ELSEVIER

CrossMark

ORIGINAL ARTICLE

Production of hydroxy marilone C as a bioactive compound from *Streptomyces badius*

Osama H. El Sayed [a], Mohsen M. S. Asker [a], Mahmoud A. Swelim [b], Ibrahim H. Abbas [b], Aziza I. Attwa [a], Mohamed E. El Awady [a,*]

[a] *Department of Microbial Biotechnology, National Research Centre, 33 Bohouth St., Dokki, Giza 12622, Egypt*
[b] *Botany Department, Faculty of Science, Benha University, Benha, Egypt*

Abstract Hydroxy marilone C is a bioactive metabolite produced from the culture broth of *Streptomyces badius* isolated from Egyptian soil. Hydroxy marilone C was purified and fractionated by a silica gel column with a gradient mobile phase dichloromethane (DCM):methanol then Sephadex LH-20 column using methanol as a mobile phase. It was subjected to many procedures such as infrared (IR), nuclear magnetic resonance (NMR), Mass spectroscopy (MS) and UV spectroscopy for elucidation of its structure. It was evaluated for antioxidant, cytotoxicity against human alveolar basal epithelial cell line (A-549) and human breast adenocarcinoma cell line (MCF-7) and antiviral activities; showed that the maximum antioxidant activity was 78.8% at 3 mg/ml after 90 min. and the IC_{50} value against DPPH radical found about 1.5 mg/ml after 60 min. Using MTT assay the effect of the pure compound on the proliferation of A-549 cells and MCF-7 cells was 443 μg/ml and 147.9 μg/ml, respectively, while for detection of antiviral activity using Madin–Darby canine kidney (MDCK) cells the maximum cytotoxicity was at 27.9% and IC_{50} was 128.1 μg/ml. The maximum concentration required for protecting 50% of the virus-infected cells against H1N1 viral cytopathogenicity (EC_{50}) was 33.25% for 80 μg/ml. These results indicated that the hydroxy marilone C has potential antitumor and antiviral activities.

1. Introduction

Among all known producers of small molecule natural products, microorganisms represent a rich source of biologically active secondary metabolites that find wide-ranging applications as antibiotics, immunosuppressant's, antiparasitics, antiviral and anticancer agents [1]. Secondary metabolites are excreted by microbial cultures at the end of primary growth and during the stationary phase of growth. Secondary metabolites are compounds neither essential for growth nor key intermediates of the organism's basic metabolism but presumably playing some other role in the life of microorganisms. They are usually found as a mixture of closely related molecules with a peculiar and rare chemical structure [2,3]. *Streptomyces* spp. are widespread in nature and continue to have a significant

role in the production of novel secondary bioactive metabolites. *Streptomyces* spp. produce many classes of secondary metabolites with great biofunctional diversity that may ultimately find application as antibiotics, antifungal, antiviral, anticancer, immunosuppressants, insecticides, herbicides etc. and diverse chemical structures, which makes them useful as pharmaceuticals and agricultural agents [4,5]. However, the search for novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to multiple chemotherapeutic drugs. In addition, the high toxicity usually associated with cancer chemotherapy drugs and their undesirable side effects increase the demand for novel antitumor drugs active against untreatable tumors, with fewer side effects and/ or with greater therapeutic efficiency [6]. About 7.6 million deaths worldwide were due to cancer with 12.7 million new cases per year being reported worldwide. Traditional treatments of cancer inaccessible to surgery are multi-modal comprising chemotherapy, immunotherapy, hormonal therapy and radiotherapy. Although, there have been improvements in cancer patient survival by employing cytotoxic chemotherapeutic regimens, this approach is encumbered by poor accessibility of medications to tumor site and non-specific cytotoxicity to normal cells [7]. The clinical treatment methods against cancer include: surgery, which is local excision of tumor; radiotherapy, which eliminate tumor by exposing to radiation; chemotherapy, which relies on drug targeting tumor cells; combined modality therapy, which includes all three former treatments together; and immunotherapy, which evokes an immune response against tumor [8]. Natural products have not only yielded new and effective drugs, but they have also provided insight into new mechanisms of action, and cancer treatment would be immeasurably poorer without the insights and the compounds provided from nature. The reasons for the effectiveness of natural products are at least twofold. In the first place, there is a high correlation between the properties of drugs and those of natural products. Second, natural products usually have built-in so; it can bind to complex proteins and other three-dimensional biological receptors [9].

So, this work aimed at isolation of a bioactive secondary metabolite from *Streptomyces badius* and usage of several techniques to identify this metabolite. Therefore, it was tested as antioxidant, anticancer and antiviral compound.

2. Materials and methods

2.1. Fermentation and extraction of bioactive compounds

S. badius (M7) isolated from Egyptian soil capable to give highest antioxidant and antitumour activities [10] was selected for production of the bioactive compound. Every one liter Erlenmeyer flask contained 250 ml of ISP2 medium (g/L): malt extract 10; yeast extract 4; glucose 20 at pH 7.0 [11] and was inoculated with a piece having 0.5 cm diameter of a well-grown agar subculture of the strain [12]. Thirty flasks were kept at 28 °C using a rotary shaker (150 rpm) and harvested after 7 days. After harvesting and centrifugation at 5000 rpm for 15 min, the supernatant of each flask was extracted by ethyl acetate, and then separated from the water phase using a separating funnel. Ethyl acetate extracts were concentrated in vacuum tell dryness, yielding 4.5 g of a reddish brown crude extract.

2.2. Purification of bioactive compounds

The obtained extract was applied to column chromatography on silica gel G$_{254}$ eluted by a gradient of dichloromethane (DCM) and methanol and the drops at the bottom of the column were collected and the fractions were analyzed using TLC. The fractions collected at the end of the fractionation process on the silica gel column showed varied biological antitumour activity using Ehrlich Ascites Carcinoma cells (EACC). The fraction have high antitumour activity was loaded on the top of the Sephadex LH-20 column and methanol was the mobile phase. Every individual fraction of bioactive residue was collected at the end of fractionation process on a Sephadex LH-20 column. These fractions showed varied biological antitumour activity. Fraction have high antitumour activity was also subjected to separate and purify using preparative TLC. The plates were prepared by slurring 30 g of silica gel Type 60 F$_{254}$; (Merck) in 60 ml distilled water. The compounds were detected from their UV absorbance at 254 and 366 nm and by spraying the TLC plates with anisaldehyde/sulfuric acid reagent giving different colored reactions with many structural elements [13].

2.3. Spectral measurements

The elucidation of the chemical structures of the promising active compound had been done using several instruments as infrared (FTIR), mass, nuclear magnetic resonance (NMR) and UV spectroscopy. An IR spectrum of the pure compound was performed with KBr pellets, 2.0 mg sample and 200 mg KBr using the FTIR-UNIT Bruker Vector 22 Spectrophotometer. NMR (^{1}H and ^{13}C NMR) were obtained on JEOL-ECA 500 MHz spectrometer using TMS as internal standard, the chemical shifts were expressed in d (ppm) and coupling constants J in Hz. Mass spectra were recorded on thermo scientific-trace GC Ultra, USA. Coupled with single quadrupole MS, temperature (0–300 °C) and UV spectroscopy were recorded on a Shimadzu UV-2401 PC double beam spectrophotometer using 1.0 cm quartz cells according to Paterson and Kemmelmeier 31. These experiments were carried out in central services laboratory at National Research Centre, Egypt.

2.4. Biological activity

2.4.1. Antioxidant activity
The free radical scavenging activity (RSA) of the active secondary metabolite was assessed by the decoloration of chloroform solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical (violet color) according to [14]. The scavenging activity of free radical by active compound was evaluated spectrophotometrically at 517 nm. The scavenging activity was calculated as follows:

$$\text{Scavenging ability}(\%) = \left(A_{517\,\text{of control}} - A_{517\,\text{of sample}} / A_{517\,\text{of control}} \right) \times 100.$$

2.4.2. Assay of the antitumor activity against different cell lines
Cytotoxicity against the two tumor cell lines, human breast Aden carcinoma cell line (MCF-7) and human alveolar basal

epithelial cell line (A-549), which were purchased from ATCC, USA, were used to evaluate the cytotoxic effect of the tested samples using MTT cell viability assay according to [15]. Cells (0.5×10^5 cells/well), in serum-free media, were plated in a flat bottomed 96-well micro plate, and treated with 20 μL of different concentrations of the tested sample for 48 h at 37 °C, in a humidified 5% CO_2 atmosphere. After incubation, media were removed and 40 μl MTT solution/well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 μl of acidified isopropanol/well and plate was shacked at room temperature, followed by photometric determination of the absorbance at 570 nm using micro plate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by < 100% relative viability. Percentage of relative viability was calculated using the following equation:

$$[Absorbance\ of\ treated\ cells/Absorbance\ of\ control\ cells] \times 100$$

Then the half maximal inhibitory concentration (IC_{50}) was calculated from the equation of the dose–response curve.

2.4.3. Antiviral assays

The antiviral activity of the compounds was determined against A/Puerto Rico/8/1934 (PR8) H1N1 virus. Stock solutions of the test compounds were prepared in DMSO. Cells grown to confluence in 96-well plates were infected with 100 μL of 100 TCID50 viruses. After an adsorption period of 1 h at 37 °C, virus was removed and serial dilutions of the compounds were added. Maintenance DMEM with 2% FBS was added (100 μl/well). The cultures were further incubated at 37 °C for 3 days, until complete CPE was observed in the infected and untreated virus control. The determination of the anti-H1N1 virus activity of the compounds was based on virus-induced cytopathogenicity of H1N1 infected MDCK cells, measured at day 4 post virus infection by the MTT colorimetric method [16,17]. An absorbance of formazan was detected by a multi-well plate reader at 540 nm with 620 nm reference wavelength. The results were expressed as the 50% effective concentration (EC_{50}). The 50% effective antiviral concentration (EC_{50}) was defined as the compound concentration required for protecting 50% of the virus-infected cells against viral cytopathogenicity.

3. Results

3.1. Isolation of hydroxy marilone C

After evaporation of the organic phase 4.5 g of brown oily extract was obtained. The extract was fractionated by silica gel column yielding five fractions (F1, F2, F3, F4 and F5). Each single fraction was assessed to determine the antitumor activity (by Ehrlich ascites carcinoma). Fig. 1 showed that F3 was the highest fraction showing an inhibition ratio to the Ehrlich cells of 67.05%. F3 was fractionated by Sephadex LH-20 column using methanol as a mobile phase to yield six fractions (F3F1, F3F2, F3F3, F3F4, F3F5 and F3F6). All six sub-fractions screened using Ehrlich cells showed that all
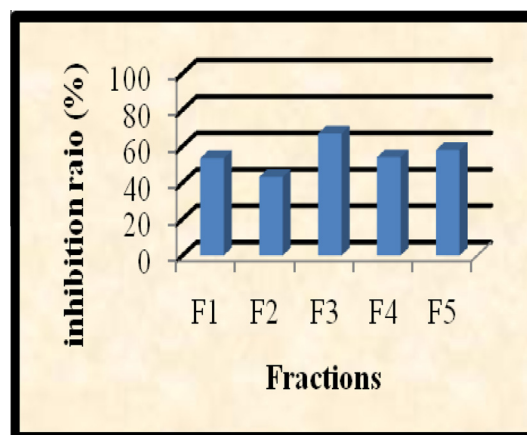

Figure 1 Antitumor activity of *Streptomyces badius* (M7) secondary metabolites fractionated from silica gel column.

sub-fractions which separated from fraction F3 have antitumor activities in different percents except F3F4 which has no activity. Thus, sub-fraction F3F1 was the highest fraction showing an inhibition ratio to the Ehrlich cells of 69.98% (Fig. 2). Sub-fraction F3F1 was separated by preparative thin layer chromatography. Fig. 3 showed that sub-fraction F3F1b was the highest fraction showing an inhibition ratio to the Ehrlich cells of 67.57%. So, it was selected as an active purified compound for further experiments.

3.2. Structure elucidation of hydroxy marilone C

The active sub-fraction F3F1b isolated from *S. badius* (M7) was obtained as a light yellow oily compound insoluble in water while soluble in methanol, hexane, ethyl acetate, chloroform and dichloromethane. The isolated compound gave a positive reaction (purple color) with concentrated H_2SO_4. It appeared as a single spot on the TLC Merck Kiesel gel 60 F_{254} (R_f: 0.62 with solvent system DCM:methanol 9:1) under long wave length UV light (365 nm). It was investigated by IR, NMR (^1H and ^{13}C NMR), MS and UV spectroscopy. The ultraviolet (UV) absorption spectrum recorded a
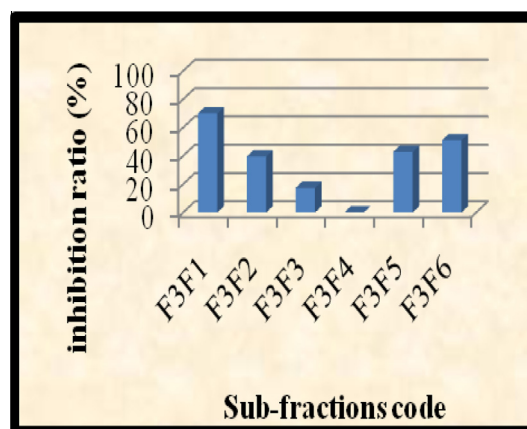

Figure 2 Antitumor activity of F3 sub-fractions from Sephadex LH-20 column.
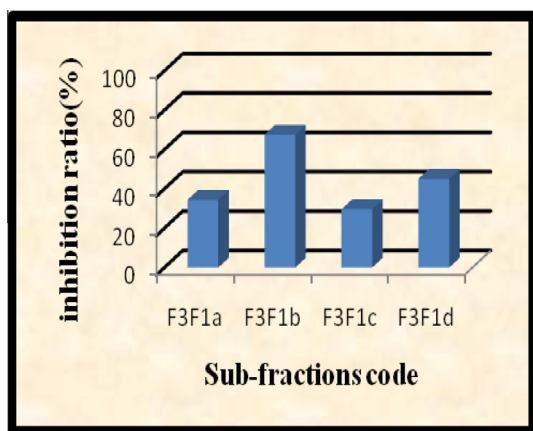
Figure 3　Antitumor activity of sub-fractions from preparative TLC.

maximum absorption peak at $\lambda_{max} = 228$ with dichloromethane, $\lambda_{max} = 207$ nm with methanol and $\lambda_{max} = 242$ with chloroform as shown in Fig. 4.

Fig. 5 showing the Mass spectrum revealed that the molecular formula is $C_{20}H_{26}O_5$ and the molecular weight is 346 g/mol. and showed a fragment at m/z 279.3 due to loss (C_5H_7) from the side chain followed by a fragment at m/z 261.3 (279–OH&H) and the base beak at m/z 57.14 resulting from cleavage of ring (A) after loss OH to give an ionic fragment $[C_2O_2H]^+$. The ^{13}C NMR and ^1H NMR spectrum were obtained at 500 MHz in $CDCl_3$ solution listed in Table 1.

The IR spectrum of the sub-fraction (Fig. 6) showed characteristic bands corresponding to 3425 cm^{-1} indicated as (OH), 1730 cm^{-1} indicated as (C=O), 1633 cm^{-1} indicated as (C=C), 1116 cm^{-1} indicated as gemdimethyl and 1453 cm^{-1} indicated as (OCH$_3$).

The obtained data, UV, IR, MS, ^{13}C NMR and ^1H NMR suggested that the isolated compound is very close to marilone C, but the OH group shown at 3425 cm^{-1} at IR spectrum and

7.5 ppm at ^1H NMR indicated the presence of the OH group on the furan ring (A) at position 1. So, from these available data the suspected structure of this compound was shown at Fig. 7.

3.3. Biological evaluation of hydroxy marilone C

DPPH free radical scavenging activity technique was used to determine quantitatively the antioxidant activity at different times (30, 60 and 90 min.). By increasing the concentrations of the compound from 100 to 3000 µg/ml the total antioxidant activity was increased. Maximum antioxidant activity was 78.8% at 3000 µg/ml after 90 min. The IC$_{50}$ value against DPPH radical was found to be about 1500 µg/ml after 60 min. Fig. 8.

The effect of the hydroxy marilone C on the proliferation of A-549 cells and MCF-7 cells was studied after 48 h of incubation. As shown in (Figs. 9 and 10), respectively, the treatment of MCF-7 cells was more cytotoxic-sensitive than treatment of A-549. As shown in (Fig. 11) the calculated IC$_{50}$ for cell line MCF-7 was 147.9 µg/ml, while the other cell line A-549 showed a weak cytotoxic level as concluded from the high calculated IC$_{50}$ values – indicating low anti-tumor affinity – to be 443 µg/ml.

The cytotoxicity of different concentrations of hydroxy marilone C by increasing the concentrations from 20–80 µg the cytotoxicity of the pure compound is increased and showed maximum cytotoxicity at 27.9%. Also, the concentration required to cause a microscopically detectable alteration of normal cell morphology (IC$_{50}$) was 128.1 µg/ml (Table 2).

The antiviral activity of the hydroxy marilone C was determined against A/Puerto Rico/8/1934 (PR8) H1N1 virus and the concentration of hydroxy marilone C required for protecting 50% of the virus-infected cells against viral cytopathogenicity (EC$_{50}$). The maximum EC$_{50}$ of the hydroxy marilone C was 33.25% for 80 µg/ml as effective concentration of the hydroxy marilone C.
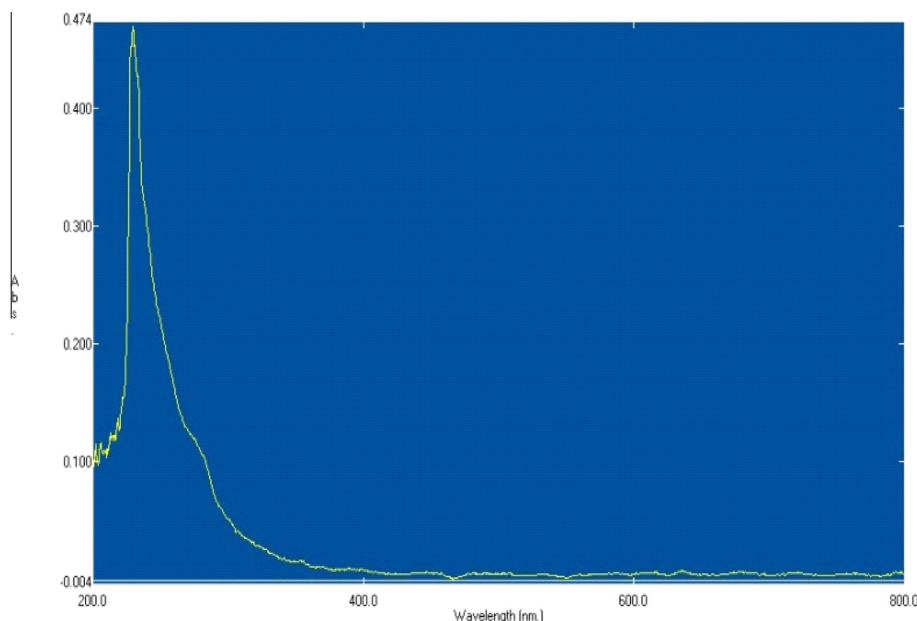

Figure 4　UV spectrum of purified sub-fraction F3F1b with chloroform.

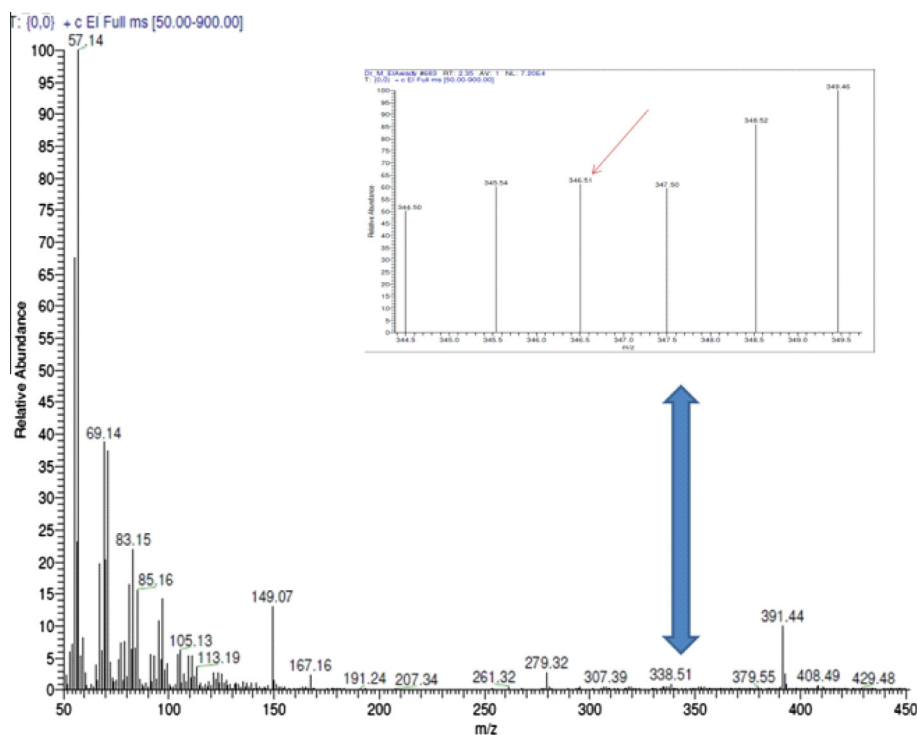**Figure 5** MS spectrum of purified sub-fraction F3F1b.

Table 1 ^{1}H NMR and ^{13}C NMR spectrum data.

Position	^{1}H NMR δ, ppm	^{13}C NMR δ, ppm	Position	^{1}H NMR δ, ppm	^{13}C NMR δ, ppm
1	7.53	78.49	1\	4.65	65.32
2	–	128.31	2\	5.34	122.67
3	–	153.18	3\	–	141.43
4	–	124.44	4\	2.28	40.64
5	–	159.94	5\	2.29	29.43
6	7.24	0.87	6\	5.12	125.81
7	–	126.53	7\	–	132.90
8	–	168.02	8\	1.24	23.26
9	3.48	60.85	9\	1.41	20.03
10	2.56	10.46	10\	0.87	22.78

4. Discussion

There are two reasons for the effectiveness of secondary metabolites, the first, there is a high correlation between the properties of drugs and those of secondary metabolites. Second, secondary metabolits usually have built-in chirality, and are thus uniquely suited to bind to complex proteins and other three-dimensional biological receptors [18]. The ability of *Streptomyces* species to produce antibiotics or related secondary metabolites has attracted great interest among researchers [19]. Ability is generally ascribed to the existence of the clusters of the *Streptomyces* strains, which encodes enzymes for a lot of secondary metabolic pathways [20].

However, secondary metabolite production in microbes is also strongly influenced by nutritional factors and growth conditions [21]. The data obtained in the present study comprise the isolation of hydroxy marilone C as a major bioactive compound produced fot the first time from *S. badius* M7. Marilones are phthalide derivatives which are organic chemical compounds which are a class of structurally very diverse secondary metabolites with more than 180 naturally occurring compounds or phytochemical compounds classified under lactones [22,23]. Phthalides are known to provide health benefits by stimulating various enzymes in the body [24]. Also, it exhibits an equally broad spectrum of bioactivity [25].

Some phthalides having anticancer activity can enhance the sensitivity of drug resistance of tumor cells against chemotherapy and decrease the drug-resistance of tumor cells and enhance notably several chemotherapy agent induced apoptosis of tumor cells [26]. Such phthalides isolated from *Angelica sinensis*, have recently been found to be cytotoxic against several brain tumor cell lines and leukemia cells and colon cancer HT-29 [27] and they show anticancer activity against the HeLa cancer cell line [28], while, phthalides isolated from *Alternaria porri* were highly cytotoxic toward HeLa/cervical cancer and KB/oral cancer cells [29].

A large scale-fermentation of the strain followed by working up and purification using a series of chromatographic techniques led to isolation of hydroxy marilone C which was identified using intensive studies of different spectroscopic means such as ^{1}H NMR and ^{13}C NMR, MS, UV and IR which gave data similar to Almeida et al. [30] and suggested marilone C as a name in these results.

Hydroxy marilone C was evaluated for antioxidant, cytotoxic activity against two tumor cell lines (A-549 and
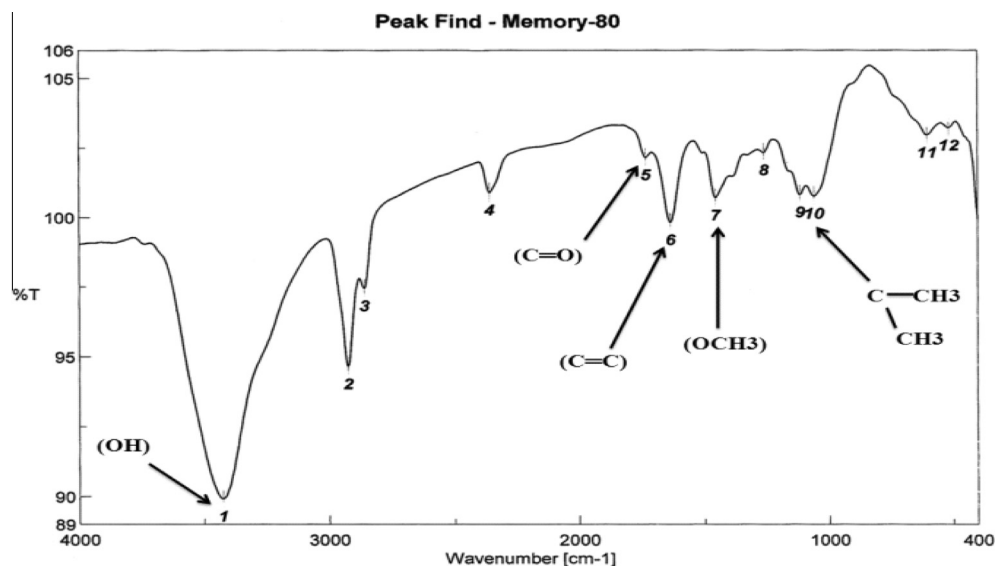
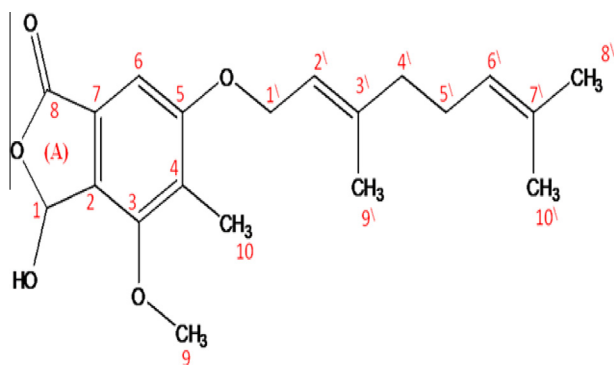**Figure 6**   IR spectrum of purified sub-fraction F3F1b.

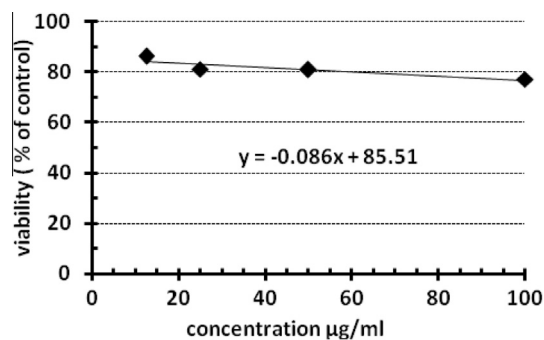**Figure 7**   The suggested structure of purified sub-fraction F3F1.

Figure 9   Cytotoxic effect of different samples against A-549 cells using MTT assay ($n = 4$), data are expressed as the mean value of cell viability (% of control).

Figure 8   Scavenging activity of pure compound at different times.

Figure 10   Cytotoxic effect of different samples against MCF-7 cells using MTT assay ($n = 4$), data are expressed as the mean value of cell viability (% of control).
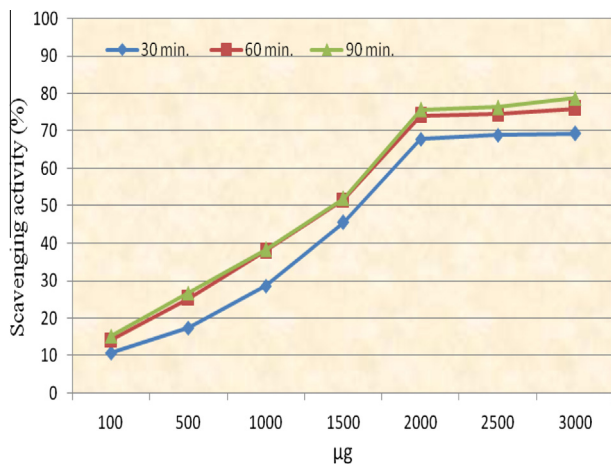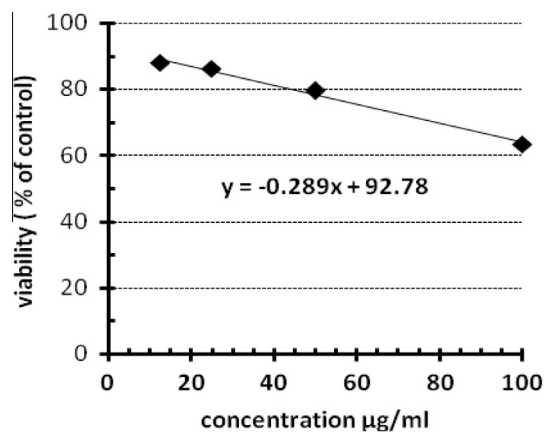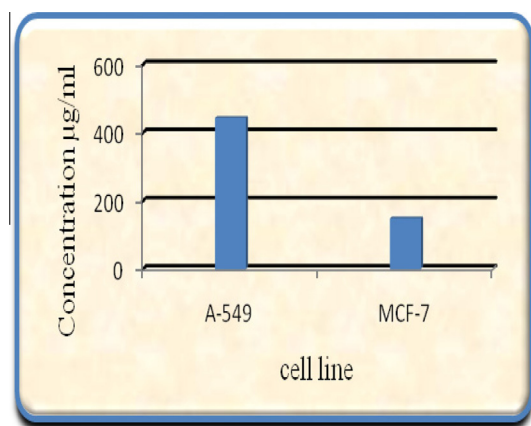
Figure 11 IC50 at MCF-7 and A-549 cells using MTT assay.

Table 2 Cytotoxicity at different concentrations and IC_{50} for pure compound.

Concentration (µg/ml)	Cytotoxicity (%)
20	0
40	2.9
80	27.9

MCF-7) and antiviral activities. The total antioxidant activity of the hydroxy marilone C was increased by increasing the hydroxy marilone C weight from 100 to 3000 µg, while, IC_{50} value against DPPH radical was found to be about 1500 µg after 60 min. The DPPH assay used to determine antioxidant activity was produced from *Streptomyces* sp. [31,32]. The treatment of MCF-7 cells with hydroxy marilone C was more cytotoxic-sensitive than treatment of A-549. The calculated IC_{50} for the cell line MCF-7 was 147.9 µg/ml, while, the other cell line A-549 showed IC_{50} indicating low anti-tumor affinity to be 443 µg/ml for hydroxy marilone C. These results agree with Almeida et al. [30] who showed that marilone C which was tested for cytotoxic activity toward three cancer cell lines (NCI-H460/lung, MCF7/breast and SF268/CNS) showed antiproliferative activity with an average IC_{50} of 26.6 µM. Wang et al. [33] isolated five new derivatives *Streptomyces* sp. FXJ7.328 and showed that these derivatives have antiviral activity against influenza A (H1N1) virus with an IC_{50} value of 41.5 ± 4.5 µM. Hydroxy marilone C concentration required for protecting 50% of the virus-infected cells against viral cytopathogenicity (EC_{50}) was 17.5% with 40 µg as effective concentration while the maximum EC_{50} was 33.25% with 80 µg as effective concentration. These results mentioned that *S. badius* have an antiviral activity against A/Puerto Rico/8/1934 (PR8) H1N1 virus.

So, the obtained data in this study showed *S. badius* (M7) have the ability to produce bioactive compounds such as hydroxy marilone C (phthalide derivative) for the first time as a bioactive compound. This compound offers health benefits and its antioxidant activity, anticancer activity and antiviral activities are recorded.

References

[1] S.P. Manly, R. Padmanabha, S.E. Lowe, in: W.P. Janzen (Ed.), High Throughput Screening: Methods and Protocols, vol. 190, Humana Press, New Jersey, 2002, pp. 153–168.

[2] D.L. Hawksworth, P.M. Kirk, B.C. Sutton, D.N. Pegler, Ainsworth and Bisby's Dictionary of the Fungi, eighth ed., International Mycological Institute, Egham, United Kingdom, 1995, pp. 641–655.

[3] M.E. Zain, H.H. El-Sheikh, H.G. Soliman, A.M. Khalil, J. Saudi Chem. Soc. 15 (3) (2011) 239–246.

[4] H. Laatsch, Antibase, A Data Base for Rapid Structural Determination of Microbial Natural Products, Wiley-VCH, Weinheim, Germany, 2010.

[5] A.S. Anderson, E.M. Wellington, Int. J. Syst. Evol. Microbiol. 51 (2001) 797–814.

[6] A.L. Demain, S. Sánchez, J. Antibiot. 62 (2009) 5–16.

[7] IARC, International Agency for Research on Cancer: Cancer Incidence and Mortality Worldwide, IARC, Lyon, France, 2010.

[8] A.B. Miller, B. Hoogstraten, M. Staquet, A. Winkler, Cancer 47 (1981) 207–214.

[9] M. Feher, J.M. Schmidt, J. Chem. Inf. Comput. Sci. 43 (2003) 218–227.

[10] Mohsen M.S. Asker, Mahmoud A. Swelim, Ibrahim H. Abbas, Aziza I. Attwa, Mohamed E. El Awady, Osama H. El Sayed, J. Biol. Chem. Environ. Sci. 9 (1) (2014) 423–435.

[11] T.G. Pridham, P. Anderson, C. Foley, H.A. Lindenfelser, C.W. Hesseltine, R.G. Benedict, Antibiot. Ann. (1956) 947–953.

[12] M.M. Nagia, M.M. El Metwally, M. Shaaban, S.M. El Zalabani, A.G. Hanna, Org. Med. Chem. Lett. 2 (2012) 9.

[13] B.P. Lisoboa, J. Chromatogr. 16 (1964) 136–151.

[14] W. Brand-Williams, M.E. Cuvelier, C. Berset, Lebensm. Wiss. Technol. 28 (1995) 25–30.

[15] M.B. Hansen, S.E. Nielsen, K. Berg, J. Immunol. Methods 119 (1989) 203–210.

[16] R. Pauwels, J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, E. DeClarcq, J. Virol. Methods 20 (1988) 309–321.

[17] A.E. Rashad, A.H. Shamroukh, R.E. Abdel-Megeid, W.A. El-Sayed, Synth. Commun. 40 (2010) 1149–1160.

[18] J.Y. Ortholand, A. Ganesan, Curr. Opin. Chem. Biol. 8 (2004) 271–280.

[19] G.L. Challis, D.A. Hopwood, Proc. N. Y. Acad. Sci. 100 (II) (2003) 14555–14561.

[20] S.O. Mura, H. Ikeda, J. Ishikawa, A. Hanamoto, C. Takahashi, M. Shinose, Y. Takahashi, H. Horikawa, H. Nakazawa, T. Osonoe, T. Kikuchi, T. Shibai, Y. Sakaki, M. Hattori, Proc. N. Y. Acad. Sci. 98 (21) (2001) 12215–12220.

[21] J.R. Tormo, J.B. Garcia, M. DeAntonio, J. Feliz, A. Mira, M.T. Diez, P. Hernindez, F. Peliez, J. Ind. Microbiol. Biotechnol. 30 (l0) (2003) 582–588.

[22] K.R. Arun, M.C. Uma, S. Ghantasala, C. Jyothi, R.K. Rajender, Adv. Synth. Catal. 353 (2011) 401–410.

[23] Z. Ye, G. Lv, W. Wang, M. Zhang, J. Cheng, Angew. Chem. 122 (2010) 3753–3756.

[24] D. Rayabarapu, H. Chang, C. Cheng, Chem. Eur. J. 10 (2004) 2991–2996.

[25] G. Lin, S.K. Chan, H.S. Chung, S.L. Li, in: A. Rahman (Ed.), Studies in Natural Products Chemistry, Elsevier, Amsterdam, Netherlands, 2005, pp. 611–669.

[26] F. Chen, C.N. Shanghai, T. Wang, Use of phthalide derivatives. Untied state patent, No. US8,445,532 B2, 2013

[27] W.L. Kan, C.H. Cho, J.A. Rudd, G. Lin, J. Ethnopharmacol. 120 (2008) 36–43.

[28] C. Chen, R.L. Yang, J. Med. Biol. Res. 46 (2013) 643–649.

[29] P. Phuwapraisirisan, J. Rangsan, P. Siripong, S. Tip-Pyang, Nat. Prod. Res. 23 (2009) 1063–1071.

[30] C. Almeida, S. Kehraus, M. Prudêncio, Beilstein J. Org. Chem. 7 (2011) 1636–1642.

[31] K. Zhong, X. Goa, Z. Xu, H. Goa, S. Fan, I. Yamaguchi, L. Li, R. Chen, Curr. Res. Bacteriol. 4 (2) (2011) 63–72.

[32] T. Revathy, M.A. Jayasri, K. Suthindhiran, Am. J. Biochem. Biotechnol. 9 (2013) 282–290.

[33] P. Wang, L. Xi, P. Liu, Y. Wang, W. Wang, Y. Huang, W. Zhu, Mar. Drugs 11 (2013) 1035–1049.