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RESEARCH ARTICLE

In vitro anti-proliferative activities of the sterols and fatty acids isolated from the Persian Gulf sponge; *Axinella sinoxea*

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Abstract

Purpose Marine sponges are rich sources of anticancer metabolites. *Axinella sinoxea* is a less studied sponge, found in the Larak Island's waters, of the Persian Gulf. In the present study, we have explored the cytotoxic properties and chemical constituents of *A. sinoxea*.

Methods Repeated silica gel flash column chromatography of methanol extract of the *Axinella sinoxea* sponge, yielded fatty acid and sterol fractions. These fractions were analyzed by GC-MS and their anti-proliferative activities were evaluated by MTT assay against three human cancer cell lines including MOLT-4, MCF-7 and HT-29 as well as NIH/3 T3 fibroblast cells. The sterol-rich fractions were pooled and purified by HPLC and its sub fractions' cytotoxic activities were evaluated by MTT assay against MOLT-4 and NIH/3 T3 cells.

Results The GC-MS spectral analysis of a fraction eluted with hexane: diethyl ether (90: 10), resulted in the identification of twelve fatty acids, including five linear chain saturated fatty acids; tetrdecanoic acid (**1**), pentadecanoic acid (**3**), hexadecanoic acid (**5**), heptadecanoic acid (**7**), and octadecanoic acid (**10**); one branched chain isoprenoid fatty acid, 4,8,12-trimethyltridecanoic acid (**2**); four monoenoic fatty acids; 9-hexadecenoic acid (**4**), 7-methyl-6-hexadecanoic acid (**6**), 9-octadecenoic acid (**8**) and 11-octadecenoic acid (**9**) and two polyunsaturated fatty acids; 5,8,11,14-eicosatetraenoic acid (**11**) and 4,7,10,13,16,19-docosahexaenoic acid (**12**). Spectral analysis of a non-polar fraction eluted with hexane: diethyl ether (85: 15), resulted in the identification of eight steroids including: cholesta-5,22-dien-3β-ol (**13**), cholest-5-en-3β-ol (**14**), ergosta-5,22-dien-3β-ol (**15**), ergost-5-en-3β-ol (**16**), stigmasta-5,22-dien-3β-ol (**17**), γ-sitosterol (**18**), 33-norgorgosta-5,24(28)-dien-3β-ol (**19**) and stigmasta-5,24(28)-dien-3β-ol (**20**). Fatty acids-containing fraction was active against HT-29 cell line with IC_{50} 26.52 ± 8.19 μg/mL, while the steroids-rich fraction was active against the three above mentioned cell lines with IC_{50} values of 1.20 ± 0.24, 4.12 ± 0.40 and 2.47 ± 0.31 μg/mL, respectively. All of the above-mentioned fractions and sub-fractions were inactive (IC_{50}s > 50 μg/mL) when assayed against normal fibroblast cells.

Conclusion The present study suggests *A. sinoxea* as a potential natural source of cancer chemotherapeutics.

Keywords Fatty acids · Steroids · Cytotoxic · Marine sponge · *Axinella sinoxea* · Persian gulf

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Introduction

Many researchers have investigated marine organisms for potential biologically active metabolites. Various classes of metabolites, including fatty acids, lipid-containing fatty acids (FAs) and sterols are reported in marine organisms, especially from sponges and gorgonians [1–3]. Fatty acids as hydrophobic metabolites are widely distributed in the nature and play an important role as a barrier in separating the cell contents from the extracellular medium in living organisms [1, 4]. Steroids

are metabolic derivatives of triterpenes that have two main biological functions: structural role, such as cholesterol, which are important components of cell membrane, and physiological roles such as signaling molecules and activate steroid hormone receptors [5].

Marine sponges are the ancient multicellular organisms, which contain a wide variety of common and unusual FAs, and steroids ranging from simple to complex structures. These organisms are known as biological habitats for a wide variety of microorganism from various taxonomic groups including: bacteria, cynobacteria, microalgae, fungi, and protozoa. Their symbionts can occupy up to 40% of the sponge tissues [3, 6, 7]. Therefore, the total FAs and steroid contents of a sponge is the sum of FAs and sterols of the sponge cells and symbiotic organisms [4, 6].

The lipid compositions of twenty genera of marine sponges from the Demospongiae class were analyzed and shown to contain unusually high levels of C_{24}-C_{30} (34–79%), and the sponges specific FAs [8]. Some sponges' lipids are known as biologically active constituents [3, 4, 7, 9]. For instance, mixtures of $\Delta^{5,9}$-unsaturated fatty acid (UFAs) have cytotoxic activity against mouse Ehrlich carcinoma cells and a hemolytic effect on mouse erythrocytes [10]. Additionally, the cytotoxicity of FAs isolated from marine sponges were evaluated on human glioma (U87) and neuroblastoma (SH-SY5Y) cell lines, and showed that FAs are potential cytotoxic metabolites [9].

The genus *Axinella* belonging to the class of Demospongiae, order Axinellida and family Axinellidae, are widely used for drug discovery. Funel et al. investigated the cytotoxic activity of steroids from the Indian Ocean sponge; *Axinella* cf. *bidderi* and showed that extruded epoxy sterols have strong cytotoxic activity against human tumoral cell lines including: ovary (IGROV-ET), pancreas (PANC1) and lung cell lines (NSCLC N6-L16) [11]. The steroids extracted from *Axinella carteri* exhibited the potential of being agonistic toward nerve growth factor (NGF) [6].

Despite the long Iranian coastline of the Persian Gulf, there have been few reports on the screening of its sponges for anticancer metabolites; therefore as part of continuing investigation for finding cytotoxic metabolites from the Persian Gulf sponges, we reported steroids as cytotoxic metabolites in *A. sinoxea* for the first time.

Material and methods

Instruments and reagents

GC-MS analyses were performed using an Agilent 7890A GC coupled to HP-6890 mass spectrometer operating in EI mode at 70 ev. The GC was equipped with a HP-5 MS (J & W Scientific column, 30 m × 0.25 mm i.d., 0.25 μm film thickness). Reversed-phase HPLC analyses were performed using a Knauer analytical HPLC with a K-1001 pump and a four channel K-2600 UV detector set at λ 210, 254, 320 and 365 nm. The HPLC column was Eurospher-100-5 C4, 250 × 4.6 mm, Knauer, Germany. The open and flash column chromatography (FCC) separations were performed using silica gel 60 (0.063–0.200 mm particle size), and (0.040–0.063 mm particle size), respectively. The TLC analyses were performed on silica gel 60 F_{254} pre-coated aluminum plates (0.25 mm film thickness). The adsorbents were purchased from Merck Chemical Company, Darmstadt, Germany.

Animal material

Axinella sinoxea [12] was collected by scuba diving in July 2015, at a depth of 7–12 m from the Larak Island in the Persian Gulf. The sponge samples were placed immediately in plastic bags containing seawater and transferred to the laboratory on ice and then stored at −20 °C. Identification of the sponge sample was done by M. Nazemi, based on scanning and optical microscope studies on skeletal slides and dissociated spicule mounts by the keys of sponge guide, John N.A. Hooper [13]. Parts of the sponge were stored in 70% ethanol and kept in Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Science museum, as a voucher specimen; 94–7–2-1/1.

Extraction of the sponge material

The sponge (775 g fresh weight) was cut into small pieces (approximately 1 cm) and extracted by methanol (2 × 4 L) for 4 days at room temperature in the dark, to yield a dry syrup after evaporation of the solvents in reduced pressure at 40 °C by rotary evaporator.

Isolation and purification of the sponge's chemical constituents

The methanol extract was subjected to silica gel column chromatography (CC; 50 × 4 cm glass column; 100 g silica gel, 70–230 mesh; Merck chemical company). The column was eluted with *n*-hexane and then the polarity of the mobile phase was increased to pure dichloromethane (DCM) followed by methanol to afford 30 fractions. Fractions 24 and 25 were eluted by dichloromethane: methanol (95: 5) and were mixed based on their similarity in TLC analyses (Fig. 1a). FCC of the fractions F24 and F25 resulted in yielding 21 sub-fractions. The sub-fraction F6,7-F24,25 eluted by hexane: diethyl ether (90: 10) (31 mg) was a pale yellow oil, which was detected as a gray spot ($R_f = 0.5$) on silica gel F_{254} TLC using *n*-hexane: diethyl ether (6:4)mobile phase and after reagent spray with vanillin-sulfuric acid solution followed by heating at 110 °C, while the sub-fraction F10,11,12-F24,25 eluted by hexane: diethyl ether (85: 15) (28 mg) has the same physical

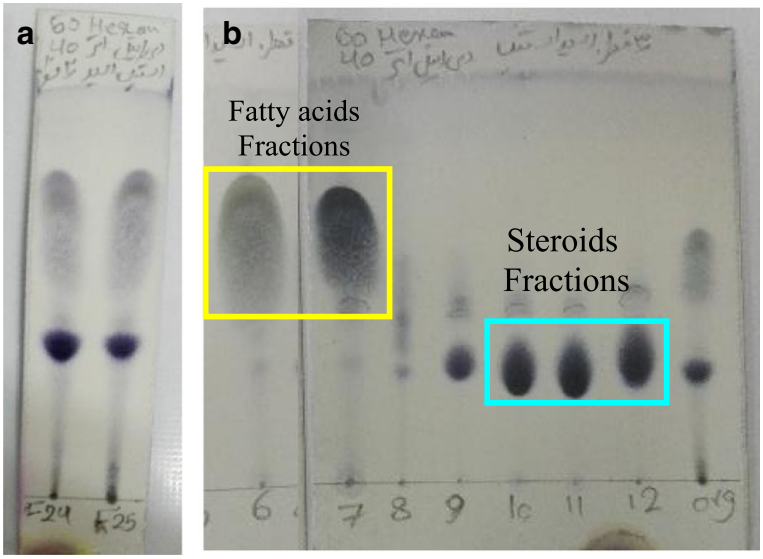

Fig. 1 **a**: Silica gel TLC analyses of the semi purified fractions containing fatty acids and steroids; **b**: silica gel TLC analyses of the separated steroids from the fatty acids fractions resulted from repeated FCC on silica gel of *A. sinoxea* (the last right hand side spot is the original F24 + F25). The TLCs were sprayed with 1% vanillin in 10% sulfuric acid/ethanol reagent followed by heating at 110 °C

appearance, but was detected as a dark-violet spot ($R_f = 0.5$) on the above mentioned TLC analyses conditions (Fig. 1b).

The sub-fractions (F6,7-F24,25) and (F10,11,12-F24,25) could not be analyzed by normal- or reversed phase chromatography and therefore were subjected to GC-MS analyses for detection of its major constituents (Figs. 2 and 3). For further purification of the steroid containing fractions, reversed-phase (RP-4) HPLC analyses were performed using a Knauer analytical HPLC with a K-1001 pump and a K-2600 UV detector set at λ 210 nm [14]. The HPLC column (Eurospher-100-5 C4, 250 × 4.6 mm, Knauer, Germany) was eluted with acetonitrile (solvent B; 95%) in ultrapure water (solvent A; 5%). The flow rate of the mobile phase was set at 1 mL/ min. In the resulting HPLC chromatogram four major peaks were detected, which were then collected by repeated HPLC analyses from the outlet of the column and named as FH2; FH3; FH4 and FH5 (Fig. 4). Cholesterol was used as an external standard for quantification of steroids. The composition of the collected peaks after removal of their solvents and GCMS analyses were determined.

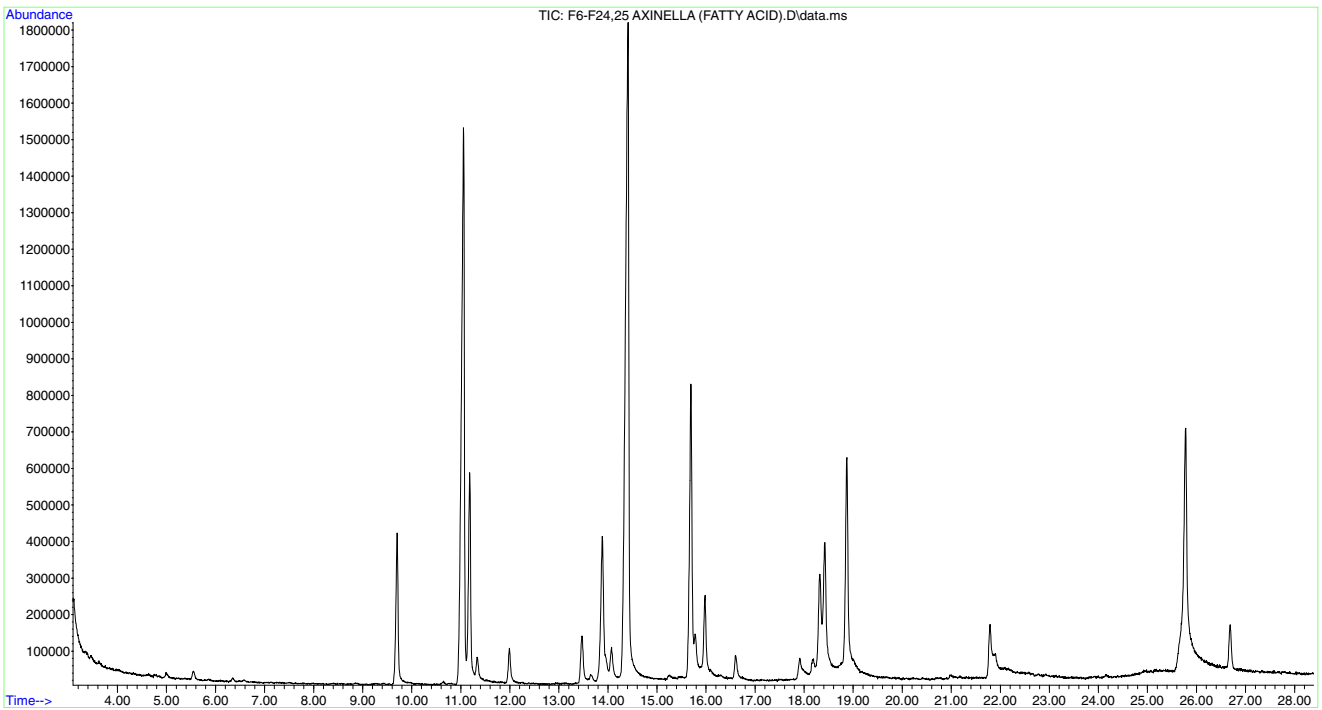

Fig. 2 GC-MS chromatogram of fatty acid fraction (F6,7-F24,25) isolated from *A. sinoxea*

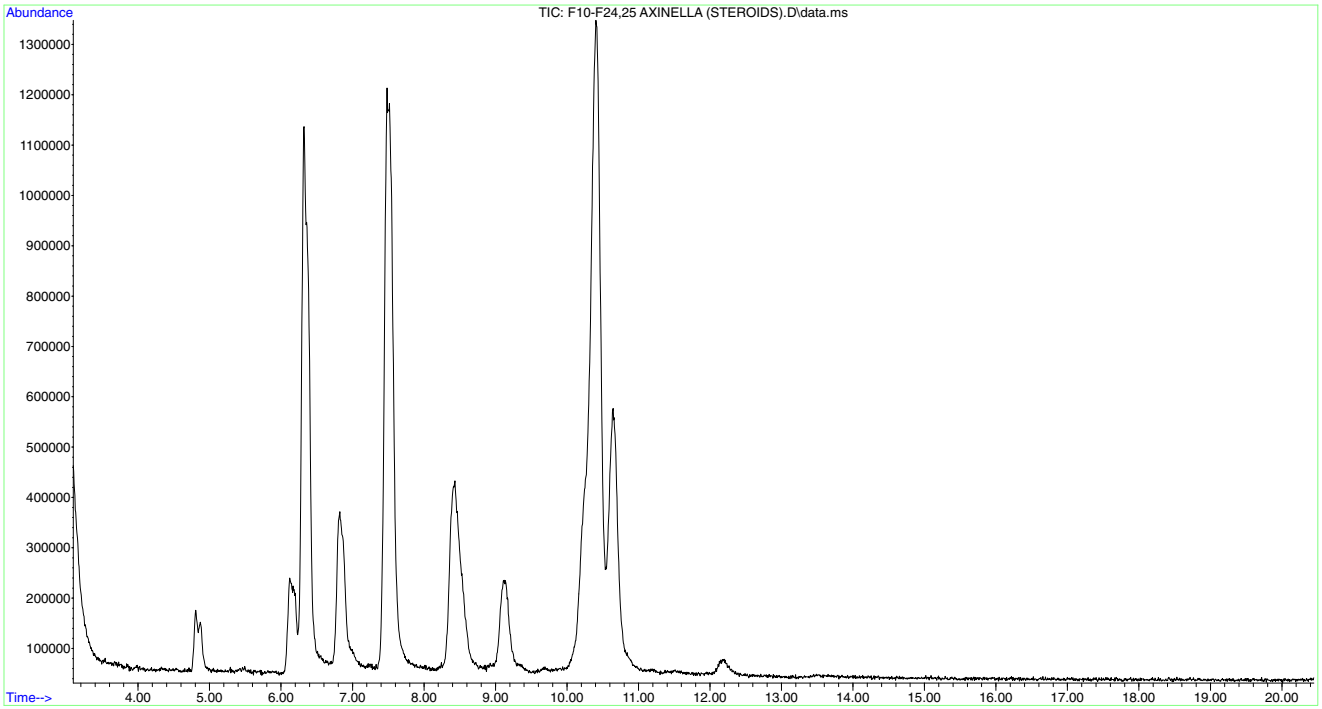

Fig. 3 GC-MS chromatogram of steroidal fraction (F10,11,12-F24,25) isolated from *A. sinoxea*

Fatty acid methyl trans-esterification

In order to modify the performance of GC analyses and quantify the FAs in the sub-fractions F6,7-F24,25, was transformed to its methyl ester derivatives. Chemical transformation to a less polar methyl ester, prior to GC-MS is an essential method, which reduces polarity and increases volatility, and simultaneously, thermal stability of FAs metabolites [15]. Briefly, 500 μL of a solution of 20% BF_3 in MeOH was added to 3 mg of F6,7-F24,25 in a sealed tube and then heated in hot water bath (70 °C). After 1 hour, 1 mL distilled water was added to the above solution, and later the mixture was extracted with n-hexane (1 mL × 3). The organic layers were mixed and dried on anhydrous Na_2SO_4 and evaporated under nitrogen stream. The residue was dissolved in 1 mL pure hexane and then subjected to GC-MS analysis (Fig. 5) [16].

Synthesis of the steroid's trimethylsilyl derivatives

The steroid contents in the sub-fractions F10,11,12-F24,25 was detected by GC-MS. The steroidal compounds were transformed to their trimethylsilyl derivatives to modify its GC-MS analyses. Briefly, 200 μL of derivatization reagent; N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) in 1% trimethylsilyl chloride (TMCS) was added to 2 mg of F10,11,12-F24,25 and vortexed. After 1 h heating in a hot water bath at 60 °C, the reaction mixture was evaporated under a stream of nitrogen. Total of 200 μL DCM was added and the resulting solution was subjected to GC-MS analysis (Fig. 6) [17, 18].

Gas chromatography–mass spectrometry (GC-MS)

To analyze the fatty acids, the GC-oven temperature was programmed from 150 °C for 4 min, rose to 250 °C at 4 °C/min and kept for 10 min at the final temperature. Helium (He) was used as the carrier gas with a flow rate of 1 mL/min and the injector temperature was set at 260 °C in split mode (1:20). The injection volume was 0.2 μL. To analyze the steroids, the oven temperature was set at 265 °C for 20 min, then with a ramp of 5 °C/min rose to 300 °C, and kept for 10 min at the final temperature. The carrier gas was the same as mentioned earlier but the injector temperature was set at 300 °C with a split ratio of 1:10 and 0.1 μL of the sample was injected on GC-MS [17].

Cell lines and culture

MOLT-4 (human lymphoblastic leukemia, Cell bank number: C149) and MCF7 cells (human breast adenocarcinoma, Cell bank number: C135) were obtained from National Cell Bank of Iran, Pasteur Institute, Tehran. HT-29 cell line (human colorectal adenocarcinoma, Cell bank number: IBRC C10097) and NIH/3 T3 cells (mouse embryonic fibroblast, Cell bank number:

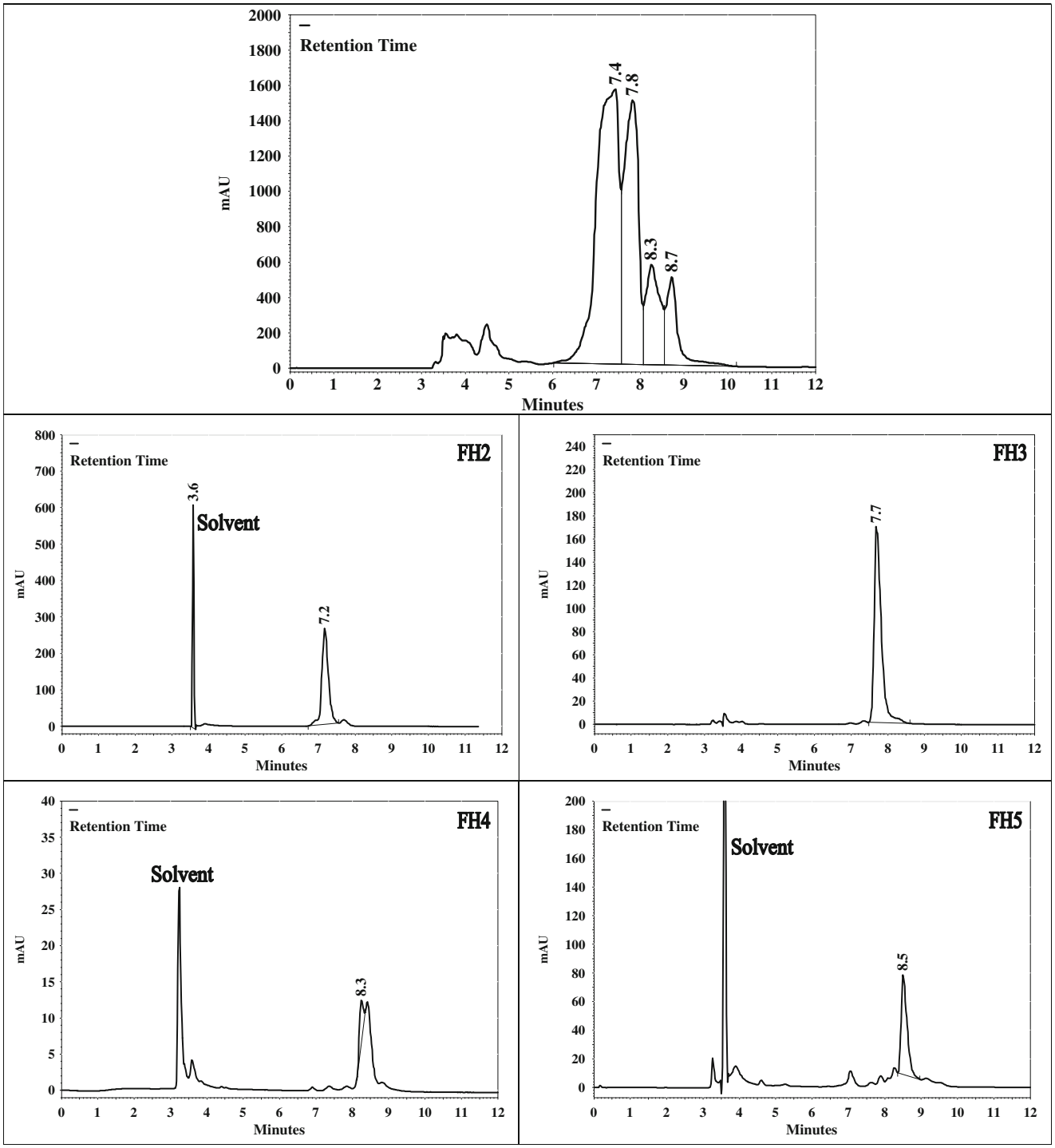

Fig. 4 HPLC chromatograms of steroidal fraction (F10,11,12-F24,25) of MeOH extract of *A. sinoxea*

IBRC C10100) were purchased from Iranian Biological and Genetic Resources Center, Tehran. MOLT-4 and MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% *v*/v fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL), and HT-29 cells were cultured in DMEM medium supplemented with 20% v/v fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) and L-glutamine at 37 °C in humidified air containing 5% CO_2.

Cytotoxicity assay

In vitro cytotoxic activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

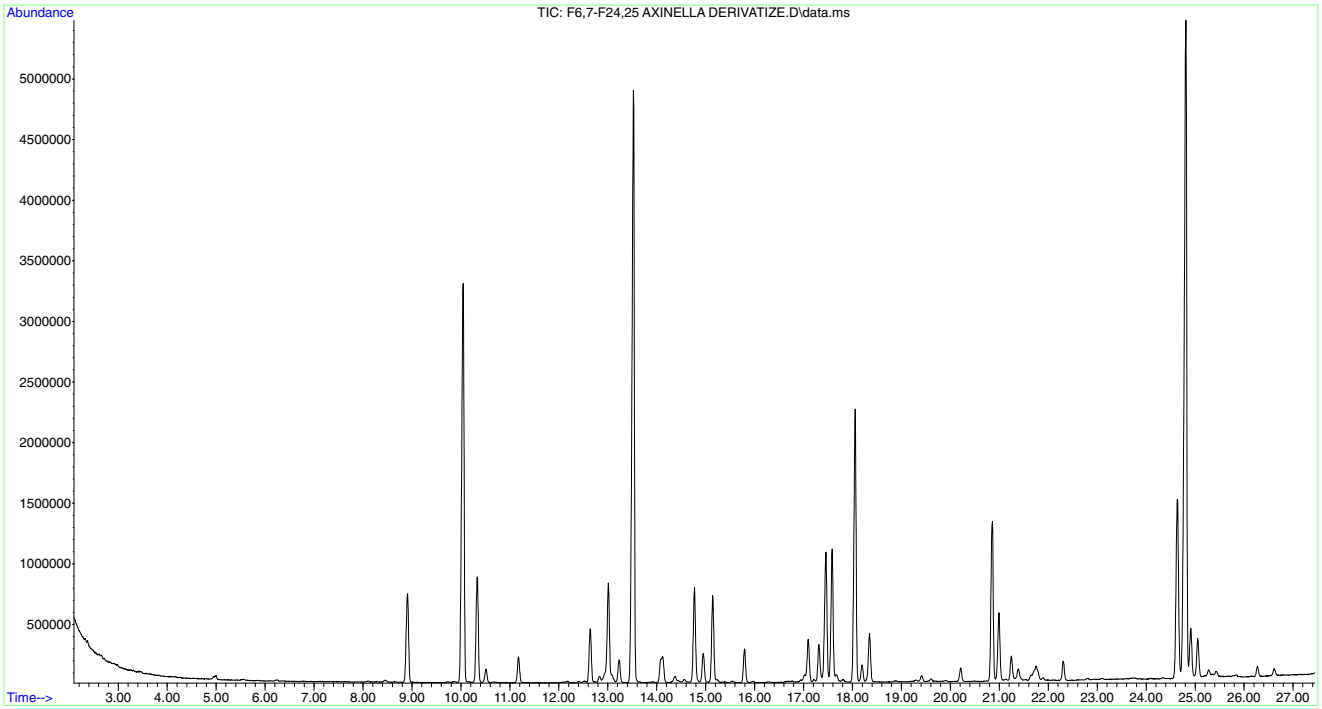

Fig. 5 GC-MS chromatogram of fatty acids fraction isolated from *A. sinoxea* after methyl esterification: it modified both resolution and the shape of peaks

reduction assay. MTT is a colorimetric assay that can be used to evaluate cytotoxic or anti-proliferative effect of different compounds or extracts [19].

The fatty acid's fraction (F6,7-F24,25; 20 mg) and steroid's fraction (F10,11,12-F24,25; 20 mg) and the resulting purified sub-fractions (each 20 mg) were dissolved in 1 mL DMSO, and then diluted in growth medium at least 400 times. All of the prepared fractions were tested against MCF-7, HT-29, MOLT-4 and NIH/3 T3 cells, while the purified sub-fractions were tested against the last two cell lines.

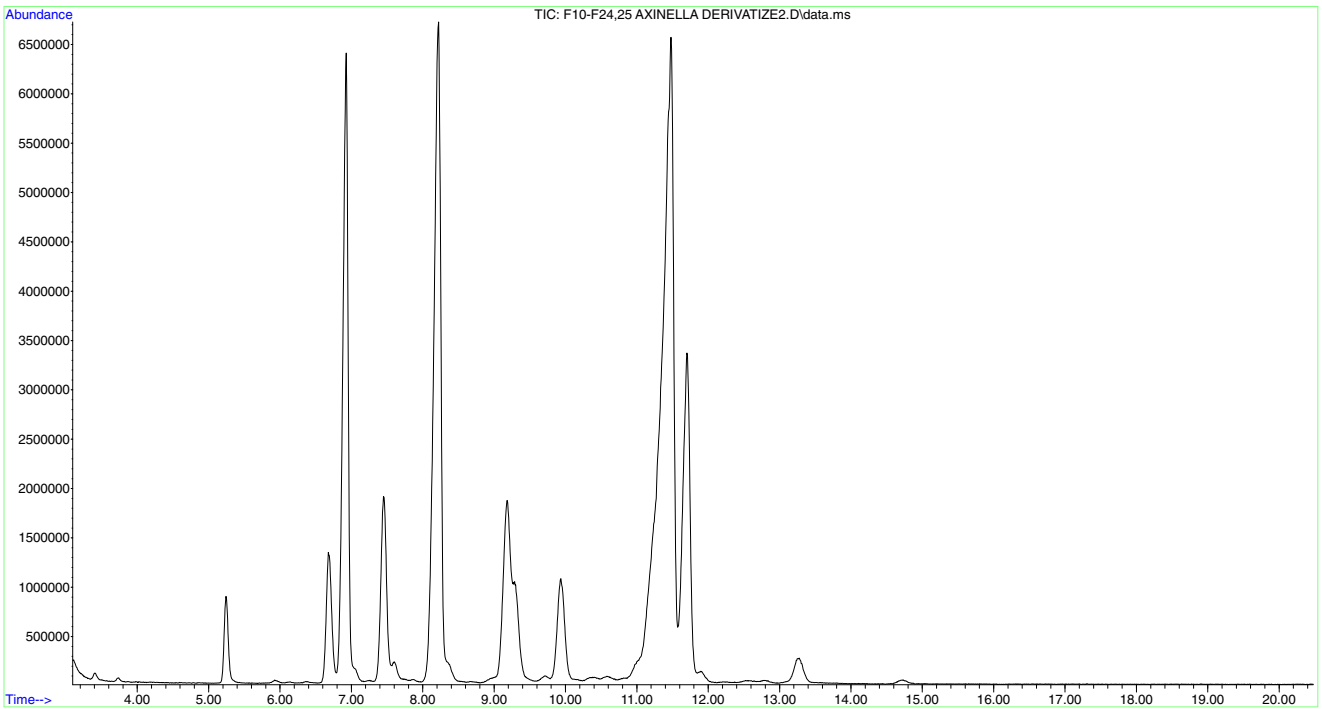

Fig. 6 GC-MS chromatogram of steroids fraction isolated from *A. sinoxea* after trimethylsilyl derivatization

Cells were seeded in 96-well plates at the density of 20,000 cells/mL (MOLT-4 cell line), 30,000 cells/mL (MCF-7 cell line), 10,000 cells/mL (HT-29 cell line) and 40,000 cells/mL (NIH/3 T3 cell line) in 100 μL medium and incubated for 24 h at 37 °C. Then, four different concentrations of each fraction or cisplatin (as positive controls) were added to the wells in triplicate and the plates were incubated at 37 °C for 72 h. Afterwards, the media (80 μL) of each well was replaced by 80 μL MTT solution diluted in RPMI without phenol (concentration of 0.5 mg/mL) and incubated at 37 °C. After 3 h of incubation and formation of formazan crystals, the media was removed and 200 μL DMSO was added to each well to dissolve the crystals. Finally, absorbance was measured at a wavelength of 570 nm with background correction at 650 nm using a microplate reader (model 680, Bio-Rad, Japan) and IC_{50} (concentration that results in 50% inhibition of cell viability) was calculated with Curve Expert statistical software [20, 21].

Spectroscopic data and identification of the compounds

To characterize the FAs and steroids, we calculated the relative retention indices (RRI) and recorded the mass spectrum in the GC-MS chromatograms of the extracts and fractions, and compared them with those reported in the literature; National Institute of Standard and Technology (NIST) [22]; Pub Chem. NCBI [23] and Database of Pheromones and Semiochemicals (pherobase) [24].

Spectroscopic data of FAs

Tetradecanoic acid (myristic acid) (1) GC-MS retention time (R_t: 9.70 min) EIMS *m/z* (rel.int.%): 228$[M]^+$ $C_{14}H_{28}O_2$ (31.6), 199 (8.3), 185 (44.9), 171 (16), 157 (4.9), 143 (15.2), 129 (60.1), 115 (14.7), 97 (15.5), 83 (22.8), 73 (100), 69 (35.6), 67 (6.4), 60 (83.5),55 (57), 53 (4.4), 45 (8).

4,8,12-Trimethyl-tridecanoic acid (2) GC-MS retention time (R_t: 11.06 min) EIMS *m/z* (rel.int.%): 256$[M]^+$ $C_{16}H_{32}O_2$ (6.5), 227 (2.1), 213 (4.2), 199 (29.6), 194 (2.9), 181 (0.6), 171(4.84), 153 (20.7), 143 (36.9), 135 (9.8), 127 (19.8), 113 (25.9), 99 (26.1), 85 (44.6), 83 (34.2), 73 (87.4), 71 (85), 69 (51.6), 57 (100), 55 (62.2), 53 (6.0), 48 (1).

Pentadecanoic acid (Pentadecylic acid) (3) GC-MS retention time (R_t: 11.18 min) EIMS *m/z* (rel.int.%): 242$[M]^+$ $C_{15}H_{30}O_2$ (33.1), 227]242-$CH_3[^+$ (2.1), 213 (6.3), 199 (58.8), 185 (22.4), 180 (5.6), 171(14.1), 157 (16.2), 143 (23.2), 129 (54.1), 115 (17.7), 97 (29.2), 85 (33.9), 73 (100), 71 (49.1), 60 (79), 55 (71.2), 53 (5).

9-Hexadecenoic acid (palmitoleic acid) (4) GC-MS retention time (R_t: 13.89 min) EIMS *m/z* (rel.int.%): 254$[M]^+$ $C_{16}H_{30}O_2$ (5.1), 236 (20.2), 218 (1.8), 207 (3.1), 192 (7.9), 179 (3.9), 165 (5), 152 (11.4), 137 (10.1), 123 (13.4), 111 (25.3), 97 (50.5), 83 (58.2), 69 (74), 55 (100), 45 (6).

Hexadecanoic acid (palmitic acid) (5) GC-MS retention time (R_t: 14.41 min) EIMS *m/z* (rel.int.%): 256$[M]^+$ $C_{16}H_{32}O_2$ (52.1), 227 (10.5), 213 (38.65), 199 (9), 185 (19.5), 171(20), 157 (21), 143 (8.8), 129 (53.7), 115 (18.7), 97 (23), 83 (26.3), 73 (100), 69 (38), 60 (77), 57 (58.1), 45 (7).

7-Methyl-6-hexadecenoic acid (6) GC-MS retention time (R_t: 15.69 min) EIMS *m/z* (rel.int.%): 268$[M]^+$ $C_{17}H_{32}O_2$ (26.6), 256 (2.8), 235 (1.4), 221 (1.6), 213 (2.8), 185 (1.6), 168 (14.1), 153 (12.4), 138 (78), 123 (32.5), 110 (41.6), 97 (61.6), 83 (57.6), 69 (93.4), 55 (100), 53 (13.7).

Heptadecanoic acid (margaric acid) (7) GC-MS retention time (R_t: 15.98 min) EIMS *m/z* (rel.int.%): 270$[M]^+$ $C_{17}H_{34}O_2$ (34.4), 256 (3), 241 (21.1), 227 (21.7), 223 (16.7), 213 (12.3), 205 (12), 199 (2.7), 185 (24.1), 171 (19.6), 167 (6), 153 (8), 139 (13.8), 129 (48.6), 125 (18.9), 111 (27.5), 97 (42), 83 (48), 73 (50), 69 (54.7), 60 (42.3), 57 (100), 55 (78.2), 48 (2.6).

9-Octadecenoic acid (8) GC-MS retention time (R_t: 18.32 min) EIMS *m/z* (rel.int.%): 282$[M]^+$ $C_{18}H_{34}O_2$ (3.3), 264[282-$H_2O]^+$ (20.1), 246 (2.2), 222 (11.4), 207 (4.1), 194 (4.6), 180 (5), 165 (5.5), 151 (9.5), 139 (10.7), 125 (15.7), 111 (27), 97 (52.5), 83 (57.7), 69 (80), 55 (100), 45 (9).

11-Octadecenoic acid (9) GC-MS retention time (R_t: 18.42 min) EIMS *m/z* (rel.int.%): 282$[M]^+$ $C_{18}H_{34}O_2$ (3.7), 264[282-$H_2O]^+$ (19.7), 256 (1.8), 246 (1.4), 235 (3.2), 222 (6.6), 207 (5.5), 193 (3.6), 180 (4.4), 165 (5), 152 (7.5), 139 (9), 125 (10.7), 111 (27.3), 97 (55.6), 83 (57.5), 69 (77.3), 55 (100), 53 (9.2).

Octadecanoic acid (stearic acid) (10) GC-MS retention time (R_t: 18.87 min) EIMS *m/z* (rel.int.%): 284$[M]^+$ $C_{18}H_{36}O_2$ (64), 255 (8.6), 241 (38.2), 227 (9.6), 213 (5), 199 (13.6), 185 (33.7), 171 (13.2), 157 (5.1), 143 (9.6), 129 (60.3), 115 (15), 97 (31.4), 83 (42.1), 73 (100), 60 (72.5), 48 (1).

5,8,11,14-Eicosatetraenoic acid, methyl ester (11) GC-MS retention time (R_t: 21.79 min) EIMS *m/z* (rel.int.%): 318$[M]^+$ $C_{21}H_{34}O_2$ (0.5), 281 (1), 264 (0.5), 247 (1), 220 (2), 203 (5), 180 (4), 175 (5), 150 (14), 147 (9), 133 (15), 119 (20), 106 (28), 105 (35), 93 (47), 91 (74), 79 (100), 67 (69), 55 (39).

4,7,10,13,16,19-Docosahexaenoic acid (12) GC-MS retention time (R_t: 25.78 min) EIMS *m/z* (rel.int.%): 328$[M]^+$ $C_{22}H_{32}O_2$

(0.5), 312 (0.6), 299 (1), 281 (1), 259 (1), 241 (1), 228 (0.7), 215 (2), 192 (4), 173 (5), 159 (9), 145 (12), 135 (7), 131 (22), 128 (10), 119 (39), 117 (27), 105 (50), 95 (17), 93 (45), 91 (87), 80 (36), 79 (100), 77 (42), 67 (53), 55 (31).

Spectroscopic data of FAs after methyl esterification

Tetradecanoic acid, methyl ester (methyl myristate) (1) GC-MS retention time (R_t: 8.91 min) EIMS *m/z* (rel.int.%): 242[M]+ $C_{15}H_{30}O_2$ (5.5), 211 (6.2), 199 (11.9), 185 (2.9), 157 (3.3), 143 (15.6), 129 (5.4), 111 (1.9), 87 (61.3), 74 (100), 57 (14), 55 (27.4), 53 (2.3).

4,8,12-Trimethyl-tridecanoic acid, , methyl ester. (2) GC-MS retention time (R_t: 10.05 min) EIMS *m/z* (rel.int.%): 270[M]$^+$ $C_{17}H_{34}O_2$ (1.2), 241 (1.6), 213 (11.7), 197 (2.5), 171 (0.7), 157 (14.4), 153 (3.1), 127 (4.7), 111 (6.1), 97 (7.8), 87 (100), 74 (37.3), 69 (23.7), 59 (9.3), 55 (38.3), 53 (3.2).

Pentadecanoic acid, methyl ester (3) GC-MS retention time (R_t: 10.34 min) EIMS *m/z* (rel.int.%): 256[M]$^+$ $C_{16}H_{32}O_2$ (6.8), 225 (2.2), 213 (12.4), 199 (4.2), 185 (3.5) 171(3.2), 157 (4.4), 143 (15.9), 129 (6.9), 111 (2.7), 101 (6.5), 97 (8.2), 87 (67.4), 74 (100), 69 (17.8), 57 (17.1), 55 (32.5), 53 (2.6).

9-Hexadecenoic acid, methyl ester (4) GC-MS retention time (R_t: 13.01 min) EIMS *m/z* (rel.int.%): 268[M]+ $C_{17}H_{32}O_2$ (2.6), 236 (12.8), 225 (1.1), 207 (3.9), 194 (11.1), 192 (3.9), 171 (0.8), 152 (11.1), 137 (7.4), 123 (11.4), 110 (15.5), 98 (24.3), 96 (31.6), 83 (36.8), 74 (53.7), 69 (59.4), 67 (37), 55 (100).

Hexadecanoic acid, methyl ester (5) GC-MS retention time (R_t: 13.52 min) EIMS *m/z* (rel.int.%): 270[M$^+$ $C_{17}H_{34}O_2$ (9.5), 242 (0.5), 239 (6), 227 (12.3), 213 (1.8), 199 (4.6), 185 (4.8), 171 (4.7), 157 (1.8), 143 (17), 129 (6.6), 111 (2.1), 97 (6.7), 87 (68.1), 83 (8.7), 74 (100), 69 (15), 57 (16.8), 55 (30.5), 53 (2.3).

6-Hexadecanoic acid, 7-methyl, methyl ester (6) GC-MS retention time (R_t: 14.77 min) EIMS *m/z* (rel.int.%): 282[M]$^+$ $C_{18}H_{34}O_2$ (10.5), 251 (7.3), 235 (1.2), 221 (3.2), 207 (3.5), 193 (1.7), 170 (1.6), 168 (7.5), 167 (15.8), 155 (5), 151 (11.6), 138 (52.4), 123 (18.3), 109 (25.7), 97 (42.3), 81 (44.2), 69 (65.1), 67 (43.6), 55 (100).

Heptadecanoic acid, methyl ester (7) GC-MS retention time (R_t: 15.15 min) EIMS *m/z* (rel.int.%): 284[M$^+$ $C_{17}H_{34}O_2$ (10), 255 (5.6), 241 (11.7), 227 (3), 207 (3), 199 (7.5), 185 (8.5), 171 (1.5), 143 (18.3), 129 (7), 111 (6), 101 (6.1), 97 (13.6), 87 (66.3), 74 (100), 69 (24.7), 57 (44.2).

9-Octadecenoic acid, methyl ester (8) GC-MS retention time (R_t: 17.46 min) EIMS *m/z* (rel.int.%): 296[M]$^+$ $C_{19}H_{36}O_2$ (3), 264 (17.5), 246 (1.1), 222 (11.1), 207 (5), 194 (1.5), 180 (7.6), 166 (5), 152 (5), 139 (9), 125 (8.1), 111 (16.1), 97 (35), 87 (36.5), 83 (42.7), 74 (53), 69 (58.5), 55 (100).

11-Octadecenoic acid, methyl ester (9) GC-MS retention time (R_t: 17.59 min) EIMS *m/z* (rel.int.%): 296[M]$^+$ $C_{19}H_{36}O_2$ (2.5), 264 (15.7), 246 (2), 222 (92), 207 (3.4), 194 (1.5), 180 (7), 166 (5), 152 (4.5), 139 (9), 124 (6.7), 111 (13.4), 97 (31.9), 87 (36), 83 (37.5), 74 (49.3), 69 (59.8), 67 (30.5), 55 (100).

Octadecanoic acid, methyl ester (10) GC-MS retention time (R_t: 18.06 min) EIMS *m/z* (rel.int.%): 298[M$^+$ $C_{19}H_{38}O_2$ (12.3), 267 (4.7), 255 (12), 241 (2.2), 227 (0.9), 213 (2.7), 199 (7.3), 185 (3), 171 (1), 157 (2), 143 (17.2), 129 (6.2), 111 (2.7), 97 (7.7), 87 (68.7), 83 (9.8), 74 (100), 57 (20.7), 55 (32).

5,8,11,14-Eicosatetraenoic acid, methyl ester (11) GC-MS retention time (R_t: 20.85 min) EIMS *m/z* (rel.int.%): 318[M]$^+$ $C_{21}H_{34}O_2$ (0.5), 281 (1), 264 (0.5), 247 (1), 220 (2), 203 (5), 180 (4), 175 (5), 150 (14), 147 (9), 133 (15), 119 (20), 106 (28), 105 (35), 93 (47), 91 (74), 79 (100), 67 (69), 55 (39).

4,7,10,13,16,19-Docosahexaenoic acid, methyl ester (12) GC-MS retention time (R_t: 24.81 min) EIMS *m/z* (rel.int.%): 342[M]$^+$ $C_{22}H_{32}O_2$ (0.5), 281 (1), 259 (1), 241 (1), 223 (0.7), 199 (2), 173 (4), 161 (4), 159 (9), 145 (11), 135 (15), 131 (19), 119 (27), 117 (28), 105 (39), 93 (42), 91 (89), 79 (100), 77 (45), 67 (58), 55 (27).

Spectroscopic data of steroids

Cholesta-5,22-dien-3ß-ol (22-dehydrocholesterol) (13) (R_t: 6.32 min.) EIMS *m/z* (rel.int.%): 384[M]$^+$ $C_{27}H_{44}O$ (84). 369[384-CH_3]$^+$ (18). 351[369-H_2O]$^+$ (23), 341 (2), 324 (3), 300 (80), 285 (25), 271 (50), 255 (100), 253 (13), 241 (11), 229 (14), 213 (33), 207 (10), 199 (21), 187 (18), 173 (20), 159 (63), 145 (60), 133 (58), 119 (36), 111 (50), 105 (56), 91 (56), 79 (49), 69 (74), 55 (88), 43 (49).

Cholest-5-en-3ß-ol (cholesterol) (14) GC-MS retention time (R_t: 6.82 min) EIMS *m/z* (rel.int.%): 386[M]$^+$ $C_{27}H_{46}O$ (100), 371[386- CH_3]$^+$ (42), 368 (53), 353 (38), 326 (13), 302 (13), 301 (59), 276 (23), 275 (61), 255 (31), 247 (15), 231 (18), 213 (47),199 (16), 187 (17), 178 (18), 173 (17), 163 (29), 161 (40), 159 (36), 145 (41), 133 (37), 119 (32), 105 (61), 95 (17), 91 (57), 81 (47), 79 (38), 69 (26), 67 (23), 57 (37), 55 (41), 43 (76), 41 (44).

Ergosta-5,22-dien-3β-ol (brassicasterol) (15) (R_t: 7.48 min.) EIMS *m/z* (rel.int.%): 398[M^+ $C_{28}H_{46}O$ (100), 383 [398-CH_3]$^+$ (14), 380 (7), 365 (16), 355 (7), 337 (15), 314 (11), 300 (50), 285 (14), 283 (10), 271 (70), 255 (80), 241 (8), 229 (13), 213 (29), 199 (15), 187 (12), 175 (11), 161 (24), 159 (51), 147 (39), 145 (50), 133 (45), 131 (24), 121 (20), 119 (35), 117 (15), 109 (41), 107 (40), 105 (45), 95 (37), 93 (33), 91 (42), 83 (34), 81 (53), 79 (36), 69 (80), 67 (36), 57 (21), 55 (67), 43 (36) ,41 (32).

Ergost-5en-3β-ol (24-epicampesterol) (16) (R_t: 8.56 min.) EIMS *m/z* (rel.int.%): 400[M]$^+$ $C_{28}H_{48}O$ (88), 385 [400-CH_3]$^+$ (41), 382 (46), 367 (44), 315 (59), 301 (11), 289 (51), 273 (33), 255 (33), 231 (20), 213 (41), 199 (16), 191 (22), 185 (23), 178 (20), 173 (14), 171 (19), 163 (39), 161 (32), 159 (53), 149 (30), 147 (25), 145 (48), 143 (21), 135 (53), 133 (42), 131 (33), 125 (12), 123 (23), 121 (33), 120 (28), 119 (41), 117 (21), 115 (12), 109 (33), 107 (48), 105 (61), 95 (60), 93 (42), 91 (63), 85 (7), 83 (25), 81 (53), 79 (42), 71 (18), 69 (33), 67 (24), 57 (39), 55 (60), 43 (100), 41 (40).

Stigmasta-5,22-dien-3β-ol (stigmasterol) (17) (R_t: 9.12 min.) EIMS *m/z* (rel.int.%): 412[M]$^+$ $C_{29}H_{48}O$ (100), 397 [412-CH_3]$^+$ (13), 394 (7), 379 (11), 369 (27), 351 (31), 327 (12), 314 (21), 300 (34), 285 (13), 273 (22), 272 (37), 271 (52), 267 (23), 255 (61), 241 (15), 229 (13), 213 (29), 199 (19), 197 (15), 191 (15), 189 (11), 187 (10), 185 (15), 177 (9), 175 (13), 173 (15), 171 (9), 163 (16), 161 (24), 159 (54), 157 (16), 151 (18), 149 (18), 147 (38), 145 (39), 143 (18), 137 (14), 135 (34), 133 (53), 131 (27), 129 (15), 123 (39), 121 (26), 119 (43), 117 (17), 115 (10), 109 (29), 107 (40), 105 (45), 97 (45), 95 (53), 93 (42), 91 (54), 83 (73), 81 (73), 79 (47), 77 (24), 71 (5), 69 (73), 67 (39) ,65 (5), 57 (28), 55 (98), 43 (56), 41 (46).

γ-Sitosterol (clionasterol) (18) (R_t: 10.23 min.) EIMS *m/z* (rel.int.%): 414[M]$^+$ $C_{29}H_{50}O$ (100), 399 [414-CH_3]$^+$ (34), 396 (42), 381 (35), 371 (5), 354 (9), 341 (5), 329 (46), 303 (52), 273 (28), 255 (29), 231 (18), 213 (37), 199 (19), 191 (9), 187 (14), 185 (12), 178 (15), 175 (13), 173 (19), 171 (11), 163 (27), 161 (30), 159 (32), 158 (13), 157 (15), 149 (19), 147 (30), 145 (44), 143 (20), 135 (24), 133 (36), 131 (19), 123 (11), 121 (25), 119 (39), 117 (17), 111 (11), 109 (21), 107 (45), 105 (42), 97 (17), 95 (35), 93 (40), 91 (38), 85 (15), 83 (15), 81 (33), 79 (27), 77 (11), 71 (15), 69 (25), 67 (25), 57 (35), 55 (47), 53 (6), 43 (62), 41 (30).

33-Norgorgosta-5,24(28)-dien-3β-ol (19) (R_t: 10.41 min.) EIMS *m/z* (rel.int.%): 410[M]$^+$ $C_{29}H_{46}O$ (4), 396 [414-H_2O]$^+$ (3), 377 (2), 314 (17), 299 (11), 281 (27), 272 (32), 271 (100), 253 (11), 239 (7), 229 (13), 213 (15), 211 (12), 199 (8), 187 (6), 175 (6), 171 (6), 159 (15), 145 (17), 133 (15), 119 (14), 105 (19), 95 (18), 93 (19), 91 (21), 81 (29), 79 (18), 67 (16), 55 (18), 43 (12), 41 (13).

Stigmasta-5,24(28)-dien-3β-ol (20) (R_t: 10.64 min.) EIMS *m/z* (rel.int.%): 412[M]$^+$ $C_{29}H_{48}O$ (5), 397 [412-CH_3]$^+$ (2), 379 (1), 315 (23), 314 (100), 299 (22), 296 (8), 281 (36), 271 (13), 253 (7), 229 (25), 213 (12), 211 (12), 199 (5), 197 (4), 187 (4), 185 (4), 175 (4), 173 (6), 171 (4), 161 (6), 159 (12), 158 (6), 157 (5), 147 (8), 145 (12), 143 (7), 135 (7), 133 (10), 131 (7), 129 (3), 123 (5), 121 (7), 119 (12), 117 (5), 109 (7), 107 (13), 105 (14), 97 (6), 95 (13), 93 (15), 91 (17), 83 (10), 81 (15), 79 (12), 69 (17), 67 (11), 57 (5), 55 (29), 43 (8), 41 (10).

Spectroscopic data of steroids after derivatization

22-Dehydrocholesterol trimethylsilyl ether (TMS) (R_t: 6.96 min.) EIMS *m/z* (rel.int.%): 456[M]$^+$ $C_{30}H_{52}OSi$ (62). 441[456-CH_3]$^+$ (14), 367 (21), 366 (71), 351 (37), 327 (81), 282 (8), 255 (63), 215 (23), 199(8), 161 (17), 159 (32), 145 (33), 133 (30), 129 (100), 119 (31), 111 (96), 105 (31), 91 (25), 79 (21), 69 (74), 55 (62), 43 (18).

Cholesterol TMS GC-MS retention time (R_t: 7.47 min) EIMS *m/z* (rel.int.%): 458[M]$^+$ $C_{30}H_{54}OSi$ (45), 443[458- CH_3] $^+$ (16), 368 (78), 353 (42), 329 (100), 301 (5), 275 (8), 255 (18), 247 (16), 233 (4), 213 (11), 199 (5), 173 (9), 163 (10), 161 (15), 159 (18), 145 (25), 129 (89), 119 (28), 107 (24), 95 (29), 91 (19), 81 (23), 79 (15), 73 (44), 69 (15), 67 (11), 57 (23), 55 (21), 43 (24), 41 (12).

Brassicasterol TMS (R_t: 8.28 min.) EIMS *m/z* (rel.int.%): 470[M^+ $C_{31}H_{54}OSi$ (70), 455 [470-CH_3]$^+$ (14), 380 (69), 365 (29), 341 (44), 340 (19), 282 (8), 271 (11), 255 (67), 243 (6), 229 (5), 213 (19), 199 (9), 185 (6), 173 (14), 161 (17), 159 (34), 147 (23), 145 (32), 143 (17), 133 (32), 129 (93), 125 (48), 119 (32), 109 (17), 107 (27), 105 (30), 95 (23), 93 (25), 91 (25), 83 (27), 81 (35), 79 (20), 75 (39), 73 (55), 69 (100), 67 (22), 57 (19), 55 (51), 43 (18), 41 (14).

24-Epicampesterol TMS (R_t: 9.33 min.) EIMS *m/z* (rel.int.%): 472[M^+ $C_{31}H_{56}OSi$ (47), 457 [472-CH_3]$^+$ (16), 383 (25), 382 (83), 367 (40), 343 (100), 315 (4), 289 (6), 261 (13), 255 (20), 213 (12), 203 (7), 185 (6), 173 (12), 163 (11), 161 (16), 159 (19), 149 (8), 147 (15), 145 (27), 143 (13), 135 (12), 133 (19), 131 (19), 129 (90), 123 (8), 121 (25), 120 (14), 119 (26), 117 (9), 115 (5), 109 (15), 107 (26), 105 (25), 95 (30), 93 (20), 91 (19), 83 (9), 81 (25), 79 (14), 75 (33), 73 (45), 71 (18), 69 (16), 67 (12), 57 (18), 55 (21), 43 (40), 41 (13).

Stigmasterol TMS (R_t: 9.96 min.) EIMS *m/z* (rel.int.%): 484[M^+ $C_{32}H_{56}OSi$ (65), 469 [412-CH_3]$^+$ (11), 394 (54), 379 (23), 355 (28), 351 (25), 343 (10), 309 (4), 282 (9), 271

Table 1 Fatty acids composition of *A. sinoxea* detected by GC-MS

Compound	Retention time	Kovats retention index (KI)	Kovats retention index standard (pherobase.com)	Kovats retention index of FA methyl esters	(%Area of total)	Base peak (*m/z*)	Molecular weight	Reference for identification
Tetradecanoic acid (myristic acid) (**1**) (14:0)	9.70	1764	1768	1728	4.1	73	228	[22–24]
4,8,12-Trimethyl-tridecanoic acid (**2**)	11.06	1824	ND	1780	19.8	57	256	[22, 23]
Pentadecanoic acid (pentadecylic acid) (**3**) (15:0)	11.18	1829	1820	1792	5.6	73	242	[22–24]
9-Hexadecenoic acid (palmitoleic acid) (**4**) (16:1)	13.89	1947	1953	1909	4.9	55	254	[22]
Hexadecanoic acid (palmitic acid) (**5**) (16:0)	14.41	1970	1964[a]	1931	27.4	73	256	[22, 23]
7-Methyl-6-hexadecenoic acid (**6**) (17:1)	15.69	2026	ND	1986	7.3	55	268	[22, 23]
Heptadecanoic acid (margaric acid) (**7**) (17:0)	15.98	2038	2038	2002	2.0	57	270	[22–24]
9-Octadecenoic acid (**8**) (18:1)	18.32	2144	2141	2105	3.3	55	282	[22–24]
11-Octadecenoic acid (**9**) (18:1)	18.42	2148	2141[b]	2111	4.7	55	282	[22, 23]
Octadecanoic acid (stearic acid) (**10**) (18:0)	18.87	2169	2172	2132	6.4	73	284	[22–24]
5,8,11,14- Eicosatetraenoic acid, methyl ester; omega-6 (**11**) (20:4)	21.79	2307	2274[a]	2263	1.0	79	318	[22]
4,7,10,13,16,19-Docosahexaenoic acid (DHA); omega-3 (**12**) (22:6)	25.78	2508	2520[a]	2458	10.9	79	328	[22]
					Total % 97.4			

Nd not determined

[a] NIST Mass Spectrometry Data Center;

[b] pubchem.com

(9), 255 (62), 241 (5), 228 (6), 213 (19), 199 (9), 185 (8), 173 (17), 161 (17), 159 (35), 147 (28), 145 (31), 143 (16), 139 (13), 135 (11), 133 (34), 131 (22), 129 (90), 123 (9), 121 (19), 119 (30), 117 (11), 109 (18), 107 (26), 105 (28), 97 (23), 95 (27), 93 (27), 91 (27), 83 (100), 81 (46), 79 (23), 75 (42), 73 (58), 69 (55), 67 (20), 57 (17), 55 (63), 43 (19), 41 (15).

Table 2 Sterols composition of *A. sinoxea* detected by GC-MS

Compound	Retention time	Kovats retention index (KI)	Kovats retention index standard	Kovats retention index of steroids trimethylsilyl ether	(%Area of total)	Base peak (*m/z*)	Molecular weight	Reference for identification
Cholesta-5,22-dien-3β-ol, (22-dehydrocholesterol) (**13**)	6.32	3091	3085	3133	16.7	255	384	[22–24]
Cholest-5-en-3β-ol (cholesterol) (**14**)	6.82	3124	3192	3165	4.3	386	386	[22, 23]
Ergosta-5, 22-dien-3β-ol (Brassicasterol) (**15**)	7.48	3166	3115 (active phase: OV-1)	3212	20.9	398	398	[22, 23]
Ergost-5-en-3β-ol (24-epicampesterol) (**16**)	8.56	3224	3233	3264	9.2	43	400	[22, 23]
Stigmasta-5,22-dien-3β-ol (stigmasterol,) (**17**)	9.12	3253	3248 (active phase: VF-5MS)	3294	3.1	412	412	[22]
γ-Sitosterol (Clionasterol) (**18**)	10.23	3305	3290 (active phase: RTX-5MS)	3348	….	414	414	[22, 23]
33-Norgorgosta-5,24(28)-dien-3β-ol (**19**)	10.41	3312		3359	32.1	271	410	[22, 23]
Stigmasta-5,24(28)-dien-3-ol (**20**)	10.64	3321	3318	3367	10.5	314	412	[22–24]
					Total % 96.9			

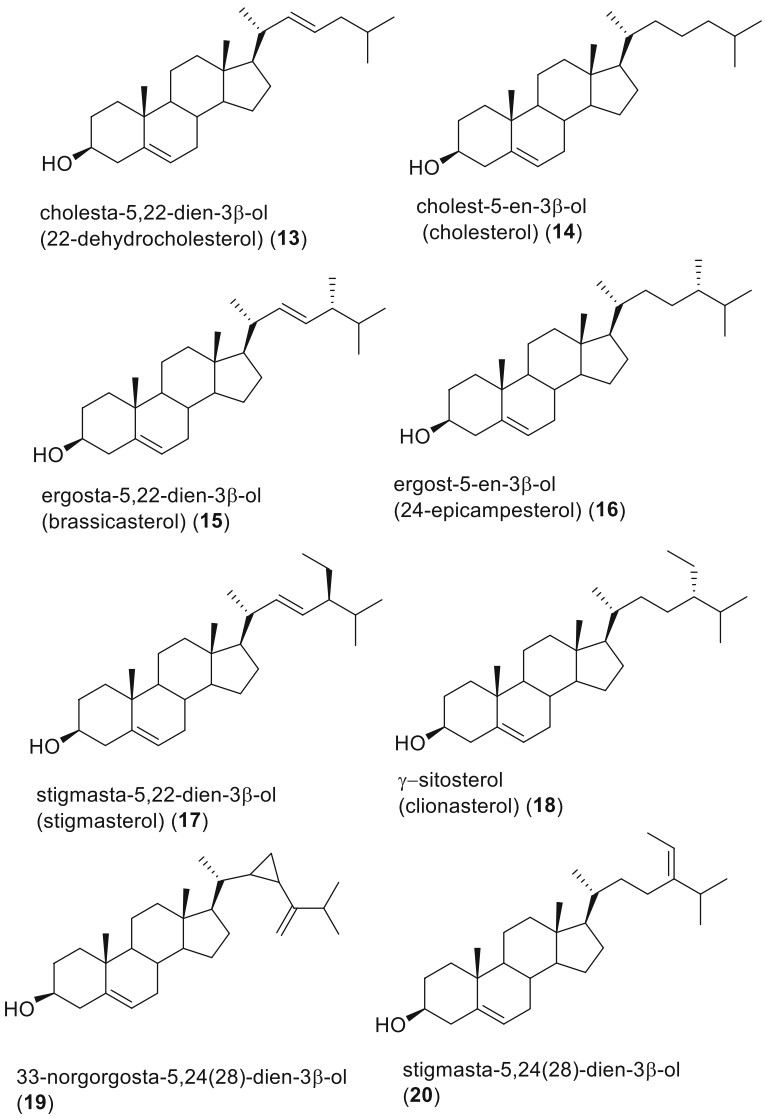

Fig. 7 Molecular structures of steroids isolated from *A. sinoxea*

γ-Sitosterol TMS (R_t: 11.20 min.) EIMS *m/z* (rel.int.%): 486[M]$^+$ $C_{32}H_{58}OSi$ (47), 471 [486-CH_3]$^+$ (14), 396 (79), 381 (41), 357 (89), 343 (6), 329 (5), 303 (5), 275 (10), 255 (20), 213 (13), 199 (6), 189 (5), 185 (5), 177 (5), 175 (8), 173 (14), 171 (5), 163 (11), 161 (17), 159 (25), 157 (10), 149 (9), 147 (19), 145 (28), 143 (18), 135 (14), 133 (23), 131 (20), 129 (100), 121 (26), 119 (29), 117 (9), 109 (15), 107 (27), 105 (26), 97 (8), 95 (31), 93 (21), 91 (20), 85 (12), 83 (9), 81 (28), 79 (15), 75 (35), 73 (52), 71 (15), 69 (21), 67 (15), 57 (28), 55 (24), 43 (40), 41 (13).

33-Norgorgosta-5,24(28)-dien-3β-ol TMS (R_t: 11.62 min.) EIMS *m/z* (rel.int.%): 482[M]$^+$ $C_{32}H_{54}OSi$ (4), 467 [482-CH_3]$^+$ (7), 386 (16), 343 (100), 296 (21), 281 (43), 253 (64), 239

Table 3 Sterols' composition of the HPLC Purified fractions from F10,11,12-f24,25, characterized by GC-MS

Fraction	Compounds
FH2	Cholesta-5, 22-dien-3β-ol (22-dehydrocholesterol) (**13**) (23.7%)*, 33-norgorgosta-5,24(28)-dien-3β-ol (**19**) (63.2%)*
FH3	Cholesterol (**14**) (11.6%)*, Ergosta-5, 22-dien-3β-ol (Brassicasterol) (**15**) (60.0%)*, stigmasta-5,24(28)-dien-3-ol (**20**) (23.5%)*
FH4	Ergost-5-en-3β-ol (24-epicampesterol) (**16**) (28.5%)*, Stigmasterol (**17**) (46.3%)*
FH5	γ-Sitosterol (Clionasterol) (**18**) (100%)*

*Area percentage (%) of the detected compounds using GC-MS

Table 4 Cytotoxic activity of fractions isolated from the *A. sinoxea*. Values are presented as mean ± S.E.M. of 3–6 experiments

	IC_{50} μg/mL		
Fractions	MOLT-4	MCF-7	HT-29
F6,7-F24,25	NA*	NA*	26.52 ± 8.19
F10,11,12-F24,25	1.20 ± 0.24	4.12 ± 0.40	2.47 ± 0.31
Cisplatin	1.89 ± 0.16	26.92 ± 3.30	23.73 ± 1.66

* Not active; $IC_{50} > 100$ μg/mL

(11), 227 (8), 215 (13), 213 (21), 211 (22), 199 (10), 197 (8), 187 (6), 185 (9), 175 (6), 173 (12), 171 (10), 169 (8), 161 (11), 159 (26), 157 (16), 147 (21), 145 (28), 143 (18), 137 (26), 133 (24), 131 (20), 129 (61), 121 (21), 119 (33), 117 (10), 109 (21), 107 (30), 105 (29), 95 (35), 93 (27), 91 (24), 81 (48), 79 (23), 75 (34), 73 (53), 69 (12), 67 (24), 55 (21), 43 (11), 41 (12).

Stigmasta-5,24(28)-dien-3β-ol TMS (R_t: 11.83 min.) EIMS *m/z* (rel.int.%): 484$[M]^+$ $C_{32}H_{56}OSi$ (6), 469 $[484\text{-}CH_3]^+$ (8), 387 (32), 386 (100), 371 (17), 355 (7), 345 (8), 296 (58), 281 (44), 257 (26), 255 (15), 253 (11), 227 (7), 213 (12), 211 (13), 199 (6), 197 (4), 187 (5), 185 (4), 175 (6), 173 (9), 171 (6), 169 (4), 161 (10), 159 (17), 158 (7), 157 (7), 147 (11), 145 (17), 143 (10), 135 (8), 133 (14), 131 (13), 129 (53), 123 (6), 121 (15), 119 (25), 117 (6), 109 (12), 107 (19), 105 (19), 97 (9), 95 (21),

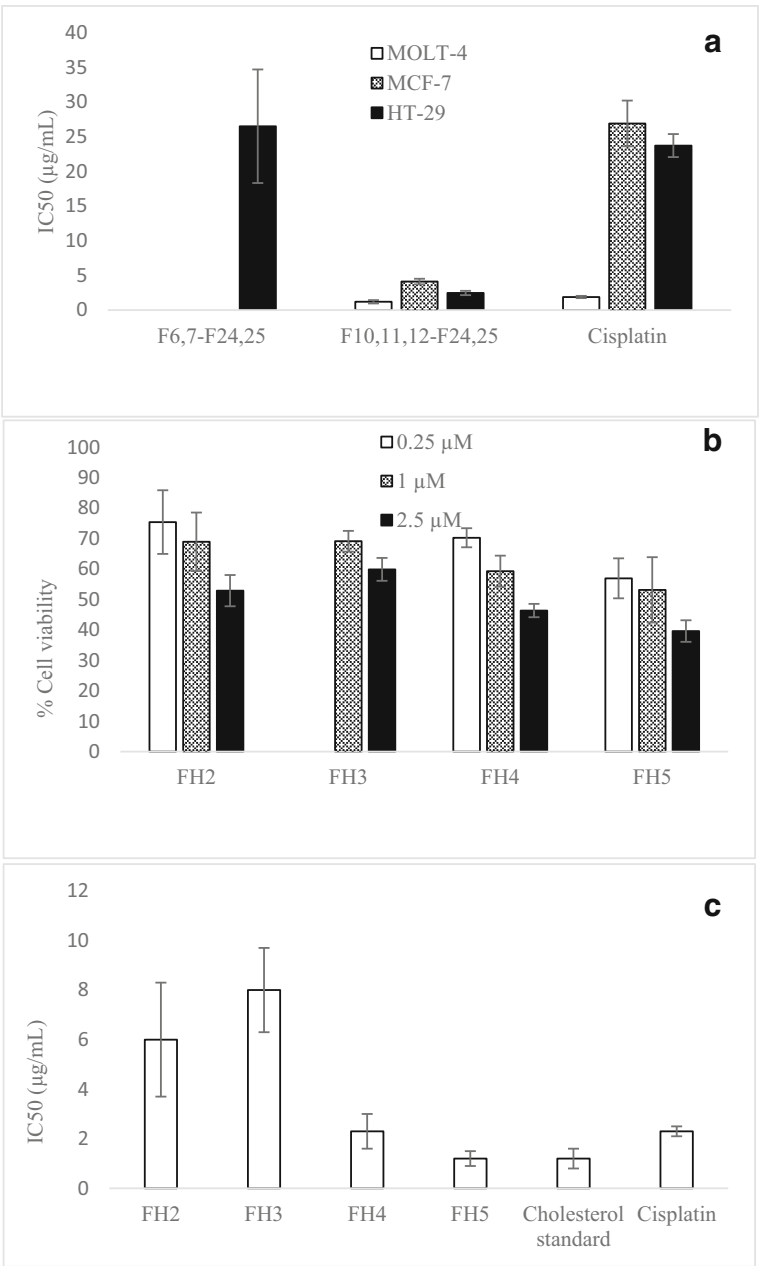

Fig. 8 Cytotoxic activity of fractions and sub-fractions measured against different human cancer cell lines. Cytotoxicity of fractions F6,7-F24,25 and F10,11,12-F24,25 was tested against three human cancer cell lines MOLT-4, MCF-7 and HT-29 cells (A); % Cell viability of MOLT-4 cell line incubated with sub-fractions obtained from purification of steroids containing fraction (F10,11,12-F24,25) by HPLC (B); Cytotoxic effects of sub-fractions of the steroids containing fraction were measured against MOLT-4 cells and expressed as IC_{50} values. Values are presented as mean ± S.E.M. of 3–5 experiments

93 (15), 91 (15), 83 (12), 81 (22), 79 (12), 75 (27), 73 (40), 69 (25), 67 (11), 55 (37), 43 (7), 41 (9).

Results

Purification of the sponges' methanol extract (12.82 g) yielded twelve fatty acids (**1–12**) and eight steroids (**13–20**). The chemical structures of the detected compounds; FAs: **1–12** (Table 1) and steroids: **13–20** (Table 2) were determined by comparing their mass spectra and GC retention indices with those reported for the authentic compounds in the spectral databases including NIST and Wiley MS libraries. The FAs of *A. sinoxea* were classified into three groups: saturated FAs including one terpenoid type FA, monoenoic and polyunsaturated FAs (PUFAs) (Table 1). The major constituents were hexadecanoic acid (palmitic acid) (**5**) (27.4%), followed by the terpenoid acid; 4,8,12-Trimethyl-tridecanoic acid (**2**) (19.8%) and 4,7,10,13,16,19-docosahexaenoic acid (**12**) (10.9%); while methyl ester of 5,8,11,14-eicosatetraenoic acid (**11**) was the minor PUFA in the sponge's extract (Table 1). The mass spectra of methyl esters of all saturated FAs exhibited the presence of ions at *m/z* $[M]^+$, $[M–29]^+$, and $[M–31]^+$ and intensive peaks at *m/z* 55, 74, 87, and 143 characteristic of the saturated FA methyl esters. Eight steroids including compounds **13–20** were detected in the sponge extract. The most abundant steroids were 33-norgorgosta-5,24(28)-dien-3-ol (**19**) (32.1%), followed by ergosta-5,22-dien-3β-ol (brassicasterol) (**15**) (20.9%) and cholesta-5,22-dien-3β-ol, (22-dehydrocholesterol) (**13**) (16.7%); while stigmasterol (**17**) (3.1%) was the minor steroids in this sponge (Fig. 7, Table 2).

The GC-MS characterized fraction; FH2 contains two of the most abundant steroids; compounds **13** and **19** while FH3 is composed of compound **15** as the major, and **20** and **14** as the minor constituents. Compounds **16** and **17** were detected as the main constituents of FH4, and finally FH5 constitutes of compound **18** (Table 3).

Cytotoxic activity

Fraction F6,7-F24,25 (fatty acids containing fraction) was active only against HT-29 cell line with an IC_{50} value of 26.52 ± 8.19 μg/ mL, while the fraction F10,11,12-F24,25 (steroids containing fraction) was active against MOLT-4, MCF-7 and HT-29 cell lines with IC_{50} values of 1.20 ± 0.24, 4.12 ± 0.40 and 2.47 ± 0.31 μg/mL, respectively (Table 4, Fig. 8).

Since the steroids containing fraction (F10,11,12-F24,25) showed the highest activity against MOLT-4 cell line, the HPLC purified sub-fractions were also tested against this cell line (Fig. 8). Amongst the tested steroids, compound **18** (FH5) and a mixture of compounds **16** and **17** (FH4) were marine originated steroids with the most potent cytotoxic activity (IC_{50} values of 1.2 ± 0.3 and 2.3 ± 0.7 μg/mL, respectively). However, the other fractions containing compounds **14**, **15** and **20** (FH3; IC_{50}: 8 ± 1.7 μg/mL) and **13** and **19** (FH2; IC_{50}: 6 ± 2.3 μg/mL) were less active compared to the standard anti-cancer drug, cisplatin. Fatty acids-containing fraction, steroids-rich fraction and sub-fractions obtained from steroids-rich fraction were inactive (IC_{50}s > 50 μg/mL) against NIH/3 T3 fibroblast cells (data not shown).

Discussion

About 400 various FAs have been reported from 250 species of marine sponges, with C_{12} to C_{32} atoms in their molecular structures. Common marine FAs are 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, amongst which 16:0 and 18:0 (1.5–33.0%) dominated in most of the sponges [3]. In addition to FAs, steroids are other biologically important marine sponge's natural products [25–28]. Therefore, it can be concluded that sponges have a great diversity of FAs and steroids among aquatic animals. The isoprenoid fatty acid, 4,8,12-trimethyl-tridecanoic acid (TMTD) have been isolated from several marine sponges. TMTD phospholipid FA is major constituent of the sponges: *Anthosigmella varians* and *Spheciospongia vesparium* (family Spirastrellidae) as well as *Cliona aprica* (family Clionidae). Therefore, TMTDs is suggested as potential biomarker of class demospongiae sponges [29, 30].

The fatty acid compositions of five sponges were investigated and showed three cyanobacterial associated sponges *Dysidea herbacea*, *Phyllospongia papyracea* and *Pseudaxinyssa* sp., had relatively higher amounts of fatty acids contents, particularly straight-chain saturated and monounsaturated fatty acids. *Pseudaxinyssa* sp. contained a significant amount of TMTD. Cyanobacteria biosynthesize C_{14} to C_{18} FAs with various degree of unsaturation. The C16:1(9) acid was the major cyanobacterial acid detected in all of the above three cyanobacteria-rich sponges; therefore, the origin of FAs are assumed to be the sponges associate cyanobacteria [31]. Also, TMTD is one of the major components of the FAs fraction of Senegalese sponges including *Cinachyrefla alloclada*, *C. kiikenthali* and *C. aff. Schdzei* (family Tetillidae) [32].

The fatty acids and steroids isolated from marine sponges have shown cytotoxic activity against different cancer cell lines. Mixtures of $\Delta^{5,9}$ unsaturation, including the rare anteiso-5,9–24:2 acid (19.5% of the total free FA), and a new iso-5,9–24:2 acid (30%) have cytotoxic activity against mouse Ehrlich carcinoma cells and a hemolytic effect on mouse erythrocytes [1]. Fatty acids containing fraction of methanol extract of *A. sinoxea* showed cytotoxic activity against human colon cancer cell line. The number of double

bonds on the carbon chain length of fatty acids influence the ability of FAs to have a cytotoxic effect. Generally, saturated FAs like stearic acid (18:0) and palmitic acid (16:0) and monounsaturated FAs, such as oleic acid (18:1) are unable to induce cytotoxic effect unlike the PUFAs [9]. The best type of cytotoxic PUFAs are those with three or more double bonds, while the saturated FAs are the weakest ones. The n-6 PUFAs with two double bonds were generally less cytotoxic agents than other n-6 PUFAs with three double bonds on their carbon chain. Amongst n-6 PUFAs, arachidonic acid (20:4) with four double bond, was one of the most potent cytotoxic agent; also n-3 PUFAs eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) showed cytotoxic activity against different cancer cell line, especially human colon cancer cells [9, 33–36]. Therefore, we can attribute the cytotoxic activity of fatty acid containing fraction from *A. sinoxea*, to two polyunsaturated fatty acid 5,8,11,14 eicosatetraenoic acid and 4,7,10,13,16,19-docosahexaenoic acid with relative percentage 1.0 and 10.9%, respectively.

Kellner-Weibel et al. showed that free cholesterol generated by the hydrolysis of cytoplasmic cholesteryl esters in model macrophage foam cells, transported to the plasma membrane by acidic vesicles, and accumulation of this compound in the pool caused cell death by necrosis and apoptosis [37]. Sundarraj et al. investigated the inhibitory effect of *Acacia nilotica* leaves extract and γ-Sitosterol on cell proliferation, apoptosis and cell cycle arrest in breast and lung cancer cells, and showed that γ-Sitosterol with induces G2/M cell cycle arrest and apoptosis through c-Myc suppression in MCF-7 and A549 cells was a potent anticancer agent [38]. In our study γ-sisosterol showed a potent cytotoxic activity against MOLT-4 cell line; hence, we can conclude that the cytotoxic activity of the steroid fraction extracted from the *Axinella sinoxea* might be related to γ-sisosterol. Generally, the major steroids that have shown cytotoxic effect in various studies were polyoxygenated steroids [39, 40], polyhydroxy sterols [41] and epoxysterols [42]. However, in the present study, all the isolated steroids from *A. sinoxea* were 3β-hydroxy sterols, which showed strong cytotoxic activity against MOLT-4, MCF-7 and HT-29 cell lines.

Conclusion

Cytotoxic activity of FAs against HT-29 cell line, and higher cytotoxicity of the steroids isolated from *A. sinoxeae* without high cytotoxicity against normal fibroblast cells, suggested the importance of sponges for further research on the isolation and characterization of marine derived natural products in order to discover a novel chemotherapeutic of cancer.

Since all of the above mentioned sponges belong to class of demospongiae, and based on their FAs profiling, it can be concluded that the FAs are plausible products of cyanobacteria. This hypothesis can be further supported by the fact that demospongiae sponges are good habitats for microalgae, especially cyanobacteria. Due to the presence of simillar composition of FAs in cyanobacteria to those reported in the sponges, they can be regarded as biomarkers.

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