



Apoptosis on non-small lung cancer cells and DPPH radical scavenging activity of the crude ethyl acetate extract of a soil bacterial species FEAI-1

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ABSTRACT

The aim of this research is to evaluate the *in vitro* apoptosis and free radical scavenging activities of the crude extract of a soil bacterial species FEAI-1. Apoptosis activity was investigated using annexin V-phycoerythrin staining on human non-small-cell lung carcinoma cell line (H1299), and an antioxidant study of the crude ethyl acetate extract was carried out by the diphenylpicrylhydrazyl (DPPH) assay. The crude ethyl acetate extract of the bacterial species FEAI-1 induced apoptosis on H1299 cell lines in a concentration-dependent manner. After a 96-hours treatment period, flow cytometry showed that the extract induced total apoptosis by 10.25%, 13.48%, 20.42%, 68.10%, and 68.80% at concentrations of 2.5, 5, 10, 20, and 40 µg/ml, respectively. The crude extracts which elicited antioxidant activity with the inhibition of DPPH radicals were 24.97% ± 0.288%, 26.29% ± 0.085%, 29.06% ± 0.123%, 33.07% ± 0.01%, 38.45% ± 0.03%, 45.33% ± 0.02%, and 53.21% ± 0.115% at concentrations (µg/ml) of 10, 20, 40, 80, 160, 320, and 640, respectively. The inhibition concentration 50 value of the extract was 510 µg/ml. These results suggest that the crude extract of the bacterial sp. FEAI-1 could be a useful natural source having therapeutic potential against cancer and diseases caused by oxidative stress. Furthermore, *in vivo* studies and compound isolation pave the way for its biomedical applications.

INTRODUCTION

Cancer is the major global health problem and the second leading cause of death after cardiovascular disease (Siegel *et al.*, 2015). Lung cancer is the most common cancer which is responsible for the largest number of cancer-related deaths globally for both men and women. It kills more people than female breast cancers, colorectal cancers, brain cancers, and prostate cancers combined (Siegel *et al.*, 2013). According to the American Cancer Society, in the USA, around 234,030 new victims of lung cancer would be identified and 154,050 people will die of the disease in 2018 (Siegel *et al.*, 2018). This is just about 26% of all cancer mortalities in the USA (Smith *et al.*, 2018). The high percentage of

lung cancer is due to the fact that both smokers and nonsmokers have the risk of lung cancer (Shopland *et al.*, 1991; Zappa and Mousa, 2016). From July 2, 2010, to July 2, 2011, a case-control survey of common cancer causes and etiology in cancer hospitals of Dhaka city was conducted with 610 subjects. The outcome of the survey was that 76 patients were suffering from lung cancer among 339 male cancer patients (Shahriar *et al.*, 2011).

Despite the significant progress achieved in anticancer therapy, typical cancer treatments (surgery, chemotherapy, immune therapy, and radiation therapy) frequently fail to attain a complete cancer recovery (Bernardes *et al.*, 2010). Moreover, still drug side effects, drug resistance, and greater systemic toxicity are the major challenges for anticancer drugs (Ko *et al.*, 2018; Ling, 1992; Longley and Johnston, 2005; Naidu *et al.*, 2004). Due to the negative consequences of radiotherapy and chemotherapy, cancer sufferers are now looking for alternative medical therapy with milder side effects (Buckner *et al.*, 2018; Rehman *et al.*, 2016). This has played an indispensable role in the searching and

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developing of myriad new approaches to prevent or treat cancer. One such strategy involves the application of bacterial secondary metabolites (Bernardes *et al.*, 2010).

The increasing rate of free radical production or reducing rate of antioxidant defense responses may increase oxidative stress (Kim *et al.*, 2008). The increased level of reactive oxygen species (ROS) inside the cells is the major etiology of a number of diseases, including cancer, neurodegenerative, and cardiovascular diseases. ROS is responsible for several kinds of DNA damage and mutations (Devasagayam *et al.*, 2004; Joseph *et al.*, 2013). Antioxidants make every effort to scavenge and prevent the generation of free radicals or to promote their decomposition and hinder the progression of these diseases (Lobo *et al.*, 2010). Metabolites of soil bacterial species having a dominant radical scavenging activity may play a crucial role in the development of therapeutic drugs (Ser *et al.*, 2016). Therefore, the objective of the current study is to explore the secondary metabolites of a soil bacterial species named FEAI-1 with apoptosis and antioxidant activity.

MATERIALS AND METHODS

Isolation of soil bacterial species

During the isolation process, we focused on isolating actinomycetes from the soil, as their secondary metabolites are well known for antibacterial, anticancer, antitumor, antifungal, antiviral, antioxidant, and immunosuppressive activities (Ajijur Rahman *et al.*, 2010; Hong *et al.*, 2009; Kawahara *et al.*, 2012; Kumar *et al.*, 2014; Qin *et al.*, 2011; Tan *et al.*, 2015). A total of 11 soil samples were collected from the Mirzapur region of Rajshahi city area in Bangladesh. At 60°C–65°C, soil samples were dried in an oven for 3 hours to decrease the number of other bacterial species, except for actinomycetes, and were then preserved at 4°C before screening. 1 gm of preserved soil was weighed and tenfold serial dilution was carried out in sterile distilled water. 0.1 ml of each solution, at dilutions of 10^{-4} – 10^{-5} , was then spread on selective media (per 1 litre: 0.4 g casein, 1.0 g starch, 0.5 g KNO_3 , 0.1 g CaCO_3 , 0.2 g K_2HPO_4 , 0.1 g $\text{Mg}_3(\text{PO}_4)_2$, and 15 g agar). After that, the Petri dishes were incubated at 30°C for 7–14 days. Starch and casein of the isolation media (Pharm, 2008) are the only sources of carbon and energy of that media. Only organisms having the ability to degrade starch and casein (mostly *Streptomyces* and molds) can survive and multiply. 50 µg/ml nystatin was added to the culture media to prevent the growth of fungi. Typical *Streptomyces* colonies were picked and purified by streaking onto an inorganic salt–starch agar media (per 1 litre: 10 g starch, 2 g $(\text{NH}_4)_2\text{SO}_4$, 2 g CaCO_3 , 1 g K_2HPO_4 , 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g NaCl, and 20 g agar), and then the pure cultures were placed into the incubator at 30°C for 7–14 days. A total of 27 bacteria were isolated from these soil samples. Among them, FEAI-1 had good antibacterial and cytotoxic activity (Khatun *et al.*, 2017). The isolated species FEAI-1 was preserved on an inorganic salt–starch agar media (Küster, 1959) at 4°C.

Identification of FEAI-1 bacteria by morphological and biochemical characterization

Morphological identification

Visual observation was performed with the help of optical microscopy to determine the morphological and microscopic characteristics. Morphological features (size, shape,

and arrangements) play an important role to distinguish and characterize bacterial species. Morphological characteristics of the bacterial species FEAI-1 were assessed according to the process provided by Shirling and Gottlieb (1966). Microscopic observation was carried out using the coverslip culture method. Firstly, using sterile forceps, two sterile coverslips were introduced into the sterile solid media of the Petri dish at an angle of 45°–50°. Then, at the free space of the plate, the strain was inoculated by the streaking method. After that, the plate was placed into the incubator for 10 days at 30°C. During the incubation period, the hyphae of FEAI-1 grew and spread on the coverslip and produced spores. After this period, coverslips were pulled out smoothly and mounted on slides. Lastly, the slides were examined under a microscope. The observed characteristics were shape, size, aerial mass color, and substrate mycelium. By direct microscopic examination of the culture surface, the characteristics of hyphae and spore chains were observed. Using 100x–700x magnifications, the chain of spores was observed. On the basis of Bergey's manual of systematic bacteriology, the aerial mass color was also determined (Locci, 1989). The plates of the bacterial species FEAI-1 were examined for Gram staining.

Biochemical characterization

Physiological characteristics, for instance, the capability of the bacterial species FEAI-1 to degrade casein, tyrosine, and xanthine as substrates, were used for genus confirmation.

Optimization of cultural conditions

Optimum nutritional conditions for the growth and formation of secondary metabolites of the bacterial species FEAI-1 were determined by culturing the bacteria in different culture media, such as ISP-1 agar, ISP-2 agar, ISP-4 agar, ISP-5 agar, nutrient agar, yeast-extract glucose agar, and starch–casein agar media. To determine optimum growth conditions and to ensure maximum production of secondary metabolites, the bacterial strain FEAI-1 was grown at different incubation temperatures (25°C, 30°C, 35°C, and 40°C) and different incubation periods (3, 4, 5, 7, 10, and 15 days).

Extraction of secondary metabolite

At first, 500 ml inorganic salt–starch broth culture medium was prepared in a conical flask. Then, a small amount of FEAI-1 bacteria was transferred into the conical flask aseptically and the bacteria were incubated at 35°C for 15 days. After incubation, cell biomass was separated by filtration. Then, the filtrate was partitioned using the organic solvent ethyl acetate because the solubility of secondary metabolites was higher in ethyl acetate than other organic solvents. A rotary vacuum evaporator was used to evaporate ethyl acetate fraction until a semisolid mass was obtained. The semisolid mass was then air-dried to get a solid mass.

Screening for antineoplastic activity

Cell lines collection and maintenance

For evaluation of apoptosis activity, a non-small lung cancer cell line (H1299) was used. H1299 cells were collected from the MD Anderson Cancer Center. The cell line was grown

in the DMEM/F12 (1:1) medium supplemented with 10% heat-inactivated FBS at 37°C in a humidified incubator containing 5% CO₂ and 95% air. The authenticity of this cell line was determined by the genomic short tandem repeat (STR) profile. STR was performed in the Research Animal Diagnostic Laboratory, University of Missouri (Columbia, MO), and by the Emory University Integrated Genomics Core.

Sample preparation

The extract of FEAI-1 was weighted appropriately and 100 µg/ml stock solution was prepared using dimethyl sulfoxide prior to assay.

Annexin V-phycoerythrin staining for screening apoptosis

Firstly, H1299 cells were treated with the extract (µg/ml) for 96 hours. Afterward, the cells were trypsinized and washed with cold 1 × PBS. Finally, the cells were suspended in 1 × annexin binding buffer (BD Pharmingen) and stained with annexin V-phycoerythrin (annexin V-PE; BD Pharmingen) and 7-amino-actinomycin D (7-AAD; BD Pharmingen) for 15 minutes at 25°C. The stained samples were measured with a fluorescence-activated cell sorting caliber bench-top flow cytometer. FlowJo software was used for apoptosis analysis.

Diphenylpicrylhydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging assay is a frequently used and rapid method for testing the antioxidant potential of natural compounds (Swarnalatha *et al.*, 2015). This test of FEAI-1 extract was performed according to a previous method (Swarnalatha *et al.*, 2015) with minor changes (Elmastas *et al.*, 2007). Different concentrations of 1 ml of the sample as well as standard compound (ascorbic acid) were added to 2 ml of freshly prepared 0.004% DPPH in 95% methanol. A 2 ml methanol solution of DPPH was added to 1 ml 95% methanol for control reading. All the mixtures were kept at 25°C in a dark place for 30 minutes. After that, the absorbance was measured at 517 nm on UV-visible spectrometer. The percentage of DPPH radical scavenging capacity was measured with the following formula:

$$\text{Percent inhibition} = (a-b) / a \times 100$$

where a = absorbance of control reaction and b = absorbance of test or standard samples.

RESULTS

Optimization of culture condition and identification of the selected bacterial strain

Biochemical characterizations and optimum culture conditions of the bacteria FEAI-1 are presented in Table 1 and cultural characterizations are presented in Table 2. The selected bacterium FEAI-1 belonged to the genus *Streptomyces* since it was Gram-positive and aerobic and produced well-developed substrate mycelium. Aerial hyphae were long, containing extended filamentous chains and cylindrical smooth spores. The colonies were glabrous, heaped, and folded, and the growth of the colonies was slow. The aerial and substrate mycelia possessed different colors on different culture media. In addition, it had an earthy odor. The bacterial species also had the ability to degrade

casein, tyrosine, and xanthine. The strain FEAI-1 produced yellow pigment into the culture media.

Analysis of apoptosis by flow cytometer

One of the major features of apoptotic cells is the exposure of the phospholipid phosphatidylserine (PS) to the external environment. This happened by the translocation of the PS membrane from the inner to the outer leaflet of the plasma membrane. Annexin V-phycoerythrin (PE) complex has a higher affinity for PS. Annexin V-PE binds with exposed PS in apoptosis cells (Herault *et al.*, 1999). 7-AAD has a high affinity to bind with DNA. So it is used to detect nuclear damage. Viable cells with intact cytoplasmic membranes exclude both annexin V-PE and 7-AAD, representing the lower left quadrants (Fig. 1). Lower right quadrants represented the early apoptotic cells. Here, annexin V was positive and 7-AAD was negative, which indicated that the nuclear membrane was intact but the cytoplasmic membrane was damaged. Both annexin V-PE and 7-AAD binding were positive in the upper right quadrant, which represented the late apoptotic cells or necrotic cells.

It was observed that the percentage of viable cells was decreased and apoptosis cells were increased by the increase in the crude extract treatment concentration. The total apoptotic H1299 cell populations were 10.25%, 13.48%, 20.42%, 68.10%,

Table 1. Optimum culture conditions and biochemical characteristics of the isolated bacterial strain FEAI-1.

Characteristics	FEAI-1
Gram staining	+
Morphology	hyphae formed extended filamentous chain
Colony shape	glabrous, heaped and folded
Odor	Earthy
aerial mass color	Grayish pink
Casein Fermentation	+
Tyrosine Fermentation	+
Xanthine Fermentation	+
Incubation temperature	35°C
Incubation period	10–15 days
Diffusile pigment	+

Table 2. Culture characteristics of the isolated bacterial species FEAI-1.

Medium	Growth	Spore mass	Aerial mycelium	Diffusile pigment
Tryptone-yeast extract agar (ISP-1)	+	Very low	None	-
Yeast-extract-malt extract agar (ISP-2)	+	Very low	None	+
Inorganic salt-starch agar (ISP-4)	+++	Abundant	Grayish pink	++
Glycerol-asparagine agar (ISP-5)	++	Moderate	Grayish pink	+
Nutrient agar	+	Very low	None	-
Yeast-extract glucose agar (YEGA)	+	Very low	Moderate orange pink	+
Starch- casein-nitrate—agar	+++	Abundant	Moderate pink	+

and 68.80% at concentrations of 2.5, 5, 10, 20, and 40 $\mu\text{g/ml}$, respectively (Table 3).

Most of the H1299 cells were viable (88.8%) in the untreated (control) sample, whereas only 29.1% of viable cells were observed when cancer cells were treated with 40 $\mu\text{g/ml}$ of the crude ethyl acetate extracts of FEAI-1 for 96 hours. Moreover, FEAI-1 extract induced early apoptosis and late apoptosis in a concentration-dependent manner (Table 3).

DPPH-free radical scavenging assay

The percentage of inhibition is shown in Table 4. Results revealed that FEAI-1 extract had significant radical scavenging ability. With increasing concentrations of the extract, there were slow increases in activities as follows: $24.97\% \pm 0.288\%$ at 10 $\mu\text{g/ml}$, $26.29\% \pm 0.085\%$ at 20 $\mu\text{g/ml}$, $29.06\% \pm 0.123\%$ at 40 $\mu\text{g/ml}$, $33.07\% \pm 0.01\%$ at 80 $\mu\text{g/ml}$, $38.45\% \pm 0.03\%$ at 160 $\mu\text{g/ml}$, $45.33\% \pm 0.02\%$ at 320 $\mu\text{g/ml}$, and $53.21\% \pm 0.115\%$ at 640 $\mu\text{g/ml}$. The crude extract inhibition concentration 50 (IC_{50}) value was 510 $\mu\text{g/ml}$, whereas the standard ascorbic acid IC_{50} value was 6.5 $\mu\text{g/ml}$. IC_{50} values were determined graphically (Fig. 2).

DISCUSSION

Cancer progression has been associated with an increased amount of free radicals (Reuter *et al.*, 2010). Free radicals can

induce DNA damage and mutations, which subsequently increase cancer risk (Dreher and Junod, 1996). Here, antioxidants can play a vital role to prevent the harmful effects of free radicals. Typical cancer therapies cause significant side effects (Bernardes *et al.*, 2010). Due to these reasons, nowadays, considerable attention has been paid to the identification of new compounds having anticancer and antioxidant activities (Devi, 2011; Jemimah *et al.*, 2011; Lee *et al.*, 2014; Sharma *et al.*, 2010). Bacteria and its secondary metabolites could be a promising source of anticancer and antioxidant compounds (Bernardes *et al.*, 2010; Ser *et al.*, 2016). In this research, we isolated secondary metabolites producing bacterial strain FEAI-1 from a soil sample. The bacteria were characterized on the basis of morphological and biochemical features. The isolated strain was identified as *Streptomyces* species. Then, apoptosis and free radical scavenging assay were performed.

Apoptosis is a normal genetically programmed process, which plays a crucial role in regulating the growth and development of a fetus (Biosciences, 2011; Fan *et al.*, 2005). In order to destroy cancer cells without giving inflammatory effects, this apoptosis program becomes important (Alabsi *et al.*, 2012). An apoptosis test was carried out by annexin V/7-AAD double-staining procedure. Data (Table 3) showed a concentration-dependent increase in early apoptosis and necrotic cells, with a decrease in the viable cell populations. Total apoptosis (10.25%–68.8%) was increased, respectively, with increasing concentrations (2.5–40 $\mu\text{g/ml}$) of crude extract after the 96-hours pretreatment (Table 3). A recent study reported that, after the 18-hours treatment of cancer cells HL60 with the extract (25–100 $\mu\text{g/ml}$) of *Erythrina suberosa* stem bark, the cells were stained with annexin V/PI dye. The total apoptosis was 6.60% in control cells, which increased to 31.09% after 75 $\mu\text{g/ml}$ and to 30.35% after 100 $\mu\text{g/ml}$ of extract treatment (Agrawal *et al.*, 2011).

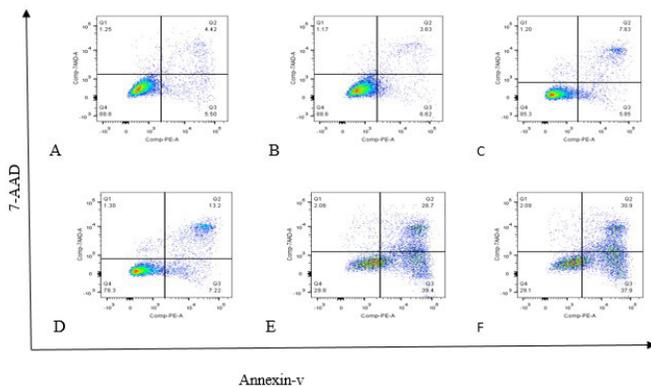


Figure 1. Contour diagram of annexin V/7-AAD flow cytometry. Flow cytometric analysis was used to determine cell apoptosis in non-small lung cancer cells (H1299) after 96 h of treatment with different concentrations of crude ethyl acetate extracts of *Streptomyces* sp. FEAI-1. (A) Untreated H1299 cells (control), (B) 2.5 $\mu\text{g/ml}$, (C) 5 $\mu\text{g/ml}$, (D) 10 $\mu\text{g/ml}$, (E) 20 $\mu\text{g/ml}$, and (F) 40 $\mu\text{g/ml}$. Lower left quadrants show viable cells. The upper right quadrants contain necrotic cells or late apoptotic cells. Lower right quadrants represent the early apoptotic cells.

Table 4. Percentage of DPPH scavenging activities of crude extract of FEAI-1 and ascorbic acid.

Concentrations of crude extract/ ascorbic acid($\mu\text{g/ml}$)	% of scavenging Mean \pm STD	
	Crude extract	Ascorbic acid
10	24.97 ± 0.288	78.98 ± 0.825
20	26.29 ± 0.085	80.84 ± 0.185
40	29.06 ± 0.123	84.27 ± 0.044
80	33.07 ± 0.01	88.23 ± 0.089
160	38.45 ± 0.03	91.34 ± 0.102
320	45.33 ± 0.02	91.59 ± 0.061
640	53.21 ± 0.115	91.85 ± 0.01

Table 3. Percentages of viable, early apoptotic, and late apoptotic/necrotic H1299 cells after 96 hour post-infection with different concentrations of the extract.

Concentrations of extract ($\mu\text{g/ml}$)	Viable cells %	Early apoptotic cells %	Late apoptotic/necrotic cell %	Total apoptosis %
Control	88.8	5.50	4.42	9.92
2.5	88.6	6.62	3.63	10.25
5	85.3	5.85	7.63	13.48
10	78.3	7.22	13.2	20.42
20	29.8	39.4	28.7	68.10
40	29.1	37.9	30.9	68.80

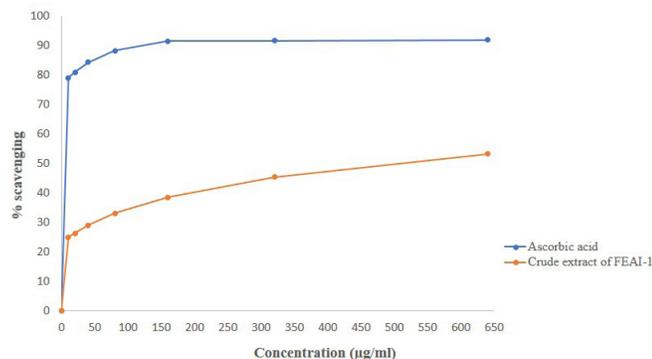


Figure 2. Comparison between antioxidant activity of the extract from *Streptomyces* sp. FEAI-1 and standard ascorbic acid in the DPPH method. The crude extract IC_{50} value was 510 $\mu\text{g/ml}$ compared with the standard ascorbic acid IC_{50} value of 6.5 $\mu\text{g/ml}$.

In our research, ascorbic acid and crude extract showed 50% radical scavenging activity at concentrations of 6.5 and 510 $\mu\text{g/ml}$, respectively, although crude extract had free radical scavenging ability but lesser than that of ascorbic acid. This may be due to the fact that ascorbic acid is a single compound but crude extract is a mixture of compounds. Some previous studies showed that the IC_{50} value of *Streptomyces* sp. MJM-10778 (Lee *et al.*, 2014) and PO-01 (Kekuda *et al.*, 2015) extracts on DPPH radicals were 92.8 and 591.69 $\mu\text{g/ml}$, respectively. Results of this research demonstrated the significant antioxidant potential of the crude extract of FEAI-1. Therefore, the results of this research indicated that the bioactive secondary metabolites of FEAI-1 could be used for cancer treatment due to its apoptosis activity and also could potentially reduce cancer risk by preventing the free radical-mediated DNA mutation.

CONCLUSION

Soil could be a good source of bioactive secondary metabolite producing bacteria having anticancer activity. The isolated bacteria of FEAI-1 showed significant apoptosis (68.80% total apoptosis was at 40 $\mu\text{g/ml}$) and antioxidant (IC_{50} value = 510 $\mu\text{g/ml}$) activity gives a possibility of being used as a therapeutic agent for cancer.

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AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

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ETHICAL APPROVALS

This study does not involve the use of animal or human subjects.

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