In-vitro bioassays on the metabolites of the fungus *Emericella nidulans* isolated from the Egyptian Red Sea algae

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Aim

There are a number of theories on which organisms provide the most interesting bioactive metabolites. In this study, we discuss the biochemical activities of the marine-derived endophyte *Emericella nidulans*, isolated from the Egyptian Red Sea algae.

Methods

The fungus *E. nidulans* was isolated as an endophyte from the Egyptian Red Sea brown alga *Turbinaria elatensis*. The fungus was identified by a morphological method and 18S rDNA sequence comparison. Chemical constituents were isolated using chromatographic techniques.

Results and conclusion

Cultivation of this fungus in Czapek's peptone media led to the isolation of five known metabolites: sterigmatocystin (**1**), emericellin (**2**), cordycepin (**3**), ergosterol peroxide (**4**), and myristic acid (**5**) from the ethyl acetate extract of the culture broth.
The structures were elucidated on the basis of NMR spectroscopic analysis and mass spectrometry. The ethyl acetate extract and the isolated compounds were tested for antimicrobial properties, activity against cancer cell lines, and inhibition of the hepatitis C virus protease.

Keywords:
anti-hepatitis C virus protease, brown algae, *Emericella nidulans*, *Turbinaria elatensis*

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Introduction

Marine-derived microbes, fungi in particular, have long been recognized as a potential source of structurally novel and biologically potent metabolites [1–3]. The fungal genus *Emericella* is one of the sexual states of *Aspergillus* [4]. Several species of this genus are saprobes, whereas others are either pathogenic or endophytic on living plants [5,6]. *Emericella* spp. have been reported as a source of a remarkable diversity of secondary metabolites with interesting biological properties, including anti-tumor indole alkaloids and quinones [7,8], neuritogenic and antimicrobial polyketides [9], cytotoxic sesterterpenes [10], aflatoxins, and sterigmatocystin [11], as well as xanthones and cyclic depsipeptides with antimicrobial, immunostimulatory, and calmodulin inhibition activities [12–15].

Within the scope of our program aiming at the isolation of bioactive natural products from marine endophytic fungi, we have isolated and identified *Emericella nidulance* from the inner tissue of the brown algae *Turbinaria elatensis* collected from the Egyptian Red Sea. The culture broth extract of the fungus was subjected to detailed chemical analysis as well as *in-vitro* bioassays for estimation of antimicrobial, anticancer, and antiviral properties.

Subjects and methods

General experimental

Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and silica gel (60–120 mesh; Qualigens, Mumbai, India) were used for column chromatography. Czapek's agar and potato dextrose broth were procured from Lab M (Lancashire, UK). Flash chromatography was carried out on silica gel (230–400 mesh). Thin-layer chromatography (TLC) was performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co., Düren, Germany). A mixture of methanol and methylene chloride (3 : 2 and 1 : 1, v/v) was used as a mobile phase for TLC analysis. Compounds were visualized as intense dark, blue, and yellow colored spots on TLC under ultraviolet (UV) light. Most of the colored spots changed after spraying with anisaldehyde/sulfuric acid followed by heating at 120°C. UV/vis spectra were recorded on a Shimadzu model UV-240 spectrometer (Shimadzu, Tokyo, Japan). NMR spectra were measured on a Varian Inova 500 spectrometer (International Equipment Trading Ltd, Vernon Hills, Illinois, USA) (^{1}H, 500 MHz; ^{13}C, 125.7 MHz). Electrospray ionization mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, California, USA). High-resolution mass spectra were recorded by

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electrospray ionization mass spectrometry on an Apex IV 7 T Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany).

Enzymes and chemicals

Sensolyte 520 HCV protease assay kit Fluorimetric (Lot #AK71145-1020), HCV NS3/4A protease, and Hepatitis Virus C NS3 protease inhibitor *2* (cat #25346) were purchased from AnaSpec Inc. (San Jose, California, USA). Becton Dickinson Falcon Microtest 384-well 120 µl black assay plates, nonsterile, no lid, were purchased from Becton Dickinson Inc. (Tokyo, Japan).

Fungal isolation and culture conditions

The brown algae *T. elatensis* was collected from the Egyptian Red Sea site at a depth of 3–6 m from the coast of Rass Mohamed (South Sina, Egypt) in March 2010. The sample was selected solely on the basis of a clean and healthy exterior and brought to the laboratory in ice. In the laboratory, the specimens were washed with sterile water and processed immediately. The sample was identified by the Coral Reef Ecology and Biology group, National Institute of Oceanography and Fisheries, Suez, Egypt.

The fungus *E. nidulans* was isolated as an endophyte using Czapek's agar containing (g/l) glucose (30), peptone (10), yeast (2), $NaNO_3$ (3), KH_2PO_4 (0.5), KCl (0.5), KH_2PO_4 (0.5), and agar (30) at 28°C. The pH of 50% seawater supplemented with penicillin benzyl sodium salt (0.02) was adjusted to 7.5 to avoid any bacterial growth. After 6–7 days, sand brown, velvety colonies were observed. The strain was identified as *E. nidulans* from the morphological features of its conidiophores and a voucher specimen of the fungus was deposited at the Microbiology Department, Assiut University, Egypt.

Stock cultures of the fungus were used to inoculate 500 ml of liquid medium in an Erlenmeyer flask (Nova-Tech International, Houston, Texas, USA) (3 l) containing biomalt broth in 50% seawater. It was then cultured at 35 ± 2°C on a rotary shaker at 200 rpm. The flask was incubated for 72 h and used as first stage inoculum. The same medium (10 l) was made in 75 Erlenmayer flasks (1 l) and inoculated with 5% of first stage inoculum. The flasks were incubated statically for 15 days at 35°C.

Identification of the endophytic isolate

The endophytic fungus was isolated from brown algae (*T. elatensis*), grown on Czapek's peptone agar at 28°C for 7 days, and morphologically characterized as *E. nidulans*. The mycelium was scraped directly from the surface of the agar culture (6 days old) and weighed. Nucleic acid was extracted and purified using the GenElute DNA isolation kit for genomic DNA (Sigma-Aldrich, St Louis, Missouri, USA) by the Chomczynski method [16]. For identification and differentiation, the internal transcript spacer regions (ITS1 and ITS4) and the intervening 5.8S rRNA regions were amplified and sequenced by electrophoretic sequencing on a 3130 genetic analyzer (Fermentas Company, Glen Burnie, Maryland, USA; Taq polymerase, deoxynucleotide triphosphates) using the GenJET sequencing kit (Signa Gen Laboratories, Gaithersburg, Maryland, USA). The DNA fragment of the ITS regions was amplified by PCR with the pair of primers ITS1 (5′-TGCCAGCMGCCGCGGTA-3′) and ITS4 (5′-GACGGGCGGTGTGTRCAA-3′), and the PCR products were assayed using the method of Kumeda and Asao [17]. DNA sequencing was carried out at Sequencer Scientific City (Borg El-Arab, Egypt).

Extraction and isolation of metabolites

Fifteen-day-old fermentation broth (10 l) was separated from the fungal mat. The liquid medium and fungal mycelia were extracted with ethyl acetate. The resultant extract was dried using a Rotavapour (Heidolph, Schwabach, Germany) with a heating water bath (≤40°C), after which defatting was carried out using *n*-hexane solvent.

The crude extract was applied to a silica gel column using *n*-hexane as the starting nonpolar eluent and by gradually increasing the polarity using ethyl acetate as a polar solvent in the eluent mixture (5, 10%, until 100% ethyl acetate), followed by 20 and 50% methanol/ethyl acetate regarding to the TLC of the crude extract as reference during the fractionation. The combined semi-fractions are further purified on a Sephadex LH-20 column with MeOH, MeOH/CH_2Cl_2 (1 : 1 and *2* : 3), and MeOH/CH_2Cl_2/*n*-hexane (*2* : 2 : 1) to yield pure compounds (**1–5**).

Sterigmatocystin (*1*)

Pale yellow crystals (26 mg); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 13.24 (1H, s, 7-OH), 7.51 (1H, t, J = 8.4 Hz, H-9), 6.83 (1H, d, J = 7.2 Hz, H-4), 6.82 (1H, d, J = 8.4 Hz, H-8), 6.76 (1H d, J = 8.4 Hz, H-10), 6.51 (1H, t, J = 2.5 Hz, H-1), 6.44 (1H, s, H-5), 5.45 (1H, t, J = 2.5 Hz, H-*2*), 4.81 (1H, dd, J = 7.2 and 2.4 Hz, H-3), 3.98 (3H, s, 6-OCH_3); ($CDCl_3$, 150 MHz): δ 181.4 (C-11), 164.6 (C-12a), 163.2 (C-10a), 162.5 (C-6), 154.9 (C-7), 154.0 (C-5a), 145.4 (C-1), 135.7 (C-9), 113.3 (C-4), 111.2 (C-10), 108.9 (C-7a), 106.5 (C-12b), 105.9 (C-11a), 105.8 (C-8), 102.5 (C-*2*), 90.2 (C-5), 56.8 (6-OCH_3), 48.2 (C-3); (+) ESI-MS: *m/z* 325 $[M + H]^+$, 347 $[M + Na]^+$, 671 $[2M + Na]^+$, and 995 $[3M + Na]^+$.

Emericellin (*2*)

White crystals (25 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 12.56 (1H, s, 1-OH), 7.46 (1H, d, J = 8.5 Hz, H-3), 7.34 (1H, s, H-5), 6.78 (1H, d, J = 8.5 Hz, H-*2*), 5.64 (1H, m, H-*2*′), 5.31 (1H, m, H-*2*′), 5.11 (2H, s, 1′-CH_2), 4.46 (2H, d, J = 7.0 Hz, 11-CH_2), 3.51 (2H, d, J = 7.0 Hz, 1′-CH_2), 2.46 (3H, s, 6-CH_3), 1.82 (3H, s, 4′-CH_3), 1.80 (3H, s, 5′-CH_3), 1.76 (3H, s, 4′-CH_3), 1.73 (3H, s, 5′-CH_3); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 184.6 (C-9), 160.1 (C-1), 153.9 (C-7), 152.9 (C-4a), 152.6 (C-10a), 142.6 (C-6), 138.6 (C-8), 136.9 (C-3), 134.3 (C-3′), 133.3 (C-3′), 121.7 (C-*2*′), 119.8 (C-*2*′), 119.6 (C-5), 118.9 (C-4), 118.1 (C-8a), 110 (C-2), 109.1 (C-9a), 72.3 (C-1′), 57.2 (C-11), 27.4 (C-1′), 25.8 (C-4′), 25.7 (C-4′), 18.2 (C-5′), 17.9 (C-5′), 17.6 (6-CH_3); ESI-MS: *m/z* 409 $[M + H]^+$ and 839 $[2M + Na]^+$.

Table 1 Anticancer results of the secondary metabolites of *Emericella nidulans*

			Solid tumors				
	Normal cells	Leukemia		Colon cancer			
Samples	CFU-GM	L1210	CCRF-CEM	HCT-116	Colon 38	Lung cancer H-125	Liver cancer HEP-G2
Ethyl acetate extract	250	–	200	0	250	100	550
Sterigmatocystin (**1**)	250	–	100	–	50	–	300
Cordycepin (**3**)	150	100	100	0	200	100	400
Ergosterol peroxide (**4**)	–	0	100	100	–	100	50

–, samples not tested against examined cell lines.

Table 2 Anti-hepatitis C virus NS3/4A protease activity for *Emericella nidulans* metabolites

Sample	HCV protease inhibitory activity IC_{50} (μg/ml)
Ethyl acetate extract	30.0 ± 2.2
Sterigmatocystin (**1**)	48.5 ± 4.2
Emericellin (**2**)	50.0 ± 3.8
Cordycepin (**3**)	24.5 ± 2.3
Ergosterol peroxide (**4**)	47.0 ± 3.4
Myristic acid (**5**).	51.0 ± 2.6
HCV-I2	1.5 ± 0.5

Results are represented as means ± SD ($n = 3$).
HCV-I2: hepatitis virus C NS3/4A; protease inhibitor 2 (positive control for HCV protease).

Cordycepin; 9-cordyceposidoadenosine; 3′-deoxyadenosine; adenine cordyceposide (*3*)

White powder (23 mg); ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.31 (1H, s, H-8), 8.12 (1H, s, H-2), 7.25 (2H, s, 6-NH_2), 5.81 (1H, d, $J = 2.5$ Hz, H-1′), 5.64 (1H, d, $J = 4.0$ Hz, 2′-OH), 4.51 (1H, m, H-2′), 4.29 (1H, m, H-4′), 3.63/3.45 (2H, m, 5′-CH_2), 2.20/1.87 (2H, m, 3′-CH_2); ^{13}C NMR (DMSO-d_6, 125.4 MHz): δ 156.0 (C-4), 152.3 (C-2), 148.8 (C-6), 138.9 (C-8), 118.9 (C-5), 90.7 (C-1′), 80.6(C-4′), 74.6 (C-2′), 62.5 (C-5′), 34.0 (C-3′); (+) ESI-MS: *m/z* 252 $[M + H]^+$, 274 $[M + Na]^+$ and 525 $[2M + Na]^+$; (+) HRESI-MS *m/z* 252.10912 $[M + H]^+$ (calc for $C_{10}H_{14}N_5O_3$).

Ergosterol peroxide; 5,8-epidioxy-5a,8a-ergosta-6,22E-dien-3b-ol (*4*)

Colorless needles (17 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.54 (1H, d, $J = 8$ Hz, H-6), 6.27 (1H, d, $J = 8$, H-7), 5.26 (1H, dd, $J = 15$ and 6.7 Hz, H-22), 5.18 (1H, dd, $J = 15$ and 6.7 Hz, H-23), 3.98 (1H, m, H-3), 2.19 (1H, dd, $J = 6.7$ and 4.5 Hz, H-20), 1.92 (1H, m, H-24), 1.82–1.21 (17H, m, remaining methines and methylenes), 1.12 (3H, d, $J = 6.5$ Hz, 21-CH_3), 0.87 (3H, d, $J = 6.6$ Hz, 25-CH_3), 0.84 (3H, s, 19-CH_3), 0.84 (3H, d, $J = 6.6$ Hz, 27-CH_3), 0.82 (3H, d, $J = 6.6$ Hz, 28-CH_3), 0.82 (3H, s, 18-CH_3); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 135.9 (C-6), 135.7 (C-22), 132.8 (C-23), 131.4 (C-7), 82.3 (C-8), 79.7 (C-5), 66.7 (C-3), 52.1 (C-14), 51.6 (C-9), 44.7 (C-13), 42.8 (C-24), 39.7 (C-20), 39.4 (C-12), 37.0 (C-4/10), 34.7 (C-1), 33.1 (C-26), 30.1 (C-2), 28.6 (C-16), 23.4 (C-15), 20.9 (C-21), 20.6 (C-11), 19.9 (C-27), 19.6 (C-28), 18.1 (C-19), 17.5 (C-25), 12.9 (C-18); (+) ESI-MS: *m/z* 451 $[M + Na]^+$, 879 $[2M + Na]^+$ and 1308 $[3M + Na + H]^+$.

Myristic acid; tetradecanoic acid (*5*)

White powder (7 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.42 (2H, m, 2-CH_2), 1.51 (2H, m, 13-CH_2), 1.28 (20H, br, 3-CH_2 to 12- CH_2), 0.85 (3H, t, $J = 2.5$ Hz, 14-CH_3); ^{13}C NMR ($CDCl_3$, 125.4 MHz): δ 179.9 (1-COOH), 34.1 (C-2), 31.9 (C-12), 29.7 (C-6/8), 29.6 (C-9), 29.4 (C-5/7), 29.2 (C-10), 29.1 (C-4), 24.7 (C-3), 22.6 (C-3), 14.1 (14-CH_3); (+) ESI-MS: *m/z* 227 $[M - H]^-$, 454 $[M - H]^-$ and 479 $[2M + Na]^+$.

Assay for hepatitis C virus protease inhibitory activity

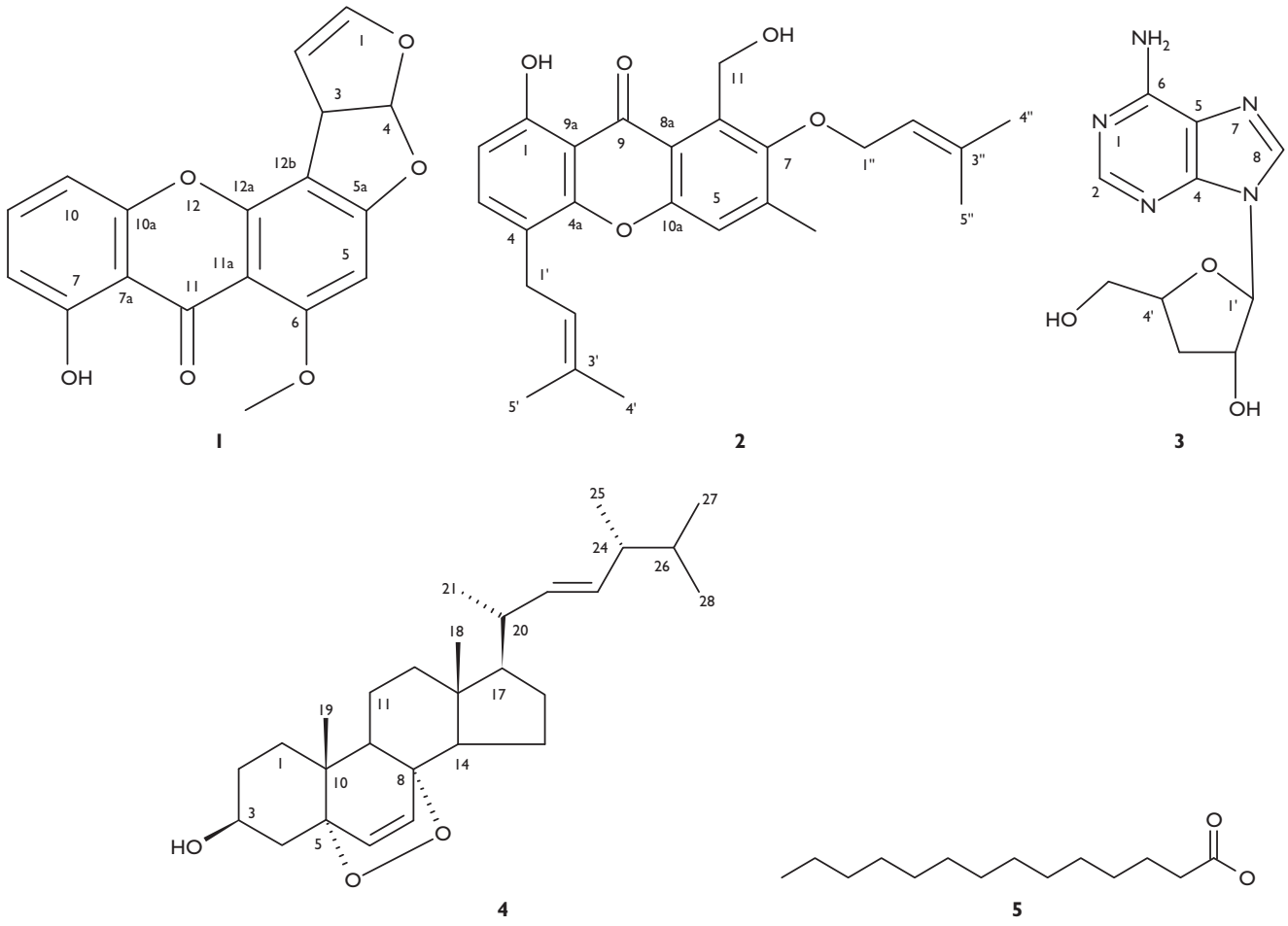
In total, 2 μl of a compound solution (DMSO as solvent) were dispensed in each well of a 384-well microplate; thereafter, 8 μl of recombinant HCV protease (0.5 μg/ml) was added to the well containing the sample and the plate was briefly agitated. Finally, 10 μl of the freshly prepared substrate [Ac-Asp-Glu-Dap (QXLTM520)-Glu-Glu-Abu-COO-Ala-Ser-Cys(5-FAMsp)-NH2; 100 × dilution of a DMSO stock solution] was added with sequential rotational shaking. The reaction mixture was incubated for 30 min at 37°C. Fluorometric analyses were carried out on an automated Tecan GENios plate reader (Tecan Group Ltd, Männedorf, Switzerland) with an excitation wavelength of 485 nm and emission wavelength 530 nm. Each test compound was analyzed in triplicates. The HCV protease percentage inhibition was calculated using the following equation:

$$\%\,\text{Inhibition} = (F_{\text{substrate}} - F_{\text{test compound}}) \times 100 / F_{\text{substrate}},$$

where $F_{\text{substrate}}$ is the fluorescence value of the substrate and enzyme without the test compound, $F_{\text{test compound}}$ is the fluorescence value of the test compound dissolved in DMSO.

Results and discussion

The culture of *E. nidulans* grown in a common liquid medium, namely Czapek's peptone broth, was extracted with ethyl acetate, and the resultant extract was subjected to chemical screening based on TLC analyses under staining with anisaldehyde/sulfuric acid reagent. Five known compounds were separated from the organic extract by Sephadex LH-20 and silica gel column chromatography and identified as sterigmatocystin (**1**), emericellin (**2**),



cordycepin (**3**), ergosterol peroxide (**4**), and myristic acid (**5**). The structures were elucidated by mass spectrometry and one-dimensional (1D) and 2D NMR spectroscopy.

In-vitro bioassays

Antimicrobial

Antimicrobial activity of the ethyl acetate extract and isolated compounds was evaluated against different bacteria and fungi. The extract showed only moderate activity against *Bacillus megaterium* (Gram-positive bacteria), whereas the isolated compounds were inactive against all tested pathogens.

Anticancer activity

The extract of the culture broth of the fungus *E. nidulans* and the isolated compounds (**1**), (**3**), and (**4**) were screened for their *in-vitro* anticancer activity against two leukemias (murine L1210 and human CCRF-CEM), four solid tumors (murine colon 38, human colon HCT-116, human lung H-125, human liver HEP-G2), as well as human normal cells (CFU-GM) using the disc diffusion assay [18]. The samples were initially prepared in DMSO and then applied to the filter disc. After 7 days of incubation with the examined cells, the cells that had survived had grown into colonies, and the zones of inhibition of colony formation were assessed. A zone of 0 implied that there was no inhibition. A zone of less than 250 implied that there was minimal activity.

From the bioassay data (Table 1), it can be seen that the ethyl acetate extract of *E. nidulance* exhibited selective anticancer activity against the solid tumor of liver cancer cells (HEP-G2) compared with leukemia (CCRF-CEM) and normal cells (CFU-GM) at a concentration of 30 μg/disc. Cordycepin (**3**) showed potent activity against the same cells with an inhibition zone difference of 250 between liver cancer cells (HEP-G2) and normal cells (CFU-GM) when 3 μg of the pure compound was applied to the filter disc, whereas sterigmatocystin (**1**) and ergosterol peroxide (**4**) showed mild to weak activity in comparison with the activity of the extract.

Hepatitis C virus NS3/4A protease inhibitory action

The ethyl acetate extract along with compounds (**1–5**) isolated from *E. nidulans* were tested for their inhibitory activity against HCV protease using HCV NS3 protease inhibitor 2 as a positive control [19,20]. The ethyl acetate extract showed potent activity against HCV NS3/4A protease with an inhibitory concentration fifty (IC_{50}) of 30.0 μg/ml (Table 2). Cordycepin (**3**) exhibited potent inhibition with an IC_{50} of 24.5 μg/ml, whereas compounds (**1**), (**2**), (**4**), and (**5**) showed a mild inhibitory effect with IC_{50} values of 48.5, 50.0, 47.0, 51.0 μg/ml, respectively.

However, it is noteworthy that this is the first report on the anti-HCV protease activity of adenosine, which warrants further investigation of other members of this widely distributed class of compounds.

Conclusion

The present study reveals that the ethyl acetate extract of the culture broth of endophytic *E. nidulans* possesses promising anti-HCV protease activity and selective anticancer activity against liver cancer cell lines, which may be attributed to the presence of adenosine class compounds.

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Conflicts of interest

There are no conflicts of interest.

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