43:86–89.

Sarma NS, Krishna MSR, Rao SR. 2005. Sterol ring system oxidation pattern in marine sponges. Mar Drugs. 3:84–111.

Sica D. 1980. Sterols from some molluscs. Comp Biochem Physiol. 65B:407–410.

Su JH, Lin FY, Huang HC, Dai CF, Wu YC, Hu WP, Hsu CH, Sheu JH. 2007. Novel steroids from the soft coral *Nepthsea chabrolii*. Tetrahedron. 63:703–707.

Sun L-L, Fu X-M, Li X-B, Xing Q, Wang C-Y. 2013. New 18-oxygenated polyhydroxy steroid from a South China Sea soft coral *Sarcophyton* sp. Nat Prod Res. 27:2006–2011.
Thao PN, Cuong XN, Luyen TTB, Quang HT, Hong Hanh TT, Kim S, Koh SY, Nam HN, Kiem VP, Minh VC, Kim HY. 2013. Anti-inflammatory components of the starfish *Astropecten polyacanthus*. Mar Drugs. 11:2917–2926.

Tian RX, Tang H-F, Li Y-S, Lin H-W, Chen X-L, Ma N, Yao M-N, Zhang P-H. 2011. New cytotoxic oxygenated sterols from the marine bryozoan *Cryptosula pallasiana*. Mar Drugs. 9:162–183.

Vijayabaskar P, Shiyamala V. 2012. Antioxidant properties of seaweed polyphenol from *Turbinaria ornata* (Turner) J. Agardh, 1848. Asian Pac J Trop Biomed. 2:S90–S98.

Wang W, Lee TG, Patil RS, Mun B, Yang I, Kim H, Hahn D, Won DH, Lee J, Lee Y, et al. 2015. Monanchosterols A and B, bioactive bicyclo[4.3.1]steroids from a Korean Sponge *Monanchora* sp. J Nat Prod. 78:368–373.

Whitson EL, Bugni TS, Chockalingam PS, Concepcion GP, Feng X, Jin G, Harper MK, Mangalindan GC, McDonald LA, Ireland CM. 2009. Fibrosterol sulfates from the Philippine sponge *Lissodendoryx (Acanthodoryx) fibrosa*: sterol dimers that inhibit PKCζ. J Org Chem. 74:5902–5908.

Wilson WK, Sumpter RM, Warren JJ, Rogers PS, Ruan B, Schroepfer GJ. 1996. Analysis of unsaturated C27 sterols by nuclear magnetic resonance spectroscopy. J Lipid Res. 37:1529–1555.