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ORIGINAL ARTICLE

Two New Xeniolide Diterpenes from the Soft Coral *Xenia umbellata*; Displayed Anti Proliferative Effects

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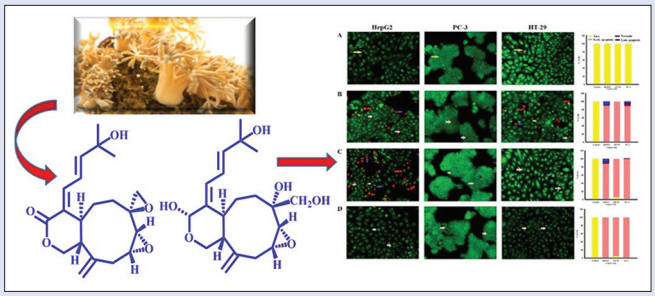
ABSTRACT

Background and Objective: *Xenia* is an octocoral genus of family *Xeniidae*. It contains 98 species and is rich of xenican-type diterpenoids. These compounds play an important role in the biological activity of *Xenia*. Different bioactivities were reported, particularly, anticancer effects. **Materials and Methods:** A specimen of a *Xenia umbellata* was exhausted with organic solvents. Then, the organic extract has been fractionated and purified employing different chromatographic procedures. The spectral information obtained from different nuclear magnetic resonance experiments, mass spectroscopy, infrared, and ultraviolet was the key to elucidate the chemical structures. The anti-proliferative activities of all compounds have been evaluated against hepatocellular carcinoma (HepG2), prostate adenocarcinoma (PC-3), and colorectal adenocarcinoma (HT-29) cells. **Results:** Two new xeniolide-type diterpenes, xeniolide L (1) and xeniolide M (2), along with two known diterpenes, xeniolide K (3) and xeniumbellal (4) were isolated. Compounds 1–4 exhibited significant cytotoxic effect with IC_{50} values ranged from 0.17 ± 0.01 to 64.7 ± 0.40 µg/mL. Compound 1 displayed late apoptotic and necrotic effects in both HepG2 and PC-3, while 2 exhibited late apoptosis in HepG2 cells. **Conclusion:** The isolated xeniolide diterpenes displayed antiproliferative effects against tumor cells (HepG2, PC-3, and HT-29). The new compounds showed late apoptotic and necrotic effects in HepG2 cells.

Key words: Anti-proliferation, apoptosis, diterpenes, octocorallia, Red Sea

SUMMARY

- Two new xeniolide diterpenes (xeniolide L and xeniolide M) along with two known diterpene (xeniolide K and xeniumbellal) were isolated from Red Sea soft coral *Xenia umbellata*. All compounds displayed anti proliferative activities against HepG2, PC 3, and HT 29 human cell lines with IC_{50} values in range between 0.17 ± 0.01 and 64.7 ± 0.40 µg/mL. Xeniolide L displayed late apoptotic and necrotic effects in HepG2 and PC 3, whereas xeniolide M exhibited late apoptosis in HepG2 cells.



Abbreviations used: AO: Acridine orange; COSY: Homonuclear correlation spectroscopy; DEPT: Distortionless enhancement by polarization transfer; EtBr: Ethidium bromide; HepG2: Hepatocellular carcinoma; HMBC: Heteronuclear multiple-bond correlation spectroscopy; HSQC: Heteronuclear single-quantum correlation spectroscopy; HT-29: Colorectal adenocarcinoma; IR: Infrared; MS: Mass spectroscopy; NMR: Nuclear magnetic resonance experiments; NOESY: Nuclear Overhauser effect spectroscopy; PC-3: Prostate adenocarcinoma; PTLC: Preparative thin-layer chromatography; SRB: Sulphorhodamine B assay; TLC: Thin-layer chromatography; UV: Ultraviolet.

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INTRODUCTION

Alcyonacea (Phylum: Cnidaria; Class: Anthozoa; Subclass: Octocorallia) includes marine invertebrates (e.g., soft corals) which live worldwide in tropical and subtropical seawaters, particularly, intertidal zones or inner reefs below the stony corals.[1] It contains 800 and 1200 soft corals and gorgonians species, respectively. *Alcyonacea* is known for its productivity of both terpenoids and steroids. The soft corals are animals, which possess allelopathic abilities of metabolite production.[2-4]

Family *Xeniidae* (*Alcyonacea*) contains 20 genera and 162 species, which live in tropical waters mainly across the Red Sea, Indian Ocean, and Pacific Ocean, in the form of yellow cylindrical of clavate colonies.[3] It has diversity of feather-like tentacles and polyps which are responsible for rhythmic pulsing motion. Thus, they are identified as pulsing *Xenia* and pom-pom *Xenia*. It is known for its productivity of terpenoids and steroidal derivatives.[5-7]

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Table 1: ^{1}H and ^{13}C nuclear magnetic resonance experiments (850 and 213 MHz, respectively) spectral data of compounds 1 and 2 in $CDCl_3$

1		2	
^{13}C	^{1}H, Multi *J* in Hz	^{13}C	^{1}H, Multi *J* in Hz
69.3	Ha 4.29, dd, *J*=11.0, 4.3	63.8	Ha 3.70, dd, 11.1, 8.5
	Hb 4.11, dd, *J*=11.0, 8.5		Hb 3.37, *J*=11.0, 5.1
168.8	-	93.4	5.56, s
141.8	-	137.4	-
33.6	3.65, dd, *J*=11.9, 6.0	34.8	3.52, dd, 11.9, 6.0
32.7	Ha 1.82, ddd, *J*=13.6, 10.2, 6.0	30.3	Ha 1.80, m
	Hb 1.77, dddd, *J*=13.6, 11.9, 10.2, 6.0		Hb 1.85, m
29.8	2.20, ddd, *J*=13.6, 10.2, 6.0	23.4	Ha 1.45, m
	1.81, ddd, *J*=13.6, 10.2, 6.0		Hb 1.85, m
54.8	-	73.5	-
55.8	3.25, d, *J*=3.4	58.4	3.00, d, *J*=4.3
58.5	3.07, ddd, *J*=11.1, 4.3, 3.4	59.1	3.11, ddd, *J*=11.1, 4.3, 4.3
32.7	2.83, dt, *J*=13.6, 4.3	28.5	Ha 2.77, dt, *J*=12.8, 4.3
	2.69, dd, *J*=13.6, 1.7		Hb 2.66, br dt, *J*=12.8, 1.7
141.2	-	143.6	-
43.6	2.76, ddd, *J*=11.9, 8.5, 4.3	52.8	2.40, dd, *J*=17.8, 12.75, 4.25
151.1	6.23, d, *J*=15.3	122.0	5.89, d, *J*=15.3
121.7	6.87, dd, *J*=15.3, 11.9	120.7	6.52, dd, *J*=15.3, 11.1
142.0	7.17, d, *J*=11.9	142.7	5.87, d, *J*=11.1
71.2	-	71.0	-
29.3	1.35, s	29.8	1.33, s
29.7	1.37, s	29.8	1.33, s
49.2	2.99, d, *J*=3.4	62.2	3.53, d, *J*=11.1
	2.73, d, *J*=3.4		3.70, d, *J*=11.1
117.7	Ha 5.25, s	118.9	Ha 5.17, s
	Hb 5.21, s		Hb 5.13, s

Xenia is an octocoral genus (*Xeniidae*), which contains 98 species. It is rich in xenican diterpenes of eight or nine macrocyclic carbon scaffold. These metabolites are characterized by having a cyclononane skeleton, which is categorized into five subclasses: xenicins, xeniolides, xeniaphyllanes, xeniaethers, and azamilides. These compounds play an important role in the biological activity of *Xenia*. Different bioactivities were reported, particularly, anticancer effects.[8]

The current results are considered as a part of continuation of our research program, which aimed at the discovery of bioactive metabolites from marine sources.[9,10] The current study has designed to evaluate the potential anti-proliferative activity of the diterpenes, which isolated from the Red Sea soft coral *Xenia umbellata (X. umbellata)* Lamarck against hepatocellular carcinoma (HepG2), prostate adenocarcinoma (PC-3), and colorectal adenocarcinoma (HT-29) human cells. The isolated compounds (1-4) showed potent activities, and consequently their apoptotic and necrotic effects were evaluated by using DNA binding dyes (Acridine orange [AO] and ethidium bromide [EtBr]). This technique was employed to measure the morphological feature of the viable, apoptotic and necrotic cells as previously published.[11]

MATERIALS AND METHODS

Animal material

X. umbellata was collected by employing 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). It was taxonomically identified by Prof. Mohsen El-Sherbiny (Faculty of Marine Sciences, King Abdulaziz University), and a voucher specimen (XC-2018-11/2) was deposited at the Faculty of Marine Sciences.

Extraction and isolation

The semi-dried soft coral (265.0 g) was exhausted with similar volumes of CH_2Cl_2/MeOH (3 L × 1 L) at room temperature. Vacuum drying of the extract yielded 21.4 g (oily residue). The extract was loaded on 60G silica gel column (100 cm × 3.2 cm) and eluted by *n*-hexane with increasing proportions of EtOAc to yield fractions, each of 50 mL. The fraction, which was eluted with *n*-hexane-EtOAc (50:50), afforded 1 and 3, while the fraction eluted with n-hexane-EtOAc (40:60), afforded compound 4. Finally, the fraction, which was eluted with *n*-hexane-EtOAc (25:75), gave 2. Preparative thin-layer chromatography (TLC) plates (20 cm × 20 cm, 0.25 mm thickness) were employed for purification of the fractions, by using appropriate solvent systems and yielded 1–4.

Characterization of the isolated compounds

Xeniolide L (1)

The portion eluted with *n*-hexane:EtOAc (50:50, v/v) (R_f = 0.32, 7.0 mg) was subjected to preparative thin-layer chromatography (PTLC) using a mixture of *n*-hexane:EtOAc (40:60). The band with R_f = 0.20 (Ultraviolet U_{254} active and develops dark blue color up on spray with sulfuric acid reagent) gave an oily material (1.9 mg, 0.00074%); $[\alpha]_D^{22}$ + 74.0 (*c* 0.02, $CHCl_3$); IR υ_{max} (film) cm^{-1}: 3432, 2961, 2924, 2852, 1704, 1632, 1459, 1461, 1187; UV λ_{max} (nm) 275; HRESIMS *m/z* = 346.1774 [M] + (Calculated *m/z* = 346.1780 for $C_{20}H_{26}O_5$); ^{1}H Nuclear magnetic resonance (NMR) ($CDCl_3$, 850 MHz) and ^{13}C NMR ($CDCl_3$, 213 MHz) [Table 1].

Xeniolide M (2)

The portion eluted with *n*-hexane:EtOAc (25:75, v/v) (R_f = 0.24, 9.1 mg) was subjected to PTLC using a mixture of *n*-hexane:EtOAc (20:80). The band with R_f = 0.15 (UV_{254} active and develops blue-violet color with sulfuric acid reagent) gave a gummy material (2.1 mg, 0.00082%); $[\alpha]_D^{22}$ + 87.0 (*c* 0.02, $CHCl_3$); IR υ_{max} (film) cm^{-1}: 3443, 2960, 2921, 2851, 1634, 1462, 1108; UV λ_{max} (nm) 230; HRESIMS *m/z* = 366.2036 [M] + (Calculated *m/z* = 366.2042 for $C_{20}H_{30}O_6$); ^{1}H NMR ($CDCl_3$, 850 MHz) and ^{13}C NMR ($CDCl_3$, 213 MHz) [Table 1].

Known natural compounds

The further isolated natural metabolites were identified as xenolide K (3) and xeniumbellal (4) after comparison of their spectral and physical properties with the published data.[3,8]

Biological activity

Determination of cytotoxic effect of isolated compounds

The cytotoxicity of the new isolated compounds was evaluated against (HepG2, PC-3, and HT-29) human cancer cells using sulphorhodamine B assay (SRB). This assay was performed as previously published.[12]

Acridine orange/ethidium bromide staining for detection of apoptosis

AO and EtBr are DNA binding dyes. They have been used for detection of the morphological features of apoptotic and necrotic cells. The staining were performed according the protocol as previously published.[13,14]

Statistical analysis

Data were presented as mean standard deviation unless otherwise indicated. Significance of the statistical analysis was acceptable to a level of $P < 0.05$. All analyses and graphs were achieved by using GraphPad Prism software, version 6.00 (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Chemistry

A specimen of a soft coral, identified as *X. umbellata*, was collected from the Red Sea. The material was exhausted with organic solvents. Then, the extract has been purified by using different chromatographic techniques. This led to isolation of four xeniolide-type diterpenes (1-4), two of them are new, xeniolide L (1) and xeniolide M (2) [Figure 1]. In this manuscript, discussion of the chemical structures of the new compounds is reported [Table 1 and Figures 1, 2]. The two known diterpenes were identified by comparing the measured spectral data with the published data of xeniolide K (3) and xeniumbellal (4).[3,8]

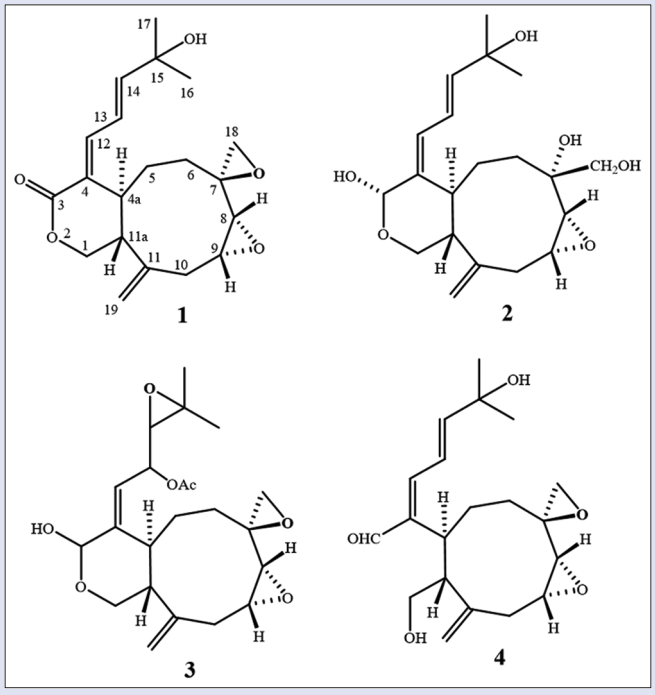

Figure 1: Compounds (1-4) isolated from *Xenia umbellata*

Compound 1 was obtained as an optically active colorless oil (a) $[\alpha]_D^{21}$ + 74 (*c* 0.02, $CHCl_3$). It was assigned that 1 has molecular formula $C_{20}H_{26}O_5$, designated by mass measurement (HREIMS *m/z* 346.1774), indicating eight sites of unsaturation. Signals due to 20 carbons in the ^{13}C NMR spectrum of 1 were assigned by interpretation of the DEPT NMR experiment, into two methyl groups, six methylenes, seven methines, and five quaternary carbons [Table 1].

It was deduced from these data that four of the eight degrees of unsaturation within **1** were due to multiple bonds (3×C = C) and a carbonyl group. This was supported by absorption band at λ_{max} 275 nm in the UV spectrum, which referred to a conjugated carbonyl group with extended diene to form dieneone moiety.[15] After association of all protons with those of the directly linked carbons by interpretation of heteronuclear single-quantum correlation (HSQC) experiment, a remaining was one proton, which should be a part of hydroxyl function. This deduction was supported by the IR absorption band at 3432 cm^{-1}. On these bases, compound 1 must have tetra-cyclic skeleton.

Extensive interpretation of ^1H-^1H COSY spectrum of 1, disclosed the presence of three spin fragments, which labeled the structure with bold black lines [Figure 2]. Fragment (a) includes a sequence of three double-bond methines and terminated with forked dimethyl groups [δ_H/δ_C 1.35, s, (3H-16) /29.3 (C-16), and 1.37, s, (3H-17) /29.7 (C-17) ppm)], which depicted on quaternary carbinol (δ_C 71.2 (C-15)). Fragment (b) starts with an oxymethylene (CH_2-1) and terminates with the relatively deshielded methylene, assigned at δ_H 2.20 (H_a-6) and 1.81 (H_b-6) [*cf. exp.* and Figure 2]. The third fragment (c) includes a characteristic oxymethylene as a part of oxirane moiety group and another substituted oxidase ring.[9] They are adjacent to each other, then, the chain terminates with the relatively deshielded methylene, assigned at δ_H 2.83 (H_a-10) and 2.69 (H_b-10) [Table 1 and Figure 2].

In HMBC spectrum, series of key correlations enable the construction of the main skeleton and confirming the ^1H-^1H COSY spin system (fragments a-c). These correlations include the remaining uncoupled moieties, to build up the planar structure of 1, particularly, the three correlation peaks of H2-18 with H_2-6, which allowed the confirmation of joining fragment (a to b); correspondingly, three cross-peaks exhibited by the exocyclic double-bond protons at δ_H 5.21, s and 5.25, s (H2-19), which enable the connection between C-11 and C-11a. These correlations which deduced from COSY and HMBC indicated the presence of the cyclononane ring.

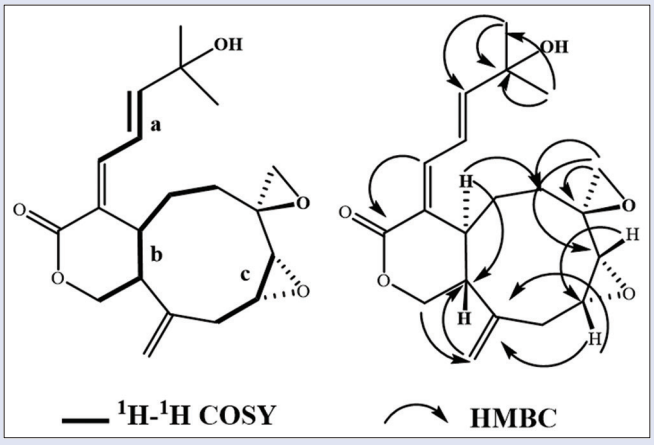

Figure 2: Selected $_1H$-$_1H$ COSY and HMBC correlations of compound 1

Further investigation of the HMBC spectrum revealed cross-peaks between the oxymethylene protons (H_2-1) and with C-11, C-4a and between H-4a and C-1, C-3, C-4, and C-11a, which indicated the structure of lactone ring and its junction to the nine-membered ring. The ^{13}C NMR assignment of C-1 and C-3 indicated that they are a part of lactone ring. The spectral data of 1 were in ful agreement with those reported for xeniolide-A,[16,17] except the appearance of two oxirane rings at positions 7, 8, and 9.

The relative stereochemistry of 1 was determined by investigating the coupling constant (*J*) values, the nuclear overhauser effect spectroscopy (NOESY) correlations, and matching them with the previously reported similar metabolites from *X. umbellata*. A *cis*-configuration was assigned to the epoxy moiety 8(9) based on small measured coupling value (3.4 Hz). A *trans*-configuration for H-4a and H-11a was concluded based on the large coupling value *J* (11.9 Hz) observed between them. The *E*-geometry was assigned to the 4 (12) double bond on the basis of the NOE cross-peaks observed between H-11 and H-12 and between H-13 and H-4a. The *E*-geometry of the Δ^{13} double bond was assigned by the large coupling constant value (*J* = 11.9 Hz) observed between H-13 and H-14. The proposed structure of the epimeric 7 (18) epoxide was elucidated based on NOESY cross-peaks and confirmed by coinciding the measured spectral data with reported diepoxy of havannahine.[18] Compound 1 is a new xeniolide diterpene, the name xeniolide L was proposed.

Compound 2 was obtained as an optically active gummy material (a) $_D^{21}$ + 87 (*c* 0.02, $CHCl_3$). It was assigned that 2 has the molecular formula $C_{20}H_{30}O_6$, designated by mass measurement (HREIMS *m/z* 366.2036), implying six unsaturation sites. Signals due to 20 carbons in the ^{13}C NMR spectrum of 2 were categorized by applying DEPT technique, as two methyl groups, six methylenes, eight methines, and four quaternary carbons (*cf. exp.*).

^{13}C NMR spectrum revealed three multiple bonds (3×C = C) and reflects the presence of tricyclic structure. The absorption band at λ_{max} 230 nm in the UV spectrum and the absence of carbonyl function indicated the presence of a conjugated diene. HSQC experiment showed the association of 27 protons to carbon atoms, the remaining protons (3H), should be a part of three hydroxyl groups. This deduction was supported by the IR absorption broad band at 3443 cm^{-1}. On these bases, compound 2 is trihydroxy tricyclic skeleton.

Extensive interpretation of ^{1}H-^{1}H COSY spectrum of 2, disclosed the presence of three spin fragments, which labeled the structure with bold black lines which labeled a, b, and c [Figure 2]. The fragment (a) includes a sequence of three double-bond methines and terminated with forked dimethyl groups (δ_H/δ_C 1.33, s, [3H-16]/29.8 [C-16] and 1.33, s, [3H-17]/29.8 [C-17] ppm]), which depicted on quaternary carbinol (δ_C 71.0 [C-15]). The remaining fragments (b and c) are closely similar to those in compound 1.

Investigation of the HMBC spectrum indicated that the chemical structure of 2 is similar to that of 1, but two positions; absence of carbonyl group at C-3, which reduced into hydroxyl function (δ_C/δ_H 93.4/5.56). The down-field chemical shift values indicated a reduced lactonic carbonyl (anomeric proton). The second position was the oxirane-ring opening at C-7 and the appearance of two hydroxyl groups; a quaternary alcohol (δ_C 73.5 [C-7] and hydroxyl methylene moiety (δ_H/δ_C 3.70 and 3.53/62.2 [C-18]).

The relative stereochemistry of 2 was established by investigating the coupling constant (*J*) values and confirmed by matching its spectroscopic data with those published from *Xenia* diterpenes and comparing the NOESY correlations of 2 with those obtained from 1. Both compounds are similar in all stereochemistry except positions 3 and 7, which are still not interpreted. The OH-3 group has β-orientation based on the

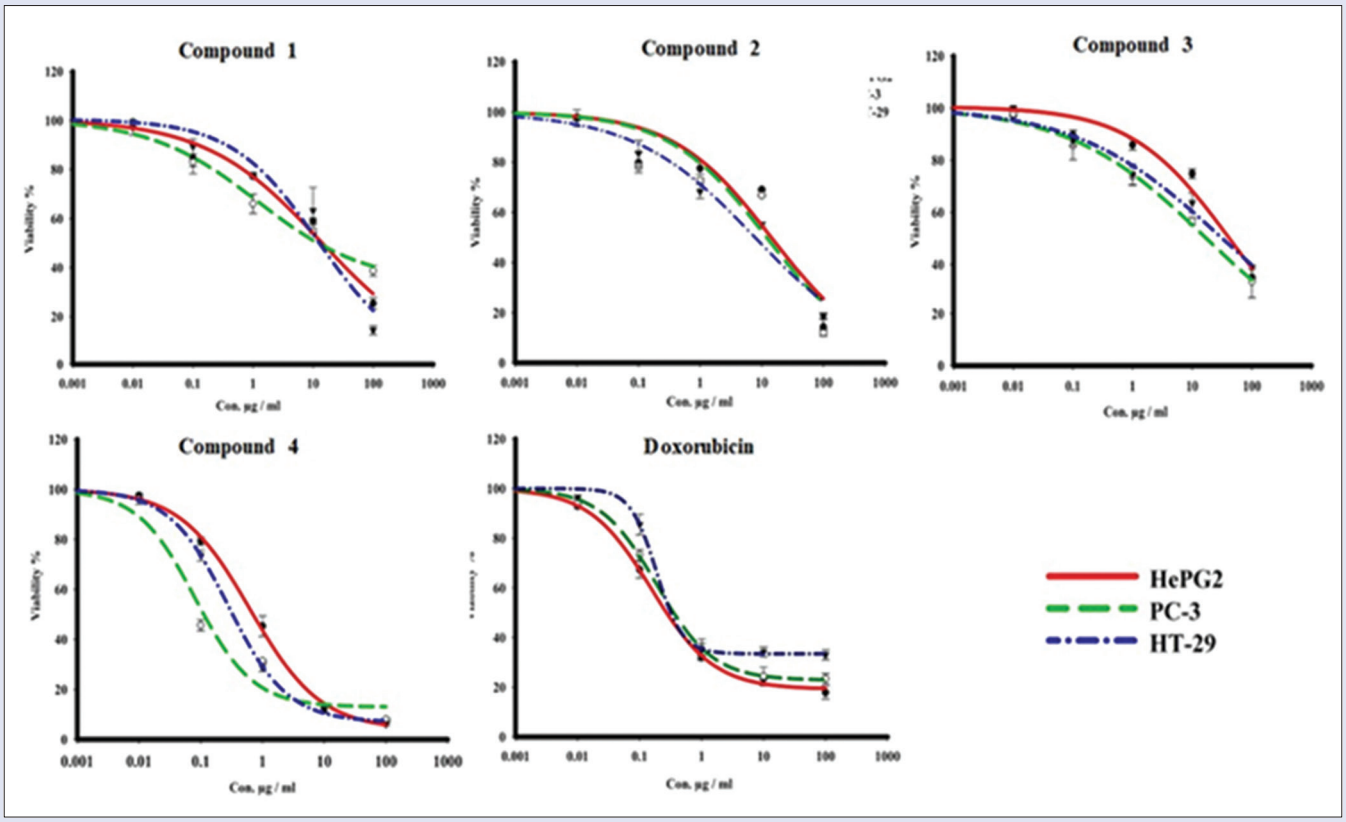

Figure 3: The dose–response curves of the compounds (1–4) cytotoxicity against hepatocellular carcinoma, prostate adenocarcinoma, and colorectal adenocarcinoma human cells

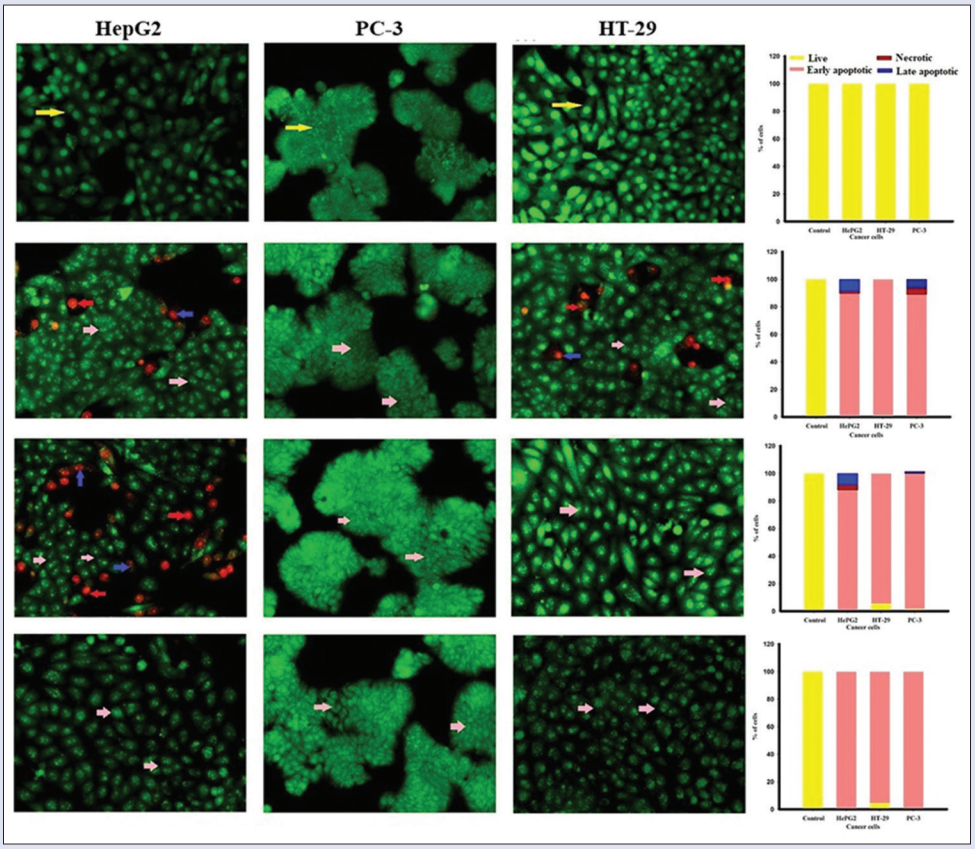

Figure 4: Cell apoptosis observed using fluorescence microscope (×200). Cells were treated with IC_{50s} of compounds 1 (b), 2 (c), 3 (d), and control (a) for 48 h and stained with acridine orange-ethidium bromide

cross-correlation between H-3 and H-4a. Similarly, OH-7 shares the same orientation based on the cross-correlation between H-18 and H-11a. **2** is a new xeniolide diterpene, the name Xeniolide M was proposed.

Biological activities

The cytotoxicity of the isolated compounds has been evaluated using SRB assay against HepG2, PC-3, and HT-29 tumor cells, employing 0.01–1000 µg/mL. Compound 1 exhibited potent cytotoxic effect against tumor cells, HepG2, PC-3, and HT-29 with IC_{50} values 36.8 ± 1.10, 24.90 ± 1.31, and 13.9 ± 2.53 µg/mL, respectively; Compound 2 exhibited potent cytotoxic effect against tumor cells, HepG2, PC-3, and HT-29 with IC_{50} values 14.7 ± 0.4, 10.9 ± 0.51, and 4.7 ± 0.52 µg/mL, respectively; Compound 3 exhibited potent cytotoxic effect against tumor cells, HepG2, PC-3, and HT-29 with IC_{50} values 46.2 ± 0.71, 48.2 ± 0.52, and 64.7 ± 0.40 µg/mL, respectively; Compound 4 exhibited potent cytotoxic effect against tumor cells, HepG2, PC-3, and HT-29 with IC_{50} values 0.57 ± 0.05, 0.17 ± 0.01, and 0.38 ± 0.01 µg/mL, respectively. Doxorubicin (positive control) displayed cytotoxicity against HepG2, PC-3, and HT-29 with IC_{50} values 0.79 ± 0.06, 1.16 ± 0.56, and 1.7 ± 0.16 µg/mL, respectively [Figure 3].

After staining the cells with AO/EtBr, the cells were appeared in the form of four colors as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), 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 that indicates the viability of the cell control. Whereas, the highly early apoptotic cell death was observed in all types of treated tumor cells. The late apoptosis is obviously observed in HepG2 cells with compounds 1 and 2, compared with other tested cancer cells. Whereas, compound 1 exhibited late apoptosis against PC-3 cells. Additionally, compounds 1 and 2 displayed necrotic effect with similar intensity against HepG2 cells however, compound 1 showed necrotic activity against PC-3 cells. The obtained results were referenced to the control that absence of any cell death manifestations [Figure 4].

CONCLUSION

The Red Sea soft coral *Xenia umbellata* was found to produce two new xeniolide-type diterpene; xeniolide L (1) and xeniolide M (2), along with two known diterpene xeniolide K (3) and xeniumbellal (4). All isolated compounds displayed anti-proliferative activities against HepG2, PC-3, and HT-29 human cell lines with IC_{50} values in range between 0.17 ± 0.01 and 64.7 ± 0.40 µg/mL. Compound 1 displayed late apoptotic and necrotic effects in HepG2 and PC 3 respectively, whereas 2 exhibited late apoptosis in HepG2 cells.

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Conflicts of interest

There are no conflicts of interest.

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