Research Article

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Rare norisodinosterol derivatives from *Xenia umbellata*: Isolation and anti-proliferative activity

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Abstract: Two new rare 30-norisodinosterol derivatives, 23,24-dimethylcholest-16-ene-3β,5α,6β,11α,20(R)-pentol 3-monoacetate (1) and 23,24-dimethylcholest-16-ene-3β,5α,6β,20(R)-tertrol 3-monoacetate (2), along with a known steroid, 3β,5α,6β,11α,20β-pentahydroxygorgosterol (3), were identified from *Xenia umbellata*. The structures of the isolated compounds were determined by analyses of the measured spectra (1D and 2D nuclear magnetic resonance, mass spectrometry, and infrared). The biosynthetic pathway of the new norisodinosterols was proposed. Compound 1 exhibited potent cytotoxicity against HepG2, PC-3, and HT-29 with IC$_{50}$ values of 4.70 ± 0.2, 5.60 ± 0.6, and 4.00 ± 0.4 μg/mL, respectively. On the contrary, compound 3 showed less potent cytotoxicity against HepG2 with IC$_{50}$ value of 22.20 ± 1.0 μg/mL. Two DNA-binding dyes have been used for the morphological detection of viable, apoptotic, and necrotic cells. The early apoptotic cell death was observed in all types of treated tumour cells. The late apoptotic cells are highly present in HepG2 cells with compound 3 compared with other cancer cells except for compound 1. The anti-proliferative activity of compounds 1 and 3 warranted further investigation.

Keywords: Red Sea, Alcyonacea, steroids, cytotoxicity, apoptosis

1 Introduction

*Alcyonacea* (Phylum: Cnidaria; Class: Anthozoa) survives worldwide in tropical and subtropical seawaters and does not have the hard calcium carbonate skeleton. Members of *Alcyonacea* inhabit the inner reefs below the stony corals [1]. They are known for their productivity of secondary metabolites such as terpenoids and steroids. The soft corals are important member of marine fauna which have cells in the form of toxic stinging nematocysts with the absence of the rigid protective skeleton of scleractinians. They also have the ability to produce toxic substances [2–4].

Family Xenidae (*Alcyonacea*) consists of 20 genera and 162 species. They live in tropical waters as the Red Sea. They present as yellow cylindrical clavate colonies [3]. They have many varieties of long feather-like tentacles and their polyps pump water into the colony, creating a rhythmic pulsing motion. They are named as pulsing *Xenia* and pom-pom *Xenia*. This genus is known for its productivity of terpenoids and steroids [5,6].

In 2018, the Saudi Cancer Registry reported a total of 24,485 diagnosed cancer cases [7]. As a part of our interest is this study, which aimed at discovering the anti-cancer metabolites from marine sources [8,9]. Thus, the present study was designed to isolate bioactive secondary compounds from *Xenia umbellata*. The anti-proliferative activity of 23,24-dimethylcholest-16-ene-3β,5α,6β,11α,20(R)-pentol 3-monoacetate (1) and 3β,5α,6β,11α,20β-pentahydroxygorgosterol (3) was evaluated against HepG2, PC-3, and HT-29. Additionally, two DNA-binding dyes, acridine orange (AO) and ethidium bromide (EtBr), have been used for the morphological detection of viable, apoptotic, and necrotic cells [10].
2 Experimental

2.1 Soft coral sample

_Xenia umbellata_ was gathered by Scuba technique at a depth of 15–20 m in October 2018, off the Red Sea coast at Jeddah, Saudi Arabia (21°29′31″N 39°11′24″E). Prof. Mohsen El-Sherbiny (Faculty of Marine Sciences, King Abdulaziz University [KAU]) identified the sample. A voucher specimen (XC-2018-11/2) was deposited in the Faculty of Marine Sciences, KAU.

2.2 Extraction and isolation

The semi-dried soft coral (265 g) was exhausted by CH₂Cl₂/MeOH (3 × 1 L, 22°C), yielding (21.4 g) an oily residue. The extract was loaded onto a 60 g silica gel column at Jeddah, Saudi Arabia – 2.2 Extraction and isolation

The fraction that eluted with n-hexane–CH₂Cl₂ (45:55) afforded compound 1. Finally, the fraction that eluted with n-hexane–CH₂Cl₂ (40:60) gave compound 3. The purification of compounds 1–3 has been done by preparative thin-layer chromatography (PTLC; normal phase silica gel).

2.3 Spectral data

2.3.1 Compound 1

Gummy material (1.6 mg, 0.00062%); [α]_D^22 = 49.6 (c 0.01, CHCl₃); infrared (IR) ν_max (film)/cm: 3,403, 2,925, 2,853, 1,730, 1,713, 1,655, 1,514, 1,246, 1,264, 1,153; ¹H nuclear magnetic resonance (NMR) (CDCl₃, 850 MHz) and ¹³C NMR (CDCl₃, 213 MHz) (Table 1); HRESIMS m/z = 520.3758 [M]+ (calculated m/z = 520.3764 for C₃₁H₅₂O₆).

2.3.2 Compound 2

Gummy material (0.5 mg, 0.0002%); [α]_D^22 = 81.1 (c 0.01, CHCl₃); IR ν_max (film)/cm: 3,387, 2,958, 2,853, 1,730, 1,713, 1,655, 1,514, 1,246; ¹H NMR (CDCl₃, 850 MHz); ¹³C NMR (CDCl₃, 213 MHz) (Table 2); HRESIMS m/z = 504.3809 [M]+ (calculated m/z = 504.3815 for C₂₃H₄₆O₃).

Table 1: ¹H and ¹³C NMR (850 and 213 MHz) spectral data of compound 1 in CDCl₃

| Carbon no. | δ_C (ppm) | δ_H (J in Hz) | Carbon no. | δ_C (ppm) | δ_H (J in Hz) |
|------------|-----------|---------------|------------|-----------|---------------|
| 1          | 34.0 (CH₂) | 1.87, m       | 16         | 124.0 (CH) | 5.48, dd (3.4, 1.7) |
|            |           | 2.01, m       |            |           |               |
| 2          | 26.9 (CH₂) | 1.67, m       | 17         | 160.1 (CH) |
|            |           | 1.85, m       |            |           |               |
| 3          | 70.9 (CH)  | 5.13, dddd (11.1, 11.1, 5.1, 5.1) | 18 | 19.4 (CH₃) | 0.98, s |
| 4          | 37.4 (CH₂) | 1.58, m       | 19         | 16.9 (CH₃) | 1.35, s |
|            |           | 2.19, m       |            |           |               |
| 5          | 76.4 (C)   |               | 20         | 75.9 (C)  |
| 6          | 76.2 (CH)  | 3.55 dd (3.4, 1.7) | 21 | 30.8 (CH) | 1.37, s |
| 7          | 34.6 (CH₂) | 1.86, m       | 22         | 49.3 (CH₂) | 1.42, m |
|            |           | 2.01, m       |            |           |               |
| 8          | 28.1 (CH)  | 2.05, m       | 23         | 31.0 (CH) | 1.40, m |
| 9          | 53.2 (CH)  | 1.45, m       | 24         | 29.8 (CH) | 1.60, m |
| 10         | 40.2 (C)   |               | 25         | 45.5 (CH) | 1.04, m |
| 11         | 68.7 (CH)  | 3.99, dt (9.4, 5.1) | 26 | 11.7 (CH₃) | 0.75, d (6.8) |
| 12         | 48.1 (CH₂) | 1.49, dd (11.9, 5.1) | 27 | 20.9 (CH₃) | 0.88 d (6.8) |
|            |           | 2.52, dd (11.9, 6.0) | |           |               |
| 13         | 48.0 (C)   |               | 28         | 14.2 (CH₃) | 0.86, d (6.8) |
| 14         | 56.2 (CH)  | 1.60, m       | 29         | 15.8 (CH₃) | 0.78, d (6.8) |
| 15         | 31.0 (CH₃) | 1.83, m       | 30         | 170.0 (C) |
|            |           | 2.08, ddd (15.3, 6.8, 3.4) | |           |               |

*All data were obtained from 1D and 2D NMR measurements. †Implied multiplicities were determined by DEPT (C = s, CH = d, CH₂ = t).
2.4 Determination of anti-proliferative effect of compounds 1 and 3

The cytotoxicity of the isolated compounds was evaluated against (HepG2, PC-3, and HT-29) human cancer cells using sulphorhodamine B assay (SRB), according to the previously published [8,9].

2.5 AO/EtBr staining for detection of apoptosis

The DNA-binding dyes AO and EtBr have been used for the morphological detection of viable, apoptotic, and necrotic cells. The procedures have been done as previously reported [11,12].

2.6 Statistical analysis

Data are presented as mean and SD. Statistical significance was acceptable to a level of \( p < 0.05 \). All statistical analyses were performed using GraphPad Prism software, version 6.00 (GraphPad Software, La Jolla, CA, USA).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

A Red Sea soft coral specimen, identified as *X. umbellata*, was extracted with a mixture of organic solvents at room temperature, yielding a viscous oily material (21.4 g). The total extract was evaluated for its cytotoxic effect against HepG2 and displayed cytotoxicity with IC\(_{50}\) (19.74 ± 1.98 \( \mu \)g/mL). The aforementioned promising anti-proliferative results directed the further chemical investigation of the *X. umbellata* extract. It was subjected to normal-phase silica gel column chromatography and PTLC to give two new steroidal derivatives, compounds 1 and 2 together with a previously identified steroid compound 3 (Figure 1).

### Table 2: \(^1\)H and \(^{13}\)C NMR (850 and 213 MHz) spectral data of compound 2 in CDCl\(_3\)

| Carbon no. | \( \delta_C \) | \( \delta_H \) (J in Hz) | Carbon no. | \( \delta_C \) | \( \delta_H \) (J in Hz) |
|------------|---------------|----------------------|------------|---------------|----------------------|
| 1          | 31.9 (CH\(_2\)) | 1.40, m              | 16         | 123.7 (CH)    | 5.48, dd (3.4, 1.7)   |
| 2          | 26.6 (CH\(_2\)) | 1.65, m              | 17         | 160.8 (C)     | 1.88, m              |
| 3          | 71.0 (CH)      | 5.17, dddd (11.1, 11.1, 5.1, 5.1) | 18         | 18.4 (CH\(_3\)) | 0.99, s             |
| 4          | 37.0 (CH\(_2\)) | 1.68, m              | 19         | 16.6 (CH\(_3\)) | 1.23, s             |
|            |               | 2.18, dd (11.9, 11.1) |           |               |                      |
| 5          | 76.0 (C)       | —                    | 20         | 75.9 (C)      |                      |
| 6          | 76.2 (CH\(_2\)) | 3.55, dd (3.4, 1.7) | 21         | 29.6 (CH\(_3\)) | 1.37, s             |
| 7          | 34.4 (CH\(_2\)) | 1.60, m              | 22         | 49.0 (CH\(_3\)) | 1.49, m             |
| 8          | 28.2 (CH)      | 1.76, m              | 23         | 30.8 (CH)     | 1.56, m             |
| 9          | 45.7 (CH)      | 1.97, m              | 24         | 29.6 (CH)     | 1.41, m             |
| 10         | 38.6 (C)       | 1.38, m              | 25         | 45.5 (CH)     | 1.80, m             |
| 11         | 21.1 (CH\(_2\)) | 1.42, m              | 26         | 11.7 (CH\(_3\)) | 0.76, d (6.8)       |
|            |               | 1.48, m              |           |               |                      |
| 12         | 36.1 (CH\(_2\)) | 2.08, m              | 27         | 21.6 (CH\(_3\)) | 0.88, d (6.8)       |
|            |               | 2.08, m              |           |               |                      |
| 13         | 47.7 (C)       | —                    | 28         | 21.0 (CH\(_3\)) | 0.86, d (6.8)       |
| 14         | 57.1 (CH)      | 1.50, m              | 29         | 15.7 (CH\(_3\)) | 0.78, d (6.8)       |
| 15         | 30.9 (CH\(_2\)) | 1.84, dd (17.0, 12.8) |           | COCH\(_3\) | 170.0               |
|            |               | 2.06, ddd (10.2, 6.8, 3.4) |           | COCH\(_3\) | 21.5               |

\(^a\)All data were obtained from 1D and 2D NMR measurements. \(^b\)Implied multiplicities were determined by DEPT (C = s, CH = d, CH\(_2\) = t).
3.1 Structure elucidation

Compound 1, \([\alpha]_D^23 = -49.6\) (c 0.01, CHCl₃), was obtained as a gummy material and had the molecular formula C₃₁H₅₅O₆, as determined by high resolution electrospray ionization mass spectrometry (HRESIMS), requiring six degrees of unsaturation. The IR spectrum of 1 showed absorptions due to hydroxyl and acetyl groups (\(\lambda_{\text{max}}\) 3,403 and 1,730/cm, respectively). The ¹³C NMR and distortionless enhancement by polarization transfer (DEPT) spectral data of 1 (Table 1) revealed the presence of 31 carbon atoms (Figure S1g–k), including six non-protonated carbons, nine sp³ methines, one sp² methine, seven methylenes, and eight methylys. The quaternary carbons were assigned to one carbonyl (δC 170.0 ppm), two oxygenated (76.4 and 75.9 ppm), and one olefinic (160.1 ppm) along with two in the upfield region (48.0 and 40.2 ppm). The methine carbon included three oxygenated (76.2, 70.9, and 68.7 ppm) and one olefinic (124.0). The total number of methine carbon counts ten after assigning eight methyls (δH 30.8, 21.5, 20.9, 19.4, 16.9, 15.8, 14.2, and 11.7 ppm) from ¹H NMR and heteronuclear single quantum correlation (HSQC) experiments (Figure S1p–r).

The ¹H NMR spectra (Figure S1a–f) displayed four tertiary methyls resonating at δH 0.98, 1.35, 1.37, and 2.03 ppm) and four secondary methyls resonating at δH 0.75 (d, J = 6.6 Hz), 0.86 (d, J = 6.8 Hz), 0.78 (d, J = 6.8 Hz), and 0.78 (d, J = 6.8 Hz)). Two unsaturation degrees were accounted as one trisubstituted carbon–carbon double bond (δH 5.48 and δC 124.0 and 160.1 ppm) and an acetyl function (δH 2.03 and δC 21.5 and 170.0 ppm). Therefore, the remaining four unsaturation degrees suggest a tetracyclic structure for compound 1. The aforementioned information, together with the methylation pattern and several other features appearing in the 1D and 2D NMR spectra, suggested the steroidal nature of 1. The nature of the side chain was determined by the interpretation of the ¹H–¹H correlated spectroscopy (COSY) spectrum (Figure S1l–o). A sequence of correlations started from the isopropyl proton resonating at δH 1.04 (H-25) with the signal at 1.60 (H-24), which in turn is correlated with a methyl at 0.86 (H-28) and a methine at 1.40 (H-23) protons, and the later methine is correlated with a methyl at 0.78 (H-29) and a methylene proton of H-22 was observed. The heteronuclear multiple bond correlation (HMBC) correlations from H-21 (1.37, s) to C-20 (75.9), C-22 (49.3), and C-17 (160.1) established the nature of the side chain as 4,5,6-trimethyl-2-heptyl-2-ol moiety (Figure S1s–v). The later deduction furnished the gross structure of 1 as 23,24-dimethylcholesterol-16-en-pentaβydroxy monoacetate. The position of the five hydroxyl groups was deduced from ¹³C NMR, DEPT, and HSQC spectra. The methine proton resonating at δH 5.13 (dd, J = 11.1, 11.1, 5.1, and 5.1 Hz) implies acetylated hydroxyl located at C-3, since this is the sole available location flanked by two methylene groups. ¹H–¹H COSY and HMBC spectra recognized the positions of the other hydroxyl groups; H-3 and H-19 are both correlated with the quaternary carbon at δC 76.4 (C-5) ppm as well as this carbon is also correlated with the proton resonating at δH 3.55 (dd, J = 3.4 and 1.7 Hz, H-6), which implies that positions 5 and 6 are both hydroxylated. The fourth hydroxyl group was decided by observing the signal at δH 3.99, which appeared as dt with J values 9.4 and 5.1 Hz. This proton could be positioned on several locations within the carboskeleton of 1; however, the HMBC correlations were observed between this proton and the two quaternary carbons at C-10 and C-13. The OH group is positioned on C-11. The fifth hydroxyl group was
deduced from the HMBC to be depicted at C-20, based on the correlations between the methyl group resonated at $\delta^H$ 1.37, s and the olefinic proton resonated at $\delta^H$ 5.48 (dd, $J = 3.4$ and 1.7 Hz) with the C-20 resonated at $\delta^H$ 75.9. The relative stereochemistry of 1 was elucidated on the basis of nuclear overhauser effect spectroscopy (NOESY) correlations (Figure S1w–y) and analyses of $J$ values. The multiplicity of the methine assignment at 5.13 ppm had the normal complexity for the 3α-carbinol proton of an A/B trans-steroid. This unusually downshifted signal is typical of 3β-hydroxysterols bearing a 5α-hydroxyl group, which esterified by acetyl moiety [13]. The downshift of the Me-19 signal at 1.35 was indicative of the β-orientation of the C-6 hydroxyl group. The large $J$-value of H-11 implies its axial orientation and hence α-OH. The strong NOESY correlations between H-3 and H-6 and between Me-19 and H-11 supported the proposed orientations. Since the spectral data of 1 coincided with the reported data and the $R$ configuration was recognized at C-20 [14]. From these data, compound 1 was concluded to be 23,24-dimethylcholesterol-16-ene-3β,5α,6β,11α,20(R)-pentol 3-monooacetate.

Compound 2, $\left[\alpha\right]_D^{20} = 81.1$ (c 0.01, CHCl$_3$), was obtained as a gummy substance and had the molecular formula C$_{31}$H$_{52}$O$_5$, as determined by HRESIMS, requiring six degrees of unsaturation. The IR spectrum of 2 showed absorptions due to hydroxyl and acetyl groups ($\lambda_{max}$ 3,387 and 1,730/cm, respectively). The $^{13}$C NMR spectroscopical data of 2 (Table 2) revealed the presence of 31 carbon signals, which were identified by assistance of the DEPT spectrum. They were categorized into six quaternary carbons, eight sp$^3$ methines, one sp$^2$ methine, eight methylenes, and eight methyls. Analysis of the spectral data of 2 and comparison with those of 1 revealed the great similarity between both structures. However, the lack of 16 mass unit in mass spectrum and the absence of signals due to CH-11 in the NMR spectra ($^1$H and $^{13}$C NMR, DEPT, HSQC, COSY, HMBC, and NOESY) in case of compound 2 allowed the determination of its structure as 23,24-dimethylcholesterol-16-ene-3β,5α,6β,20(R)-tetrol 3-monooacetate. 3β,5α,6β,11α,20β-Pentahydroxygorgosterol (3) was identified by comparing the measured spectral data with the reported [15].

### 3.2 Biological activities

**In vitro** cytotoxicity of compounds 1 and 3 was determined using SRB assay. These activities were assessed against HepG2, PC-3, and HT-29 tumour cell lines over concentration range of 0.01–1,000 μg/mL. Compound 1 showed potent cytotoxic profile against tumour cell lines HepG2, PC-3, and HT-29 with IC$_{50}$ values of 4.70 ± 0.2, 5.60 ± 0.6, and 4.00 ± 0.4 μg/mL, respectively. On the
The other hand, compound 3 showed significant cytotoxicity effect against HepG2, PC-3, and HT-29 with IC50 values 22.20 ± 1.0, ≥100, and 99.30 ± 0.9 μg/mL, respectively. Doxorubicin (positive control) displayed cytotoxicity against HepG2, PC-3, and HT-29 with IC50 values of 0.79 ± 0.06, 1.16 ± 0.56, and 1.70 ± 0.16 μg/mL, respectively (Figure 2). After staining the cells with AO/EtBr, the cells appeared in the form of four colours as follows; living cells (green nuclei), early apoptotic (bright green nuclei), late apoptotic (orange-stained nuclei), and necrotic cells (uniformly orange-stained cell nuclei). In AO/EtBr dual staining, the cells were uniformly stained green with normal, round, intact nuclei, and cytoplasm which indicate the viability of the cell control. On the contrary, the highly early apoptotic cell death was observed in all types of treated tumour cells. The late apoptotic cells are highly present in HepG2 cells with compound 3 compared with other cancer cells except for 1, while no necrotic and late apoptotic appeared with HT-29 cells after treatment with compounds 1 and 3. These results were compared with the control without manifestations of cell death (Figure 3).

### 3.3 Biosynthesis of 30-norisodinosterols carbon skeleton

Dinosterol (4), a C-30 sterol isolated from free swimming dinoflagellates, is the biosynthetic precursor to the cyclopropyl-containing sterol gorgosterol (5) (Figure 1).
Despite the reported information, gorgosterol was originally isolated from several gorgonian species and, moreover, was proved to be a symbiont product and was not isolated from the free swimming dinoflagellates [16].

The unsaturation (Δ24) was common among the initial tetracyclic precursor, lanosterol and cycloartenol, in all sterol-building organisms. The presence of such unsaturation site renders the molecules prone to decorations by reduction (e.g. formation of cholesterol) and methylation, which started from simple methylation at C-24, may extend to multiple alkylations. This situation was common among marine organisms [17]. A hypothetical biosynthetic pathway of the current isolated norisodinosterol derivatives (1 and 2) could be started with brassicasterol (6) which undergoes methylation by a methyalting agent (mainly S-adenosylmethionine). Then reduction of Δ5 and formation of Δ16 could be performed. A more evidenced route to the biosynthesis of these C-29 sterols (Scheme 1) started from compound 4, which lead to the construction of 30-norisodinosterol skeleton through the reduction of Δ25, demethylation at C-4 and then formation of Δ16. It is worthy to mention that the isolation of 23,24-dimethylcholesta-5,22-dien-3β-ol from the soft coral Sarcophyton elegans supports the speculation that dinosterol is the precursor of compounds 1 and 2 along with the symbiont production nature of these compounds [18].

The compounds isolated in the current manuscript are steroidal derivatives. They are characterized by the presence of four rings arranged in an unambiguous molecular configuration. Multi-functionality gave them unique molecular structures. Consequently, the steroids have potential diversity of bioactivity. Generally, steroids have two common biological functions: as vital components of cell membranes that alter membrane fluidity and as signalling molecules. The isolated steroids showed different chemical functionality and potent anti-proliferative activities. After further pharmacological investigation and mechanistically studies, they may be a lead of anti-cancer drug.

4 Conclusion

Xenia umbellata, a soft coral, was collected from the Red Sea and found to produce two new steroidal derivatives, compounds 1 and 2, together with a known steroid, compound 3. The chemical structures of the isolated compounds were determined by analyses of the measured spectroscopic data. The anti-proliferative activities of compounds 1 and 3 have been evaluated against hepatocellular carcinoma (HepG2), prostate adenocarcinoma (PC-3), and colorectal adenocarcinoma (HT-29) human cell lines. Compound 1 exhibited potent cytotoxic effect against tumour cell lines, HepG2, PC-3, and HT-29. Compounds 1 and 3 displayed late apoptotic effect in HepG2 cells. The anti-proliferative activity of compounds 1–3 warranted further investigation. Extra work should be carried out to establish the chemoeccological functions of nordinosterols (1 and 2) and to assess their impact on the host soft coral organism.

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Author contributions: N. O. B., A. A., and W. M. A. was in charge of the study design, supervised the experimental work, carried out collection and interpretation of the data, literature search and wrote the manuscript; and performed the cytotoxicity and apoptosis experiments. W. M. A. and A. A. equally edited the manuscript.

Supplementary materials: The following are available online. The NMR data (1H, 13C, 1H–1H COSY, HMQC, and HMBC) of compounds 1 and 2.

Conflict of interest: The authors state they have no competing interest.

Data availability statement: All data generated or analysed during this study are included in this published article (and its supplementary information files).

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