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Cytotoxic activity of methanolic extract of *Streptomyces* sp. strain KSF 83 on growth of human breast and colon cancer cells

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ABSTRACT

Aims: The attention for new and effective anticancer drugs but less toxic is increasing over time. *Streptomyces* is the most important and well-known source of their bioactive compound production with useful bioactivities. This work aimed for evaluation of the anticancer potential of methanolic extract of *Streptomyces* sp. strain KSF 83 against non-cancerous cell lines (CCD-841-CoN), breast (MCF-7, MDA-MB-231) and colon cancer cell lines (HT-29, HCT-116).
Methodology and results: The characteristic of the strain KSF 83 was identified by morphology and 16S rRNA sequencing and results confirmed that the strain belonged to the genus of *Streptomyces*. The crude substance was produced via submerged fermentation from the strain and methanol solvent was used to extract the culture filtrate. Methanolic extract possessed low toxicity against CCD-841-CoN with only 18% of inhibition activity at the 400 µg/mL. Among all tested cancer cells, the methanolic extract was able to inhibit the growth of all cancer cells tested with MCF-7 was the highest anticancer activity recorded. The methanolic extract also exhibited cytotoxicity in a range of EC_{50} of 65.79 µg/mL to 262.40 µg/mL. This study revealed the anticancer potential of *Streptomyces* sp. strain KSF 83, which could be sources of prospective anticancer drugs against breast and colon cancer.
Conclusion, significance and impact of study: The extract of KSF 83 was non-toxic toward normal cell lines and able to inhibit the growth of breast and cancer cell lines, thus it can be a potential source of the anticancer drug against breast and colon cancer.

Keywords: Actinobacteria, *Streptomyces*, bioactive compound, cytotoxicity, anticancer

INTRODUCTION

Cancer is the second leading cause of global death and the number of new cases reported are increasing every year. Globally, the five common causes of cancer death in men are lung, prostate, colorectal, liver and stomach. While in female, breast, colorectal, lung, cervical and thyroid cancer are the main cause of death (WHO, 2020). In Malaysia, the Malaysian Study on Cancer Survival (MySCan) reported that cancer was fourth most common cause of death in the country (National Cancer Institute, 2018) and there was approximately 43, 837 new cases along with 26,395 deaths due to cancer reported in 2018 (WHO, 2019). It is estimated that the number of newly diagnosed cases of cancer will be increased to more than 55,000 cases by 2030 (Khalin, 2018). In Malaysia, colorectal cancer is the most common type of cancer among men, while breast cancer was the most common form of cancer affecting women (WHO, 2019).

Cancer represents a significant burden on the public health and its treatment is still scientifically challenging. The common cancer treatments are chemotherapy, radiotherapy, hormone therapy as well as surgery, depending on the type and stage of cancer (Mansoori *et*

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al., 2017). However, most cancer patients that received the cancer treatment have experienced mild to severe side effects. The adverse effects and the increase of new cases and death are the main reasons that motivated the researchers to discover new and more effective drugs with less side effects. Natural products remain as the main source of alternative novel therapeutic agents. In recent years, there has been a positive increase in the number of newly discovered natural products from plants, animals and microorganisms with anticancer properties.

The genus of *Streptomyces* is the main members of Actinobacteria and received attention for three main reasons; abundance and importance in soil, wide phylogenetic spread, and produce multitude and diversity of bioactive secondary metabolites (Barka *et al*., 2016). They are of great interest in medicine and industry for their various bioactivities such as antimicrobial (Ahmad *et al*., 2017; Al-Ansari *et al*., 2019), antifungal (Kim *et al*., 2019), antiviral (Zhang *et al*., 2016), antiparasitic (Yao *et al*., 2014), antioxidant (Dholakiya *et al*., 2017) as well as immunosuppressive activity (Su *et al*., 2019). Until now, over 10,000 bioactive compounds have been isolated from Actinobacteria with 76% of them were extracted from *Streptomyces* (Bérdy, 2012). Presently, their bioactive compounds possessed excellent anticancer activity and acting as a source of therapeutic agents in drug discovery. Some important chemotherapy classes such as actinomycin D, ansamycin, and anthracycline were isolated from various species of Actinobacteria (Cragg and Pezzuto, 2016).

In this study, *Streptomyces* KSF 83 was isolated from primary forest soil in Jerantut, Pahang. Strain KSF 83 showed significant antiviral activity against dengue virus type-2 (DENV-2) (Unpublished data). However, there is no information available on the potential of anticancer activity of *Streptomyces* sp. strain KSF 83. Thus, the aim of this study was to investigate the anticancer activity of bioactive compound of strain KSF 83 against normal colon cell lines, breast cancer and colon cancer cell lines.

MATERIALS AND METHODS

Streptomyces sp. KSF 83 and maintenance

Strain KSF 83 (Kuala Sat Forest number 83) isolated from primary forest in Jerantut, Pahang. Initially characterization was determined by traditional morphological criteria; leathery colonies, morphology of substrate and aerial hyphae, and pigment produced (Goodfellow and Cross, 1984). The selected strain was purified using streak method on yeast-malt extract (ISP2) media and maintained on glycerol suspension (30%, v/v) at –80 °C.

16S rRNA sequencing

Strain KSF 83 was harvested at stationary phase by centrifugation (4,500 rpm) for ten minutes and the pellet was collected for DNA extraction. Genomic DNA was extracted using Quick DNA Fungal/Bacterial Kit (Zymo Research, USA) according to the manufacturer's protocol. The 16S rRNA gene fragment was amplified using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). The amplification reaction was carried out using the extracted genomic DNA as template under the following conditions; initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of amplification (denaturation at 94 °C for 1 minute, annealing at 54 °C for 1 minute, extension at 72 °C for 1 minutes) and a final extension at 72 °C for 10 minutes. The PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) according to the manufacturer's protocol.

Sequence analysis

The forward and reverse 16S rRNA gene sequences, obtained from each strain, were assembled and analysed using BioEdit Sequence Alignment Editor (Hall, 1999) and compared with those of the type strains available in EzBioCloud server (Kim *et al.* 2012). Multiple alignments of the sequences were performed using CLUSTAL-W tool in MEGA-X. Phylogenetic tree based on neighbour-joining method (Saitou and Nei, 1987) was constructed using MEGA-X (Kumar *et al*., 2018) software. Evolutionary distances were calculated using the Kimura's two parameter model (Kimura, 1980). The topology of the resultant neighbour-joining trees was evaluated by bootstrap analysis after 1000 replications (Felsenstein, 1985).

Cultural and physiological characteristic of strain KSF 83

The formation of aerial and substrate mycelium, and arrangement of the spore were done using light microscope. The characteristic of each strain such as growth, colour of colony, presence of aerial and substrate mycelium as well as diffusible pigment were determined using different type of media; yeast malt agar (ISP2), oatmeal agar (ISP3), inorganic salts–starch agar (ISP4), glycerol–asparagine agar (ISP5), Actinomycete isolation agar (AIA), *Streptomyces* agar (SA), starch casein agar (SCA) and tryptic soy agar (TSA). All media were incubated at 30 °C for 7 days. The colours of colonies were identified using the ISCC-NBS colour charts (Kelly, 1964).

The catalase test was performed with 3% of hydrogen peroxide and the oxidase test was done using oxidase reagent. The haemolytic activity were done in Columbia agar with 5% Horse blood. All plate was incubated in 30 °C for 5 days and the presence of haemolysis were identified either α-, β- and γ-haemolysis. Physiological characterization including growth temperature (20 °C to 50 °C), salt concentration (0–10%) and pH (4–10) were performed.

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Fermentation and extraction of bioactive compound

Strain KSF 83 was subjected to submerged state fermentation method to produce crude extracts. Specifically, the extraction of bioactive compounds was done according to Azman *et al*. (2017). The methanolic extract residue was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL, filtered and kept at –20 °C for further analysis.

Cell lines maintenance and growth condition

All cancer cell lines used in this study were obtained from Department of Oral and Craniofacial Science, Universiti Malaya. Breast cancer cell lines (MCF-7, MDA-MB-231), colon cancer cell lines (HT-29, HCT-116) and normal colon cells line (CCD-841-CoN) involved in this study were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic-antimycotic at 37 °C with 5% CO_2. The cancer cell lines were harvested at approximately 70% confluence and sub-cultured after 48 h.

Cytotoxicity assay

The effect of extracts on cell viability of human cancer cells lines was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent. Briefly, 5×10^3 cells/well were seeded onto a 96-well plate and allowed to adhere overnight before treatment with the methanolic extract. Different concentrations of the methanolic extracts were prepared (0–400 µg/mL) and added onto the cell. Treated cancer cells were further incubated for 48 h at 37 °C with 5% CO_2. Twenty microliters of MTS reagent were added into each well and incubated at 37 °C with 5% CO_2 for 2 h. The optical densities were measured at 450 nm with 700 nm as reference wavelength using microplate reader. The percentage of cell viability was calculated as follow:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100\%$$

Statistical analysis

All values were expressed as mean ± standard deviations (SD). The EC_{50} and statistical analysis were performed using one-way analysis of variance (ANOVA) with GraphPad Prism statistical analysis software. *p*-values ≤0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The adverse effect of drugs used in cancer treatment lead to the search for alternative drugs. Natural products from microorganisms remain as one of the main sources for the development of novel therapeutic agents. The oldest anticancer drugs known as Actinomycin-D was isolated from *Streptomyces parvulus* NBRC 13193 (Liu *et al.*, 2019). Since then, researchers are constantly searching for new anticancer drugs from the genus of *Streptomyces*.

Cultural and physiological characteristic of strain KSF 83

Earlier identification of the strain was based on the basic morphology on ISP2 media (Goodfellow and Cross, 1984). A raised convex morphology, aerial hyphae and a well-developed filamentous mycelium with spore are common characteristics of the *Streptomycetes*, shown by strain KSF 83. Therefore, strain KSF 83 was confirmed as one of the species under the genus *Streptomyces*. Further study on growth characteristic and morphology of strain KSF 83 was done in other seven different media besides ISP2 and the evaluation was made after 7 days of incubation period. Overall, strain KSF 83 showed good growth on four culture media (ISP2, SA, SCA, TSA) and no pigment produced in all media tested (Table 1).

Strain KSF 83 belongs to aerobic Gram-positive bacteria. It can grow at 20 °C to 40 °C (optimum 30–35 °C) and on medium with pH 5–8 (optimum 6–7). Study for NaCl tolerance showed strain KSF 83 was able to grow in media with NaCl concentration of less than 2%. In addition, strain KSF 83 showed sensitivity to seven antibiotic including kanamycin, tetracycline, gentamicin, chloramphenicol, ampicillin, ciprofloxacin and streptomycin. However, the strain was resistance to penicillin G, cefuroxime sodium, cefotaxime, meropenem, amoxicillin, and imipenem (Table 2).

16S rRNA gene sequence and phylogenetic analysis

The accession number for the almost complete 16S rRNA gene sequence for strain KSF 83 (1400 bp) is MT256079. The identification of 16S rRNA gene sequence using EzBioCloud server discovered that strain KSF 83 has highest sequence similarity to strain *Streptomyces rubrisoli* FXJ1.725T (98.79%), *Streptomyces ferralitis*

Table 1: Cultural and physiological characteristic of strain KSF 83.

Media	Colour of colony			
	Growth	Aerial mycelium	Substrate mycelium	Pigmentation
SA	Good	Light yellow	Light yellow	-
SCA	Good	Yellowish white	Yellowish white	-
AIA	Moderate	Pale yellow	Pale yellow	-
TSA	Good	Light yellow	Light yellow	-
ISP2	Good	Light yellow	Dark yellow	-
ISP3	Poor	Pale yellow	Pale yellow	-
ISP4	Moderate	Light yellow	Yellowish grey	-
ISP5	Poor	Pale yellow	Pale yellow	-

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Table 2: The physiological characteristic of strain KSF 83.

Characteristic	Result of KSF 83
Catalase	-
Oxidase	-
Haemolysis	β
Growth temperature (°C)	20–40 (optimum 30–35)
NaCl tolerance for growth (%)	0–4 (optimum 0–2)
pH	5–8 (optimum 6–7)
Amoxicillin	R
Ampicillin	S
Cefotaxime	R
Cefuroxime sodium	R
Chlorampenicol	S
Ciprofloxacin	S
Gentamicin	S
Imipenem	R
Kanamycin	S
Meropenem	R
Penicillin G	R
Streptomycin	S
Tetracycline	S

*R-resistance; S-sensitive

SFOp68^T (98.50%), *Streptomyces rimosus* subsp. *rimosus* ATCC 10970^T (98.07%), *Streptomyces rubidus* 13C15^T (97.91%) and *Streptomyces cattleya* NRRL 8057^T (97.86%). Phylogenetic trees were constructed based on 16S rRNA gene sequences with the neighbour-Joining method using MEGA-X software. It showed strain KSF 83 (Figure 1) formed a distinct clade with type strains *Streptomyces rubrisoli* FXJ1.725^T and *Streptomyces ferralitis* SFOp68^T at bootstrap values of 54%.

Cytotoxicity of methanolic extract

In this study, the metabolites of strain KSF 83 was extracted using methanol as extraction solvent. The extract was then subjected to cytotoxic assay against breast (MCF-7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells. A normal colon cell (CCD-841-CoN) was used as a control to investigate the toxicity level of methanolic extract towards normal cell. Overall, a dose-dependent response was observed in all types of cancer cell lines as well as non-cancerous cell line.

The cytotoxicity of KSF 83 extract is represented in Figure 2, demonstrating the cell viability of non-cancer and cancer cell lines after exposure to methanolic extract for 48 h. The methanolic extract was less toxic on normal cells (CCD-841-CoN) than on the investigated cancer cell lines, where there is no significant reduction of the cells except at the concentration of 200 µg/mL and 400 µg/mL. Interestingly, the methanolic extract was active against all the human cancer cell lines tested where the percentages of cell viability recorded ranging from 29.84 ± 2.06% to 96.35 ± 2.27%. The corresponding EC_{50} values of the methanolic extract was calculated and presented in Table 3.

Table 3: EC_{50} of the methanolic extract strain KSF 83 against cancer cell lines after 48 hours using MTS assay.

Test compound	EC_{50} values in different cell lines (µg/mL)			
	HT-29	HCT-116	MCF-7	MDA-MB-231
Methanolic extract KSF 83	262.40	65.79	230.40	260.10

*EC_{50} values represent the concentration that gives half-maximal response.

This study found that HT-29 and HCT-116 cells responded differently after exposure to different concentrations of methanolic extract. Cytotoxic assay against colon cancer cell lines showed the highest cytotoxicity recorded was at 65.79 µg/mL against HCT-116 and the highest inhibition occurred at a concentration of 100 µg/mL, where only 41.95 ± 0.94% of cancer cells survived (Figure 2b). Among all cancer cell lines tested, HCT-116 recorded the lowest EC_{50}, 65.79 µg/mL. On the other hand, the methanolic extract demonstrated least effective (EC_{50} value of 262.4 µg/mL) against HT-29 cell line. However, at the highest concentration tested (400 µg/mL), it significantly inhibited the growth of cells at 57.88 ± 1.96% (Figure 2).

In breast cancer cell lines, a dose-dependent response was observed in both type cancer cell lines. Significant cytotoxicity was demonstrated on MCF-7 cells when treated with the methanolic extract with EC_{50} of 230.4 µg/mL as compared to MDA-MB-231 (EC_{50} of 260.1 µg/mL). Among all concentration tested, the highest inhibition for MCF-7 and MDA-MB-231 were occurred at 400 µg/mL of extracts where the percentages of cell viability recorded were 29.84 ± 2.06% and 35.59 ± 5.87%, respectively.

The breast and colon cancer cell lines used in this study have different genetic composition of the cell. The HT-29 cells have mutant p53, while HCT-116 cells carry wild-type p53 (Yeung *et al*., 2010). In addition, MCF-7 are reportedly p53 wild-type, while MDA-MB-231 are p53 mutant (Kaabinejadian *et al*., 2008). However, the anticancer effect of the methanolic extract on different cancer cell lines were not due to their p53 status. The different anticancer effect of the methanolic compounds on different cancer cell lines may due to the different type of compounds in the crude.

CONCLUSION

In this study, the molecular and phylogenetic analyses demonstrated that strain KSF 83 is a member under the genus of *Streptomyces*. The methanolic extract of *Streptomyces* sp. strain KSF 83 proved to have significant anticancer activity against all human breast cancer (MCF-7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells line tested. Additionally, the extract was less toxic against normal colon cell line, CCD-841-CoN. Overall, the bioactive compounds from *Streptomyces* sp. strain KSF 83 could be a potential source for the development of

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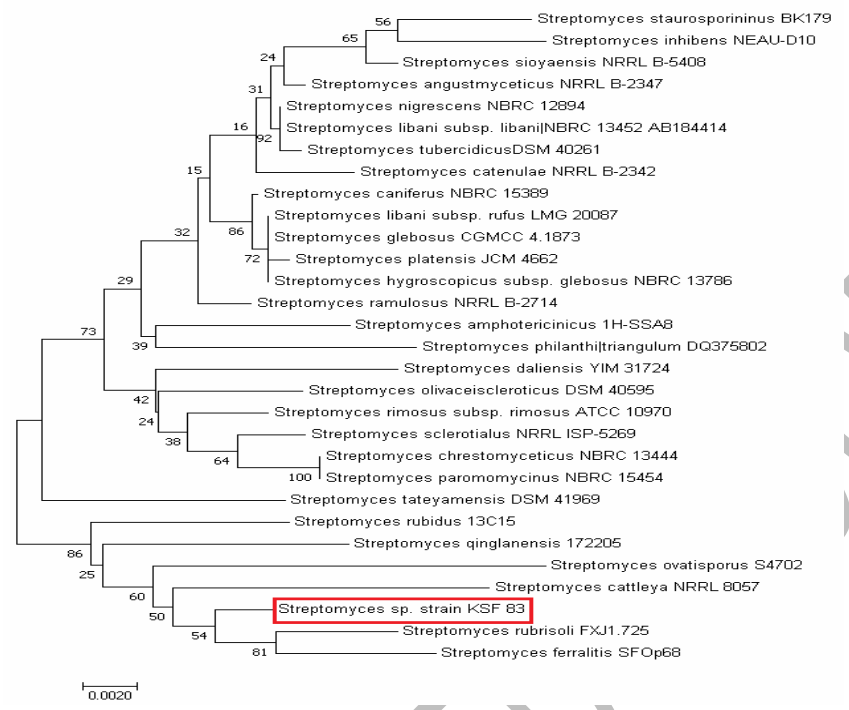

Figure 1: Phylogenetic tree based on the 16S rRNA gene sequences using neighbour-joining methods for the strain KSF 83 and its closely related type strains. Number of branch nodes are bootstrap values (1000 resampling). Bar, 0.002 substitution per site.

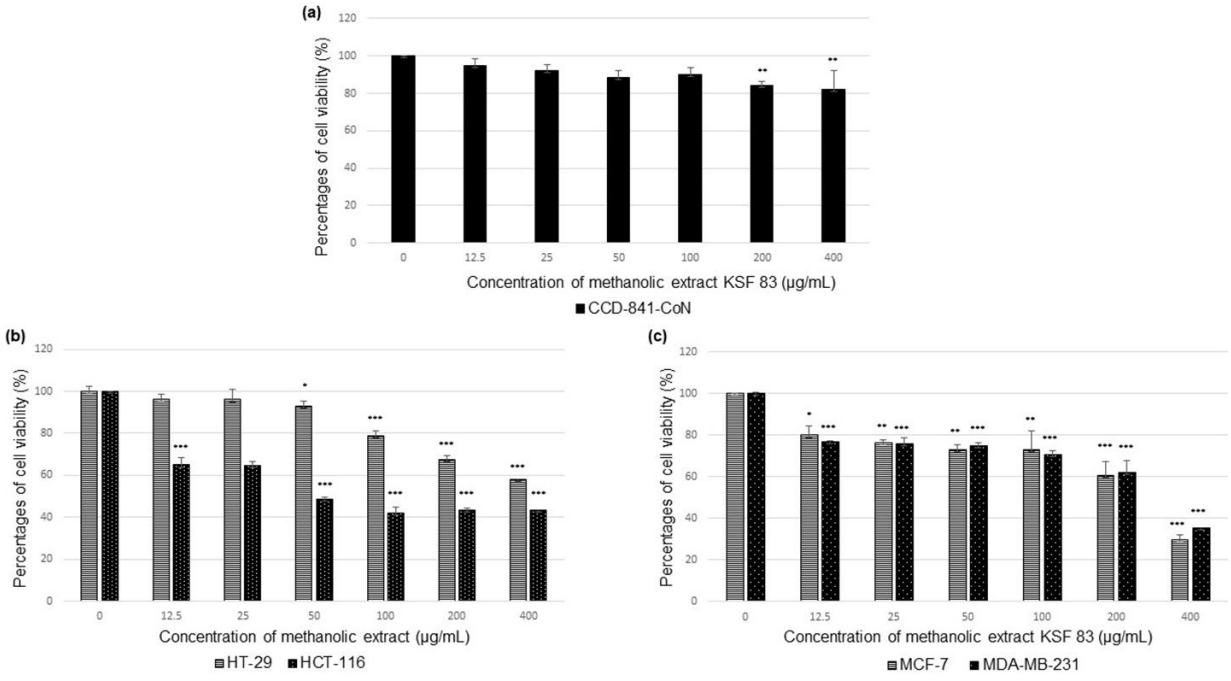

Figure 2: Cytotoxic effect of methanolic extracts against non-cancerous and cancer cell lines (a) Normal colon cell line (CCD-841-CoN) (b) Colon cancer cell lines (HT-29, HCT-116) (c) Breast cancer cell lines (MCF-7, MDA-MB-231) after 48 hours treatment. Data are presented as mean ± S.D. from all independent experiments. Asterisks indicate statistically significant differences between the means of the values obtained with treated *versus* untreated cells (***p<0.0001).

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alternative anticancer drugs. In future, the chemical composition of methanolic extract and the potent bioactive compound should be profiled.

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