AMERICAN UNIVERSITY OF BEIRUT

IN VITRO EVALUATION OF THE CYTOTOXICITY OF STREPTOMYCES MCH11 EXTRACT ISOLATED FROM LEBANESE SOIL ON A549 LUNG CANCER CELLS

by
DALIA WARDANY IBRAHIM SAIF

A thesis
submitted in partial fulfillment of the requirements
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I like to dedicate my thesis to my Mom that always supports and protects me through her prayers during my journey. Also, I want to thank my brothers and my lovely nieces, and my nephew. I would like to show my gratitude to my friends (Grace, Judith, Rana, Esraa, Neama, Nour, and Stanley) who placated and supported me in challenges I had been through. I thank all my friends in Egypt and Lebanon for their prayers and absolute support.

ABSTRACT
OF THE THESIS OF

Dalia Wardany Ibrahim Saif for Master of Science
Major: Biochemistry

Title: *In Vitro* Evaluation of the Cytotoxicity of *Streptomyces* MCH11 Extract Isolated from Lebanese Soil on A549 Lung Cancer Cells

Bacterial extracts possess many therapeutic attributes such as antibacterial, antifungal, antiviral, antioxidant, and anticancer activities. Streptomyces is the largest genus of Actinobacteria that provides various compounds with different activities, including anticancer.

Our study aims to explore the cancer-suppressive activity of extract from *Streptomyces* MCH11, which was isolated from soil in Machghara, a city in Lebanon's West Bekaa, on A549 lung cancer cells, to investigate the molecular mechanisms of cell death, and to identify the bioactive compounds in this extract.

Our results suggest a potential anticancer activity of the extract cultured in medium C on A549 cells. MTT assay showed that the extract significantly reduced the viability of A549 cells. In addition, it induced the apoptosis of cells as determined via the Annexin V-PI assay and confirmed by the demonstration of cleaved PARP-1 and cleaved caspase-9 by western blot. Thus, the extract may act by activating the intrinsic apoptotic pathway. The involvement of caspase-8 and the extrinsic pathway could not be verified. Furthermore, the scratch assay showed that the extract inhibited the metastatic potential of A549 cells after treatment with the specific dose of 0.03 mg/ml and that was accompanied with a downregulation in the protein expression of β-catenin by western blot.

Our bio-guided fractionation and sub-fractionation (using thin-layer chromatography and flash chromatography) revealed that the observed cytotoxic effect of the extract was related to the ethyl acetate fraction and probably to the sub-fraction B of the ethyl acetate fraction. Upon confirmation of the sub-fractionation analysis, the bioactive compound/s will be isolated, and the structure of this compound/s will be identified by Liquid Chromatography-Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR). This would pave the way for evaluating these compounds in the treatment of lung cancer.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

ABBREVIATIONS

A)
(ADP) Adenosine diphosphate
(Apaf-1) Apoptosis protease activating factor-1
(APC) Adenomatosis polyposis coli
(ALK) Anaplastic lymphoma kinase

B)
(Bad) Bcl-2 associated agonist of cell death
(BAK) Bcl-2 homologous antagonist/killer
(BAX) Bcl-2-associated X protein
(Bcl-2) B-cell lymphoma 2
(Bcl-xL) B-cell lymphoma-extra Large
(BH3) Bcl-2 homology domain 3
(Bid) BH3 interacting-domain death agonist
(BIM) Bcl-2-interacting mediator
(BRAF) B-Raf proto-oncogene
(BSA) Bovine serum albumin

C)
(C) cytosine
($CaCO_3$) Calcium carbonate
(c-FLIP) Cellular FLICE inhibitory protein
(CK 1) Casein Kinase 1
(CO_2) Carbone dioxide

D)
(3D CRT) Three-dimensional conformal radio therapy
(4DCT) Four-dimensional computed tomography
(DCM) Dichloromethane
(DISC) Death-inducing signaling complex
(DMEM) Dulbecco's modified Eagle's medium
(DMSO) Dimethyl sulfoxide
(DNA) Deoxyribonucleic Acid
(DPPH) Diphenyl picrylhydrazyl
(DR4 and DR5) Death receptor 4 and 5

E)
(ECL) Enhanced chemiluminescence
(ECM) Extracellular matrix
(EDTA)Ethylenediaminetetraacetic acid
(EGFR) Epidermal growth factor receptor
(ELISA) Enzyme-linked immunosorbent assay
(ERK1/2) Extracellular signal-regulated kinase

F)
(FADD) Fas-associated death domain

(FBS) Fetal bovine serum
(FDA) Food and Drug Administration
($FeSO_4$) Iron (II) sulfate

G)
(GAPDH) Glyceraldehyde 3-phosphate dehydrogenase
(G) Guanine
(GSK3β) Glycogen synthase kinase-3 beta

H)
(h) Hour
(HDAC) Histone deacetylase
(HER2) Human epidermal growth factor receptor 2
(HRP) Horseradish peroxidase

I)
(IARC) International Agency for Research on Cancer
(IC50) Half-maximal inhibitory concentration
IGRT (image-guided radiotherapy)
(ISP3) International *Streptomyces* Project 3

J)
(JNK) c-Jun N-terminal kinase

K)
(KH_2PO_4) Potassium dihydrogen phosphate

L)
(LC) Lung cancer
(LC-MS) Liquid Chromatography–Mass Spectrometry
(LUAD) Lung adenocarcinoma
(LUSC) Lung squamous carcinoma

M)
(MCH11) Machghara 11
(MCL1) Myeloid cell leukemia-1
(MENA) Middle East and North Africa
(MET) Mesenchymal Epithelial Transition
($MgSO_4.7H_2O$) Magnesium sulfate heptahydrate
(MMPs) Matrix metalloproteinases
(MOMP) Mitochondrial outer membrane permeabilization
(MT1-MMP) Membrane-type 1 matrix metalloproteinase

N)
(NaCl) Sodium chloride
(NAT-F) Neoantimycin F

(NCCN) National Comprehensive Cancer Network
(NMR) Nuclear Magnetic Resonance
(NNK) Nicotine-Derived Nitrosamine Ketone
(NOXA) NADPH oxidase activator
(NSCLC) non-small-cell lung carcinoma
(NTRK) Neurotrophic tyrosine receptor kinase

O)
(OD) Optical density

P)
(p53) Protein 53KD
(PARP) poly (ADP-ribose) polymerase
(PBS) Phosphate-buffered saline
(PD-1) Programmed death receptor-1
(PD-L1) Programmed death-ligand
(pH) potential hydrogen
(PI) Propidium iodide
(p38 MAPK) p38 mitogen-activated protein kinase
(PUMA) p53 upregulated modulator of apoptosis
(PVDF) Polyvinylidene difluoride

R)
(RET) Rearranged during transfection
(RhoA) Ras homolog family member A
(ROS1) c-ros oncogene 1
(RT) Radiotherapy

S)
(SABR) Stereotactic ablative radiotherapy
(SBRT) Stereotactic body radiation therapy
(SCLC) Small-cell lung carcinoma
(SDS) Sodium dodecyl sulfate

T)
(TBS) Tris-buffered saline
(TGS) Tris-Glycine-SDS Buffer
(TKIs) Tyrosine kinase inhibitors
(TLC) Thin-Layer Chromatography
(TNF) Tumor necrosis factor
(TNM) Tumor, Node, Metastasis
(TRAIL) TNF-related apoptosis-inducing ligand
(TRADD) TNF receptor-associated death domain

U)
(US) United States
(UV) Ultraviolet

V)
(VEGF) Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

The International Agency for Research on Cancer (IARC) has reported 19.3 million cases of cancer and around 10 million cancer deaths worldwide in 2020, and this number is anticipated to reach 28.4 million cases in 2040 (Sung et al., 2021). Lung cancer (LC) is still the primary cause of cancer death worldwide (Sung et al., 2021). The choice of treatment depends on the tumor stage. Unfortunately, most lung cancer cases are diagnosed at late stages, that is after the tumor had invaded and metastasized (Perlikos, Harrington, & Syrigos, 2013). Consequently, surgery and radiotherapy are not effective in this case, and other treatments such as immunotherapy and targeted therapy are used with limited success. Thus, developing alternative novel treatments is an urgent need to overcome these limitations. Natural products have always been a source of chemotherapeutic and antimicrobial agents especially from bacterial extracts and their secondary metabolites (Abdelghani et al., 2021).

A. Lung Cancer

1. Epidemiology

According to the World Health Organization, 1.80 million lung cancer cases died worldwide in 2020. A Lebanese study analyzed lung cancer data from 2005 to 2015 and identified it as the second most frequent cancer in Lebanon, representing 9.2% of all new cancer cases (Salhab, Fares, Khachfe, & Khachfe, 2019). Lebanon has the highest female and the second-highest male LC incidence in the Middle East and North Africa (MENA) region, mainly caused by a substantial increase in the smoking rate (Salhab et al., 2019). Previous studies showed that cigarette smoke has many

carcinogens that cause genetic alterations and eventually lead to lung cancer (X. Wang et al., 2021). Although smoking is the chief culprit of lung cancer, other factors such as occupational exposure to carcinogens (asbestos or nickel), environmental pollution (Field & Withers, 2012), domestic fuel exposure, genetic susceptibility, and some inflammatory diseases increase the risk of having lung cancer (Corrales et al., 2020).

2. Risk Factors

a. Tobacco Smoke

Smoking is the major risk factor for all types of lung cancer. Tobacco smoke comprises more than 60 carcinogenic compounds leading to DNA damage and mutations in human cells (Q. Yang, Hergenhahn, Weninger, & Bartsch, 1999; Zhao, Albino, Jorgensen, Traganos, & Darzynkiewicz, 2009). It has been proven that three main tobacco smoke carcinogens: benzo(*a*)pyrene (BP), 4-(methyl nitrosamine)-1-(3-pyridyl)-1-butanone (NNK), and aldehydes trigger mutations in human and mice DNA and prevent DNA repair (Weng et al., 2018). In addition, tobacco smoking adds from 1000 to 10000 mutations per cell, decreases the telomere length, which affects the mitotic activity, and increases cell-to-cell heterogeneity (Yoshida et al., 2020). According to an American comparative modeling approach, lung cancer mortality would decline by 79% between 2015 and 2065, if smoking cessation continues to increase in the US (Jeon et al., 2018). Therefore, more efforts in the control of tobacco smoking should be implemented.

b. Genetic Susceptibility

A pooled analysis showed that smokers with a family history of lung cancer have a 1.5-fold increase in lung cancer risk. Although non-smokers have a lower susceptibility to getting lung cancer, the risk is higher if they have an afflicted sibling, so the genetic factor is independent of smoking. An increase in the risk of lung cancer was observed among individuals having many relatives diagnosed with this cancer, especially at an early age (Matakidou, Eisen, & Houlston, 2005).

c. Diet and Alcohol

It is reported in some case-control studies that vegetables and fruits may protect against lung cancer, especially cruciferous vegetables (Fund & Research, 2007; Lam et al., 2009). Lung cancer risk may be elevated by a high intake of fried or well-done red meat (Sinha et al., 1998). This might be due to the production of heterocyclic amines when meats are cooked at high temperatures (Sinha et al., 2000). Although drinking alcohol does not increase the risk of lung cancer independently (Bagnardi et al., 2011), it does when it is associated with smoking (Bandera, Freudenheim, & Vena, 2001; Korte, Brennan, Henley, & Boffetta, 2002).

d. Ionizing radiation

It is reported that survivors of atomic bombs and patients treated with radiotherapy are more likely to have lung cancer due to their exposure to ionizing radiation (Virtanen, Pukkala, & Auvinen, 2007). In addition, lung cancer risk is increased by exposure to radioactive radon that releases α-particles, so underground mine workers are in danger due to consistent exposure to this radiation (Lubin, 1994). Nine European countries had participated in 13 case-control studies on exposure to

residential radon at home, it was found that it has a detrimental effect, especially on smokers and it is the cause of 2% of all cancer death in Europe (Darby et al., 2005).

3. Categories of Lung Cancer by Histology

Lung cancer is categorized into two main histological groups: small-cell lung carcinoma (SCLC) or non-small-cell lung carcinoma (NSCLC). SCLC originates from neuroendocrine cells, while NSCLC originates from bronchial epithelial cell precursors (Sutherland & Berns, 2010). NSCLC is the dominant group, accounting for 85% of all lung cancer, while small-cell lung cancer only accounts for 15% (Sher, Dy, & Adjei, 2008). NSCLC is subcategorized into four histotypes: Lung adenocarcinoma (LUAD) which accounts for 40% of all lung cancers; Lung squamous carcinoma (LUSC) which represents 25% to 30% of all lung cancers; Large cell carcinoma accounts for 15% of all lung cancers, and mixed histotypes which are rare (Ruiz-Cordero & Devine, 2020).

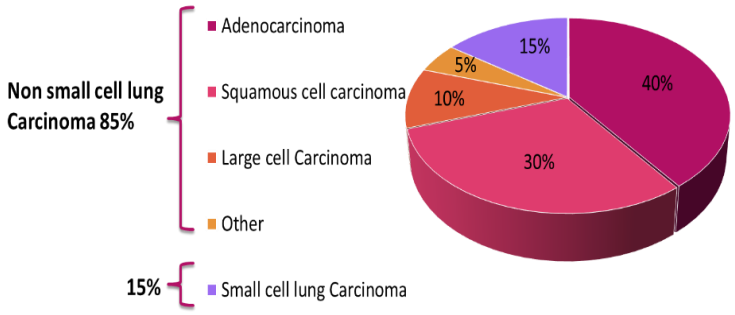


Figure 1 Main categories of lung cancer by histology. Modified and retrieved from (Ruiz-Cordero & Devine, 2020).

4. NSCLC Stages (TNM)

The international classification of tumor staging is the TNM classification system; T refers to the size of the primary tumor, N to the involvement of regional

lymph nodes, and M to the presence or absence of metastasis. The international classification for NSCLC stages is:

i. Stage I (T1N0M0): the primary tumor is limited to the organ of origin with a size ranging between 2 to 5 cm and without the involvement of lymph nodes.

ii. Stage II (T2N1M0): the primary tumor size ranges between 2 to 7 cm and the first station of lymph nodes are either involved or not.

iii. Stage III (T3N2M0): has a large primary tumor, spreading to surrounding regions such as the heart, diaphragm, and chest wall. Different lymph nodes are involved.

iv. Stage IV (T4N3M+): the primary tumor diameter is more than 10 cm with distant metastasis and involvement of extensive lymph nodes. The most common metastatic site is the liver (Tanoue, 2020).

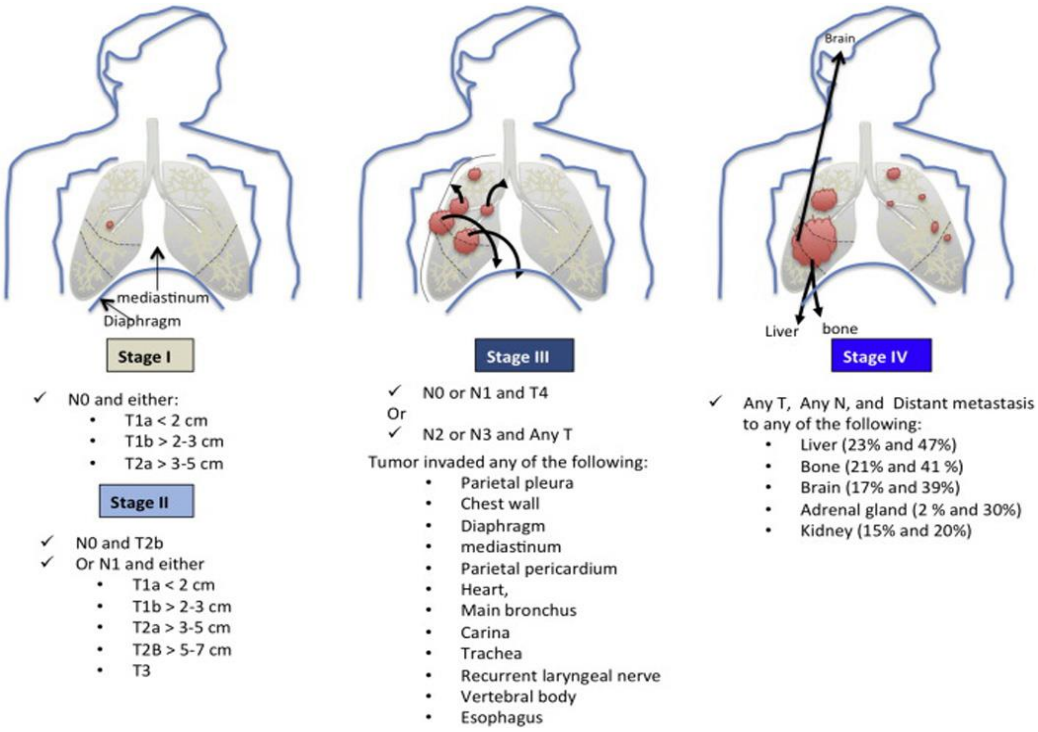


Figure 2 NSCLC stages. Retrieved from (Lemjabbar-Alaoui, Hassan, Yang, & Buchanan, 2015).

5. NSCLC Treatments

The choice of treatment for NSCLC depends on the tumor stage. In this section, we elaborate on its standard and emerging treatments.

a. Surgery

Surgery is implemented for stages I and II through lobectomy or sublobar resection (Duma, Santana-Davila, & Molina, 2019). Unfortunately, recurrence has been witnessed among patients who had a surgical procedure. For example, sublobar resections increase the rate of lung cancer relapse by 3 fold in a trial with 247 patients (Ginsberg & Rubinstein, 1995). Therefore, lobectomy is preferable compared to sublobar resections; however, its use is only limited to the early stages (T1-2 N0) of NSCLC. 5-year survival for surgery was decreased with late stages, for clinical stage IA is 77–92%, 68% for stage IB, 60% for stage IIA, and 53% for stage IIB (Goldstraw et al., 2016). Surgery recurrence is mainly caused by micro-metastatic cells (Uramoto & Tanaka, 2014).

b. Chemotherapy

i. Neoadjuvant Chemotherapy

Neoadjuvant chemotherapy is beneficial in the early stages of the disease and eases a complete resection. Although neoadjuvant chemotherapy improved the survival overall compared to surgery alone for stage II and III NSCLC, it did now show any advantage compared to adjuvant chemotherapy (Maclean et al., 2018).

ii. Adjuvant Chemotherapy

Adjuvant chemotherapy is used for stages II and IIIA of the disease after surgery. Scientists pooled analysis of 5 studies assessing adjuvant cisplatin-based chemotherapy; the survival advantage was very modest and was shown to be around 5.4 % (Pignon et al., 2008). Unfortunately, over 40% of patients with stage IIIA of NSCLC experience cancer relapse, even if they have undergone complete resection and adjuvant chemotherapy (Arriagada et al., 2004; Douillard et al., 2006).

c. Radiotherapy

Radiotherapy (RT) uses high energy of beams to destroy cancer cells by damaging their DNA. It is used to treat or act as an analgesic for NSCLC, especially for patients who do not respond to surgery or chemotherapy (Zappa & Mousa, 2016). In the early stages of NSCLC, stereotactic body radiation therapy (SBRT) provides concentrated radiation, which is more effective than photons, protons, and carbon ions; it grants greater 2-year overall survival rates, and it has a lower cost (Grutters et al., 2010). Radiotherapy has had an important role in lung cancer treatment. Three-dimensional conformal RT (3D CRT) was used since the 1990s to create 3-dimensional images for tumors and nearby organs; it allows beams of radiation to cover the tumor mass and reduce the risk of affecting other organs (Armstrong, 1998). In addition to the improvement in radiation beams, capturing images was improved as well. Four-dimensional computed tomography (4DCT) was used to track the movement of tumor-induced by respiration, which minimized the likelihood of wrong RT delivery (H. H. Liu et al., 2007). Moreover, with the aid of 4DCT, 3D CRT, and IGRT (image-guided

radiotherapy), which provide images with RT to increase the precision, stereotactic ablative radiotherapy (SABR) has been improved. The latter has been accepted worldwide to be the standard treatment for early stages NSCLC in patients who are not surgical candidates (De Ruysscher et al., 2017).

d. Immunotherapy

Cancer cells express some antigens to interact with immune cells, especially T-cells, and to suppress their activities. For instance, programmed death-ligand (PD-L1) expressed on tumor cells interacts with programmed death receptor-1 (PD-1) on T-cells and this interaction inhibits tumor cell recognition by T cells (Ruiz-Cordero & Devine, 2020). Immune checkpoint inhibitors block PD-1 on T-cells, enabling them to identify and respond to antigens on tumor cells (Ruiz-Cordero & Devine, 2020). The combination between an anti-PD-1 antibody (pembrolizumab), pemetrexed, and platinum was approved as a first-line treatment for lung adenocarcinoma (Reck et al., 2016). The main disadvantage of this immunotherapeutic approach is that the number of eligible patients who can respond to immunotherapy checkpoint inhibitors is dismal (Haslam & Prasad, 2019).

e. Targeted therapy

The latest approach followed in cancer treatment is the development of targeted therapies for specific genetic modifications, including those related to immune checkpoints (Ruiz-Cordero & Devine, 2020). In addition, somatic mutations of some tyrosine kinases corresponding to genes in NSCLC had become the target of innovative targeted therapies. For instance, a mutation in the oncogenic epidermal growth factor

receptor (EGFR) activates it unceasingly. This activation could be inhibited by tyrosine kinase inhibitors (TKIs) (Ladanyi & Pao, 2008). In this context, the Food and Drug Administration (FDA) approved erlotinib and gefitinib as a first-line treatment for metastatic NSCLC with EGFR mutations. The latter treatment had increased the overall response rate to over 75% and in many clinical trials, it was superior to chemotherapy (Rosell et al., 2012; Sequist et al., 2013). Recently, targeted therapies included many oncogenes such as the anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1), which encodes for an orphan receptor tyrosine kinase related to ALK, B-Raf proto-oncogene (BRAF), which encodes for BRAF serine/threonine-protein kinase, rearranged during transfection (RET), which encodes for RET family receptor tyrosine kinase, human epidermal growth factor receptor 2 (HER2), which encodes for human epidermal growth factor receptor 2, neurotrophic tyrosine receptor kinase (NTRK), which encodes for tropomyosin receptor kinase, and mesenchymal epithelial transition (MET), which encodes for MET receptor tyrosine kinase (Bansal, Osman, Gan, Simon, & Boumber, 2016). The National Comprehensive Cancer Network (NCCN) has recommended focusing on these genes although they don't cover all types of LC (Wood, 2015). Therefore, there is an urgent need to seek other effective strategies that expand the choice of treatments to be able to overcome NSCLC.

f. Alternative natural therapies

Most of the previous traditional cancer treatments have detrimental side effects. For instance, some classic chemotherapy agents exhibited irreversible cardiovascular dysfunctions (Bayramzade, Ni, Yk, & Mm, 2020; Curigliano et al., 2012). In addition, breast cancer radiotherapy was implicated in triggering heart diseases due to the

exposure of the heart to radiation (Taylor & Kirby, 2015) and radiation therapy for late stages of NSCLC induced cardiac toxicity in many clinical studies (Dess et al., 2017; K. Wang et al., 2017). Therefore, seeking alternative drugs with minimal side effects and more selectivity to cancer cells is an unmet need. Using natural substances from plants, animals, and marine organisms led to the discovery of many agents with anti-cancer activity against a plethora of cancer types (Lichota & Gwozdzinski, 2018). From 1946 to 2019, natural products have provided more than half of all approved medicines (Newman & Cragg, 2020).

Many natural products exhibit anticancer activity against lung cancer through several mechanisms that include targeting the tumor microenvironment (Y. Yang, Li, Wang, & Di, 2021). Many plant extracts had an anticancer activity such as a Chinese herb called *Scutellaria barbata*, which exhibited a cytotoxic impact on A549 (human non-small lung cancer) cells and induced apoptosis (Yin, Zhou, Jie, Xing, & Zhang, 2004). Furthermore, a mixture of flavonoids isolated from Korean Citrus aurantium L. showed anti-metastatic effects on A549 cells where it induced apoptosis and resulted in the cleavage of caspase-3 (Park et al., 2014). In the current project, we focus on bacterial extracts from Streptomyces and their derived compounds as described below.

B. Overview of Key Events Related to Cancer

Some of the hallmarks of cancer include sustaining cell proliferation, evading growth suppressors, resisting cell death or apoptosis, inducing angiogenesis, activating invasion, and metastasis (Douglas Hanahan & Weinberg, 2000). These hallmarks ensure continuous progression for cancer cells.

1. Apoptosis

Apoptosis, or programmed cell death, is a highly regulated biological process occurring normally during the development and aging of cells, as a defense mechanism in the immune system (Elmore, 2007), and in response to DNA damage (Norbury & Hickson, 2001). The balance between cell death and cell survival is normally preserved. If there is a disturbance in this balance that favors less apoptosis, one outcome might be the development of cancer. Cancer cells attempt to escape apoptosis by developing mutations in several apoptotic pathways (Fulda, 2010) to pursue uninhibited progression and proliferation (D. Hanahan & Weinberg, 2011).

Apoptotic cells display several morphological changes, such as blebbing of the cell membrane, flipping off the phosphatidylserine from the inner to the outer leaflet of the cell membrane, cellular shrinkage, condensation of chromatin, and fragmentation of DNA (Häcker, 2000; Saraste, 2000).

Based on the triggering signals, there are two main apoptotic pathways: the intrinsic (mitochondrial) pathway, and the extrinsic (death receptor) pathway (Figure 3):

- The *intrinsic pathway* is initiated by intracellular signals after cell exposure to some internal triggers such as an irreversible genetic mutation, high concentration of Ca+, hypoxia, and oxidative stress (Kroemer, Galluzzi, & Brenner, 2007). The intracellular signals activate pro-apoptotic BH3-only members of the Bcl-2 family such as Bim, Bid, Bad, Puma, and Noxa. These proteins bind and inhibit the anti-apoptotic Bcl-2 family proteins such as Bcl-2, Bcl-xL, Bcl-W, and Mcl-1. This event frees the pro-apoptotic Bcl-2 family proteins Bax and Bak, which oligomerize, form membrane channels, and trigger mitochondrial outer membrane permeabilization. Thus, cytochrome c is released into the cytoplasm

and along with the cytosolic apoptosis protease activating factor-1 (Apaf-1) forms the apoptosome complex. This complex induces the auto-activation and release of pro-caspase 9 (Danial & Korsmeyer, 2004). The initiator caspase-9 then promotes an activation cascade of effector caspases-3,-6, and-7, which eventually leads to apoptosis (Kuribayashi, Mayes, & El-Deiry, 2006).

- The extrinsic pathway is triggered when extracellular death ligands, produced by some lymphocytes such as natural killer cells or macrophages, bind to their death receptors expressed on the cell membrane of a target cell. This binding generally trimerizes the cell receptors, approximates the pro-caspase-8 recruited to the cytosolic domains, leading to its cleavage and activation as caspase-8 (Schulze-Osthoff, Ferrari, Los, Wesselborg, & Peter, 1998). To illustrate further, a death receptor such as Fas receptor, tumor necrosis factor (TNF) receptor, or TNF-related apoptosis-inducing ligand (TRAIL) receptor binds to its ligand; the receptor undergoes aggregation, and the adaptor molecule such as Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) is recruited to activate caspase-8 which eventually initiate apoptosis by cleaving other caspases (Walczak & Krammer, 2000).

- p53 and apoptosis

p53 is a tumor suppressor gene that functions as a transcriptional factor but also has transcription-independent activities, that is activated by cellular stress, including DNA damage, hypoxia, cellular senescence, and excessive expression of oncogenes (Fridman & Lowe, 2003). It promotes DNA repair, cell cycle checkpoints, cellular senescence, and apoptosis. Dysfunction of p53 was implicated in cellular

immortalization, so it is not surprising that it is the most mutated tumor suppressor gene in cancer (Olivier et al., 2002). Many components involved in the extrinsic and intrinsic apoptotic pathways are controlled by p53 to mitigate cellular stress. In a transcription-dependent model, p53 triggers the expression of many pro-apoptotic genes, such as Puma and Noxa, and eventually induces the intrinsic apoptotic pathway (Hage-Sleiman et al., 2017; Pietsch, Sykes, McMahon, & Murphy, 2008). In a transcription-independent model, mitochondrial p53 interacts with anti-apoptotic Bcl-2 members leading to Bax release and induction of mitochondrial permeabilization resulting in apoptosis similar to what the BH3-only proteins of Bcl-2 family do (Wolff, Erster, Palacios, & Moll, 2008). Moreover, p53 upregulates the expression of death receptors such as death receptors 4 and 5 (DR4 and DR5) involved in the extrinsic pathway (X. Liu, Yue, Khuri, & Sun, 2004). p53 also regulates Bid, thereby triggering the convergence of the intrinsic and extrinsic pathways (Sax et al., 2002).

- PARP-1 and apoptosis

The poly (ADP-ribose) polymerases (PARPs) are a group of 18 enzymes, that recognize and repair DNA strand breaks by synthesizing and transferring ADP-ribose (poly ADP-ribosylation process) to nuclear proteins and histones (Amé, Spenlehauer, & de Murcia, 2004). PARP-1 (113 KDa) is a key effector for identifying and repairing DNA lesions (Mortusewicz, Amé, Schreiber, & Leonhardt, 2007), so any defect in PARP-1 forces cells to undergo apoptosis. When apoptosis is initiated, caspases 3 and 7 cleave PARP-1 into 24-kDa and 89-kDa fragments (Germain et al., 1999). The 24-kDa PARP-1 fragment blocks DNA breaks permanently to inhibit active PARP1 from

binding to it, whilst the 89-kDa PARP-1 fragment is transferred to the cytosol (Smulson et al., 1998; C. Soldani et al., 2001).

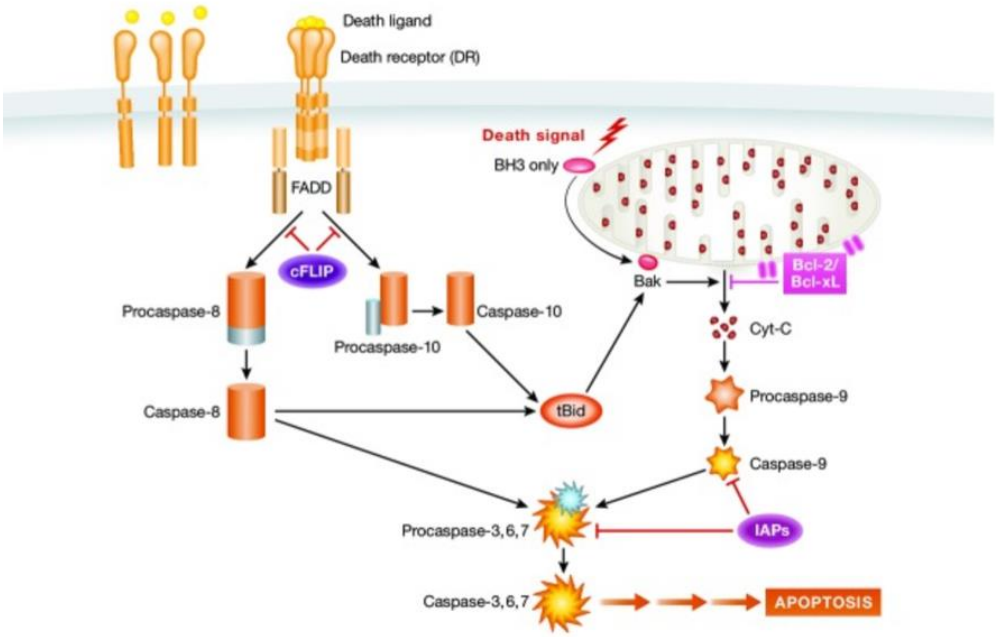


Figure 3 The extrinsic and the intrinsic apoptotic pathways. Retrieved from (Y. H. Wang & Scadden, 2015). In the extrinsic pathway, death receptors on the cell membrane bind to their ligands. Consequently, death receptors aggregate and recruit adaptor proteins and pro-caspases to create a death-inducing signaling complex (DISC). Thus, pro-caspase 8 is cleaved into active caspase-8 which in turn activates caspase-3. Then, active caspase-3 promotes the activation of the downstream caspase cascade leading to apoptosis. The anti-apoptotic regulator cellular FLICE inhibitory protein (c-FLIP) prevents the formation of DISC and activation of caspase-8. In the intrinsic pathway, an intracellular signal like DNA damage activates Bcl-2 pro-apoptotic proteins and initiates the pathway by triggering mitochondrial outer membrane permeabilization (MOMP). This leads to the release of cytochrome C that activates caspase-9, which in turn stimulates the activation of the downstream caspase cascade leading to apoptosis.

2. Cancer Cell Migration

Several biological processes, such as immune surveillance, tissue repair, and regeneration need cell migration. Many illnesses, including cancer invasion and metastasis, are driven by abnormal cell movement (Condeelis, Singer, & Segall, 2005; Yamaguchi, Wyckoff, & Condeelis, 2005). Herein, we will provide a brief overview of

two cancer hallmarks: angiogenesis or new blood vessel formation, a principal route by which cancer cells migrate from the primary tissue site and enter the circulation; and metastasis of cancer to other distant organs, a process mediated by cell migration. Then, we will elaborate on some key proteins involved in cancer cell migration.

- Angiogenesis

  Angiogenesis is the formation of blood vessels from pre-existing ones under a balance between pro- and anti-angiogenic factors and proper functions of some molecules, such as extra-cellular matrix proteins, adhesion receptors, and proteolytic enzymes (Ribatti, 2013). Cancer progression requires more access to oxygen and nutrients delivered by blood vessels. Cancer cells either seize pre-existing blood vessels of the surrounding tissues in a process named vessel co-option (Kuczynski, Vermeulen, Pezzella, Kerbel, & Reynolds, 2019) or enter the circulation of blood and lymphatic vessels after inducing angiogenesis to invade remote organs where they thrive and colonize (Polacheck, Zervantonakis, & Kamm, 2013).

- Metastasis

  Metastasis is a predominant cause of cancer-related mortality as it generally reflects a more advanced stage of cancer (Fidler, 2002). In this process, cancer cells migrate from one organ or tissue to another disconnected organ or tissue and create a secondary micro-metastasis (Wittekind & Neid, 2005). Several mechanisms and proteins help cancer cells to degrade the extracellular matrix (ECM) and penetrate the basement membrane and epithelial layer of blood or lymphatic vessels.

- Some key proteins involved in cancer cell migration:
    - Matrix metalloproteinases (MMPs):

One group of proteins that degrade the ECM is the matrix metalloproteinases (MMPs). This group consists of 21 multifunctional proteins. MMPs affect the ECM in different ways, and they alter its structure and mechanical properties (Rydlova et al., 2008). CD44 receptor which is expressed in migratory cancer cells was shown to be cleaved by membrane type 1 matrix metalloproteinase (MT1-MMP), an event that promoted cell migration (Kajita et al., 2001). MMP-1, MMP-2, and MMP-9 have been implicated in triggering metastasis in human lung cancer (Chen et al., 2008; Sauter et al., 2008).

- β-catenin:

β-catenin has dual functions. It is a signaling molecule that binds to transcription factors leading to the induction of the transcription of Wnt genes that are involved in cell growth, proliferation (Clevers, 2006), and migration (Hlubek, Spaderna, Jung, Kirchner, & Brabletz, 2004). In addition, the inactivation of Wnt/ β-catenin was shown to inhibit NSCLC metastasis (Han et al., 2021).

β-catenin has another role, it connects the cytoplasmic domain of cadherin to the cytoskeleton, this complex is pivotal for cell-cell adhesion, and cell differentiation (Kourtidis, Lu, Pence, & Anastasiadis, 2017). The dysfunction in the cadherin-catenin complex reduces cell adhesion and this was seen in neoplastic processes, leading to β-catenin accumulation in the cytoplasm and nucleus of the tumor cells (Kaszak et al.,

2020).

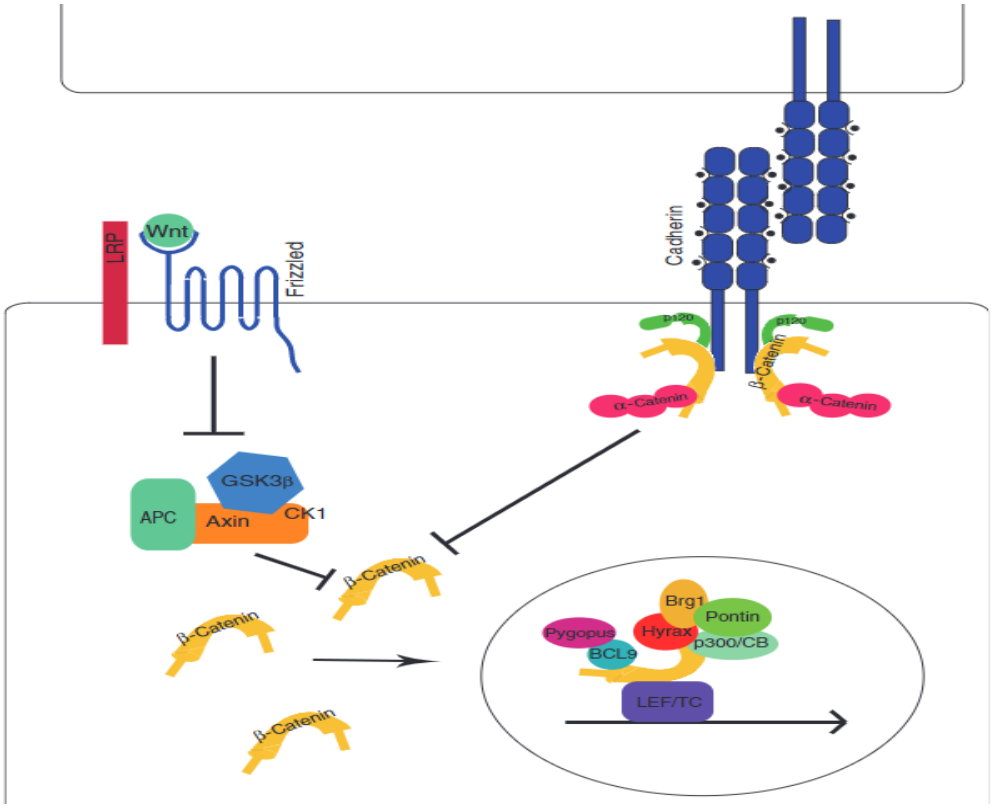


Figure 4 Regulation of β-catenin. Retrieved from (Jeanes, Gottardi, & Yap, 2008). The destruction complex (adenomatosis polyposis coli (APC), Axin, GSK3β and CK1) regulates free cytosolic β-catenin. The cytoplasmic tail of cadherin binds to p120-catenin and β-catenin. β-catenin binds to α-catenin, which links cadherins to the actin cytoskeleton. Both mechanisms limit the pool of free cytosolic β-catenin leading to inhibition of Wnt pathway.

C. Bacterial-derived Compounds and their Anti-cancer Activity

Bacteria provide various secondary metabolites with different biological activities including anticancer, antioxidant, antimicrobial, immunomodulatory, cardioprotective, and neuroprotective (Abdelghani et al., 2021). These activities were reviewed by Abdelghani et al. and are presented in figure 5.

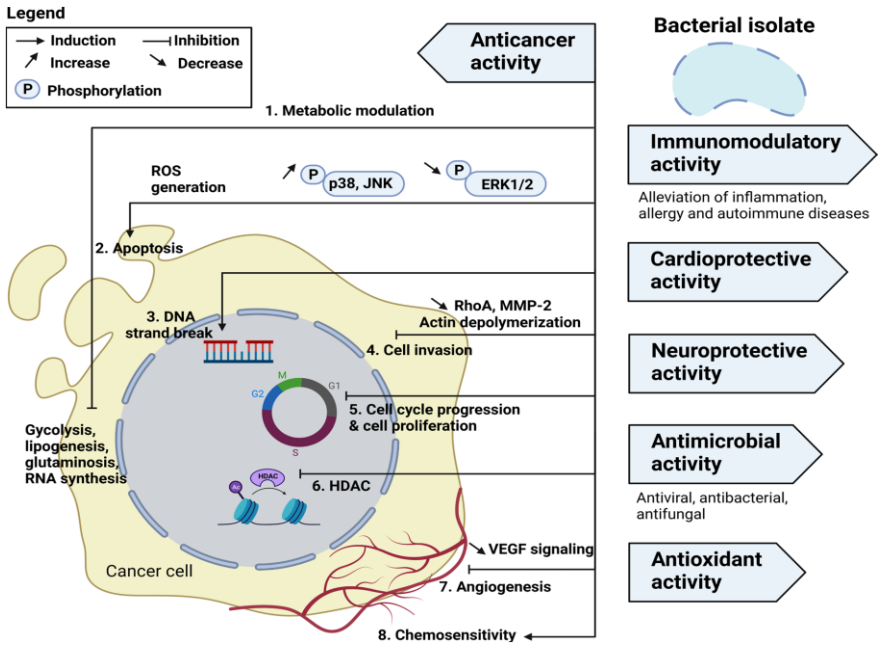


Figure 5 Biological activities of bacterial-derived compounds. Retrieved from (Abdelghani et al., 2021). Some bacterial-derived compounds can mitigate inflammation, allergies, and autoimmune diseases. In addition, some of these compounds exhibit antibacterial, antifungal, antiviral, and antioxidant activities. Furthermore, some of them have neuroprotective and cardioprotective activities. Bacterial-isolated compounds showed an anti-cancer activity through diverse mechanisms, including 1. alteration of the metabolism of cancer cells by preventing glycolysis, lipogenesis, glutaminolysis, and RNA synthesis; 2. triggering apoptosis by ROS generation, upregulation of phosphorylated p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK), and downregulation of phosphorylated extracellular signal-regulated kinase (ERK1/2); 3. inducing DNA strand break; 4. inhibiting cancer cell migration via decreasing the expression of Ras homolog family member A (RhoA) and matrix metalloproteinase (MMP) and stimulating actin depolymerization; 5. inhibiting the cell proliferation and the cell cycle progression; 6. inhibiting histone deacetylase (HDAC) enzyme; 7. inhibiting angiogenesis by downregulating vascular endothelial growth factor (VEGF); 8. inducing chemosensitivity in drug-resistant cancer cells.

Among the bacterial-derived products, we will focus on the extracts and compounds derived from the Streptomyces genus of Actinobacteria and we will elaborate more on their anticancer activities, particularly in lung cancer and NSCLC.

1. Actinobacteria and Streptomyces-derived Products

Actinobacteria are filamentous gram-positive bacteria with a high G+C DNA content (Barka et al., 2016). They are ubiquitous in both aquatic and terrestrial habitats and are one of the biggest bacterial phyla (Barka et al., 2016). Actinomycetes belong to the Actinobacteria phylum; they provide the majority of natural secondary metabolites (around 45%), 80% of which are generated by the genus Streptomyces (Bérdy, 2005).

Streptomyces is the largest genus of Actinobacteria and it was introduced by Waksman and Henrici (Waksman & Henrici, 1943). This group is mainly gram-positive bacteria with around 780 known species. The genus members produce more than 75% of antibiotics (Kinkel, Schlatter, Xiao, & Baines, 2014; Lee et al., 2014). Besides antibiotics, the Streptomyces genus provides different compounds with various biological activities including antifungal, antioxidant, anticancer, and immunosuppressive activities (Kino et al., 1987; Law et al., 2017; Rashad, Fathy, El-Zayat, & Elghonaimy, 2015). In this regard, some of the secondary metabolites of Streptomyces have been used as a source of anti-cancer drugs including doxorubicin, actinomycin D, rapamycin, mithramycin, neocarzinostatin, carzinophilin, and mitomycins (Newman & Cragg, 2007; Olano, Méndez, & Salas, 2009a). These metabolites can exhibit their anti-cancer effect by triggering apoptosis through blocking topoisomerases I or II, permeabilizing the mitochondria, inhibiting signal transduction enzymes such as proteases, altering cellular metabolism, or inhibiting angiogenesis (Olano, Méndez, & Salas, 2009b). In addition, some of these compounds induce profound perturbation in double-stranded DNA, inevitably resulting in the disruption of cell proliferation and triggering apoptosis (Wadkins, Vladu, & Tung, 1998).

2. Streptomyces-derived Products and Lung Cancer

The crude ethyl acetate extract of the bacterial strain FEAI-1, belonging to the *Streptomyces* species, elicited an apoptotic effect on H1299 non-small lung cancer cells and it exhibited an antioxidant activity by inhibition of diphenyl picrylhydrazyl (DPPH) radicals (Khatun, Haque, Anisuzzaman, & Islam, 2021a). In another study, loss of the mitochondrial membrane potential, cell cycle arrest at S and G0/G1 phase, nuclear condensation, and apoptosis were observed in PC9 and H1299 non-small lung cancer cells treated with Neoantimycin F (NAT-F); a bacterial-derived product isolated from *Streptomyces conglobatus* (L. Liu et al., 2019). This study also showed the release of cytochrome c from the mitochondria, the activation of caspase-9 and -3, and the cleavage of poly (ADP-ribose) polymerase due to an up-regulation of the pro-apoptotic protein Bax and a down-regulation of the anti-apoptotic proteins Bcl-2, Mcl-1, and Bcl-x by NAT-F (L. Liu et al., 2019). Furthermore, the ethyl acetate extract of *Streptomyces artemisiae* MCCB 248, isolated from the Arctic fjord Kongsfjorden, showed a cytotoxic effect on H460 human lung cancer cells by triggering apoptosis, DNA damage, nucleus shrinkage, along with chromatin condensation (Dhaneesha et al., 2017). Finally, Galvaquinone B derived from *Streptomyces spinoverrucosus* exerted moderate cytotoxicity and epigenetic activity against NSCLC cell lines Calu-3 and H2887 (Hu, Martinez, & MacMillan, 2012).

3. Streptomyces-derived Products and A549 NSCLC Cell Line

The A549 cell line is a non-small cell lung cancer cell line. It was developed in 1972 by Giard et al. from a patient with adenocarcinoma of human alveolar basal epithelial cells (D. J. Giard et al., 1973). These cells are used as a model for lung

carcinoma (Donald J. Giard et al., 1973). A549 cells can produce lecithin with a high percentage of unsaturated fatty acids (Lieber, Todaro, Smith, Szakal, & Nelson-Rees, 1976). A549 cells are KRAS mutant cells; KRAS gene is involved in cell growth and (Guin et al., 2013) and they are EGFR and P53 wild type (Korrodi-Gregório, Soto-Cerrato, Vitorino, Fardilha, & Pérez-Tomás, 2016).

Many bacterial extracts showed a potent cytotoxic effect on A549 cells. *Streptomyces* sp. strain 196- derived extract induced cytotoxicity and apoptosis against A549 cells, reduced their migration, and changed their cellular morphology *in vitro* (P. Kumar et al., 2021). Moreover, a flavonoid, ERINLG-4, isolated from *Streptomyces* sp. had an anti-proliferative impact on A549 cells. It induced intrinsic and extrinsic apoptosis via p53 and cytochrome c release to activate caspase-9 and caspase-3 (Balachandran et al., 2014). In addition, a milbemycin compound, extracted from *Streptomyces* sp. FJS31-2 exhibited a cytotoxic effect against A549 cells (Li et al., 2020). Furthermore, metastasis inhibition was observed in A549 cells treated with migracins that were isolated from *Streptomyces* sp (Arai et al., 2013). In addition, both Naphtomycin A that was isolated from *Streptomyces* sp. CS (Lu & Shen, 2007), and phenolic compounds found in the extract of *Streptomyces cellulosae* strain TES17 (Rani, Arora, Kaur, & Manhas, 2018), showed cytotoxic activity against A549 cells.

CHAPTER II

AIMS

Our study aims to explore the cancer-suppressive activity of *Streptomyces* MCH11 extract isolated from soil in Machghara, a city in Lebanon's West Bekaa, on A549 lung adenocarcinoma cancer cells. We will first investigate the molecular mechanisms of cell death induced by the extract. We will next address whether it has an inhibitory effect on A549 cells' migration. Finally, we will identify the bioactive compounds in this extract. Our specific aims are as follows:

1. We will assess A549 cells' viability upon treatment with the extract using the MTT assay.
2. We will study the mechanism of cell death induced by the extract using the Annexin V/PI assay and by western blot analysis of cleaved PARP-1 and cleaved caspases.
3. We will evaluate the effect of the extract on A549 cell migration using the scratch assay and we will correlate the findings with the protein expression of β-catenin using western blotting.
4. We will carry out bio-guided fractionation and sub-fractionation, to determine the sub-fraction/s with the most prominent cytotoxic effect on A549 cells to be used in the future for the identification of the bioactive compound or compounds.

CHAPTER III

MATERIALS AND METHODS

A. Molecular and Cellular Biology Methods

1. Cell Culture

A549 is a human lung adenocarcinoma epithelial cell line that was purchased from the American Type Culture Collection center (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin antibiotics (Sigma). Cells were passaged using 1 ml trypsin with Ethylenediaminetetraacetic acid (EDTA) and preserved under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, and 37°C).

2. Cell Viability Measurement by MTT Assay

A549 cells were seeded in 96-well plates at a density of 5000 cells per well. On the next day, cells were treated with DMSO as control or with the crude bacterial extract *Streptomyces* MCH11 cultured in medium C, fractions, or sub-fractions of the extract and incubated for 24 or 48 hours. At each time point, 30 µl of MTT tetrazolium dye was added and the plate was incubated for 3 hours. Then, MTT was discarded, and the formazan crystals were dissolved with 100 µl of DMSO. Finally, the plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 595 nm wavelength.

3. Migration Assay - Cell Wound Closure Assay

A549 cells were seeded in a 24-well plate at a density of 500,000 cells per well with a DMEM medium that was deprived of FBS but supplemented with 1% penicillin-

streptomycin antibiotics. On the second day, when cell confluency reached 90%, cells were treated with different concentrations (0.001, 0.01, and 0.03 mg/ml) of the crude bacterial extract *Streptomyces* MCH11 cultured in medium C for 24 and 48 hours. Control cells were treated with (0.001, 0.01, and 0.03 mg/ml) of DMSO. A scratch was made in each well by a 10 µl micropipette tip. The change in the width of the scratch was monitored and corresponding images were captured (4X magnification) at 0 h, 24 h, and 48 h via a bright-field microscope (Make-Nikon ECLIPSE Ti-S) and were analyzed using ImageJ software. The percentage of wound closure was calculated with the below equation:

Wound Closure %= $[(A_{t=0} - A_{t=\Delta t}) / A_{t=0})] \times 100\%$. The equation was adapted from (Suarez-Arnedo et al., 2020) where $A_{t=0}$ is the initial wound area and $A_{t=\Delta t}$ is the area after n hours of creating the scratch in μm^2.

4. Protein Extraction and Quantification

A549 cells were seeded at a density of 500,000 cells in a T25 flask. On the following day, cells were treated with different concentrations of the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C (0.01, 0.03, and 0.06 mg/ml) for 24 and 48 hours in the case of caspases and PARP-1, and with (0.001, 0.01, and 0.03 mg/ml) for 24 and 48 hours in the case of β-catenin. Control cells were treated with 0.1% DMSO or with the same concentrations as the extract. At each time point, cells were collected, centrifuged, and re-suspended in lysis buffer [Tris-HCl 0.25 M (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 2 mg bromophenol blue] containing protease inhibitors. Samples were denatured for 5 minutes at 95°C and then placed on ice for 20 minutes. Afterward, samples were centrifuged and supernatants

were kept at -20°C for quantification. Later, samples were quantified using the Detergent Compatible Bio-Rad Protein Assay in which proteins interact with reagents A, S, and B (Folin's reagent). The absorbance was read at 750 nm on the ELISA plate reader. The concentrations of the proteins were calculated according to a standard curve using bovine serum albumin (BSA) (Amresco).

5. Western Blot

Protein expression levels were analyzed using 10% and 12% acrylamide gels. Samples were prepared with 1:1 volume ratio of proteins to loading buffer [Tris-HCl 0.25 M (pH 6.8), 4% SDS, 20% Glycerol, 2 mg bromophenol blue, and 5% β-mercaptoethanol] and run using Tris-Glycine-SDS Buffer (TGS 1X) running buffer [TGS 10X: 30 g Tris (hydroxymethyl)-aminomethane, 144 g glycine and 10 g SDS]. The migration was performed at 70 V for the stacking gel and 120 V for the resolving gel. Following the migration, a transfer to a polyvinylidene difluoride (PVDF) membrane was done in transfer buffer (TGS 1X with 20% methanol) for 90 minutes (10% and 12%) at 100 V. Then, the membrane was blocked overnight to prevent nonspecific binding using 5% fat-free milk prepared in Tris-buffered saline (TBS 1X) [TBS 10X: 12 g Tris (hydroxymethyl)-aminomethane and 87.8 g NaCl, pH.8] with 0.1% Tween. Following blocking, the membrane was incubated at 4°C overnight with 3 ml of primary antibody in 5% milk-TBS 1X 0.1% Tween. The membrane was then washed for 10 minutes with TBS 1X 0.1% Tween for three cycles and incubated at room temperature for one hour with 10 ml of the horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch, Europe; 1/5000) diluted in 5% milk-TBS 1X 0.1% Tween. The bands were developed using enhanced

chemiluminescence (ECL) western blotting reagent (GE health care, UK). Protein bands were quantified by ImageJ software and normalized to the expression of GAPDH or β-tubulin.

Table 1 List of primary antibodies

Antibody Name	**Product code**	**Supplier**
GAPDH	Sc-47724	Santa-Cruz Biotechnology
β-tubulin	3385769	Sigma-Aldrich
β-catenin	C2206	Sigma-Aldrich
Caspase 8	ab25901	Abcam
Caspase 9	ab 202068	Abcam
PARP-1	ab 191217	Abcam

6. Annexin V/ PI Apoptosis Assay

Cells were seeded in a 24-well plate at a density of 30,000 cells per well. After 24 hours, cells were treated with different concentrations (0.01, 0.03, and 0.06 mg/ml) of the crude bacterial extract *Streptomyces* MCH11 cultured in medium C for 24 and 48 hours. Control cells were treated with DMSO having the same concentrations as the extract. At each time point, cells were collected by centrifugation at 2000 rpm for 10 minutes and then washed with PBS. Using Annexin-V-FITC staining Kit (Miltenyibiotec, Germany), pellets were incubated with 20 µl of incubation buffer and 2 µl of Annexin-V-FITC labeling agent for 15 minutes in the dark at room temperature. Then, 500 µl of incubation buffer were added to the tubes, and the tubes were centrifuged at 2000 rpm for 10 minutes. Pellets were resuspended in 100 µl of

incubation buffer and 1 µl of propidium iodide solution. Cells were read by flow cytometry (Guava easyCyte, millipore) and 10,000 events were counted by the Incyte software. Alive cells are negatively stained for Annexin V and PI. Early apoptotic cells are Annexin V positive and PI negative. Late apoptotic cells are both Annexin V and PI positive. Necrotic cells are Annexin V negative and PI-positive.

B. Microbiological Methods

1. Soil Sample Processing and Bacterial Isolation

Soil samples were collected from Machghara, a village in the Lebanese district of West Bekaa. The samples collection and the related microbiological methods were performed by the group of Dr. Antoine Abou Fayad at the American University of Beirut. The samples were then dried at 37º C for 7 days. Then, 3 grams of each sample were heated in 100 mL of sterile distilled water at 55º C for 30 minutes. Afterward, a serial dilution was conducted with various dilution factors and with a final volume of 1 mL, and 30 µL of each dilution solution was streaked on a soil agar plate and an International *Streptomyces* Project 3 Medium (ISP3) agar plate. When colonies started to appear, each was taken from the soil plates by an autoclaved toothpick and streaked on an ISP3 plate. This process was repeated several times until a pure culture was obtained from each initial colony. After purification, the strains were stored in a 50% aqueous glycerol solution at -80º C. Soil agar is the medium required for isolating and growing soil microorganisms; it was prepared from Machghara soil, with adding some nutrients such as starch and bacteriological agar. ISP3 is a medium designed for isolating Streptomyces species. A mixture of 18 grams of bacteriological agar, 20 grams

of oats, 2.5mL of trace elements, and a liter of distilled water was prepared for ISP3 agar. Both agars (soil and ISP3) were kept for one week in the incubator at 28ºC.

2. First and Second Seeds of Bacteria

The first seed was performed by inoculating 35 µL of the glycerol stock in 5 mL of ISP3 broth and incubated for 2 days in a shaker-incubator at 28ºC. Subsequently, 1 mL of this initial seed was inoculated in 10 mL of ISP3 broth and kept again for 2 days in a shaker-incubator at 28ºC. This culture was referred to as a second seed.

3. Preparation of Medium C

Medium C that acts as a stress-inducing environment was prepared with the following components: $CaCO_3$ (0.1g/L), Glycine (2.5g/L), NaCl (1g/L), KH_2PO_4 (1g/L), $FeSO_4$ (0.1g/L), $MgSO_4.7H_2O$ (0.1g/L), and Tween 60 (20g/L). The pH of medium C is 7.

4. Production of the Small-Scale Crude Extract

1 mL of the second seed was used to inoculate 50 mL of medium C and the resulting culture was incubated in a shaker incubator at 28ºC for 7 days. 1 mL of a polymeric adsorbent (Amberlite XAD 16N resin) was added to the media on the last day of incubation to adsorb the metabolites, and the mixture was then incubated in the same shaker at 28ºC for 3 hours. Afterward, the mixtures were centrifuged at 4000 rpm for 20 minutes at a temperature of 4ºC. The pellet containing the resins and the cell mass was transferred to a new flask after discarding the supernatant. To extract the metabolites from the resins, the pellet was resuspended in 30 mL of acetone and 10 mL of methanol, and the mixture was incubated on a shaker at room temperature for 2

hours. Later, glass wool was used to filter the resins and the liquid phase was allowed to dry in a chemical hood for 2 days or was directly dried via a rotary evaporator. Subsequently, the dried extract was dissolved in 1 mL of DMSO and stored at -20ºC.

5. Upscale Secondary Metabolite Production

The first and second seeds, as well as the production media (medium C), were prepared similarly to the small-scale production but in a greater amount to increase the yield of the secreted metabolites. This was followed by incubation in a shaker incubator at 150 rpm and 28°C for 14 days. On the last day of incubation, a sterile resin suspension was added to the culture overnight and then the secondary metabolites were collected via acetone/methanol similar to the small-scale production. Then, the resulting crude extract was subjected to liquid-liquid partition.

C. Bio-guided Fractionation

1. Liquid-liquid Separation

180 mL of methanol and 20 mL of water were added to the dried upscale crude extract and the mixture was transferred to a separatory funnel. Afterward, 200 mL of hexane were added to the mixture inside the funnel. The funnel was properly closed and shaken well. The funnel was then placed back on a holder allowing the two phases to separate. The water/methanol phase separates to the bottom while the hexane phase is present on top. The mixture of methanol and water was drained into a beaker and then the hexane was transferred into a separate beaker. Two more washes were performed on the water/methanol phase with 150 mL of hexane using the same technique. All the hexane fractions were collected in the same beaker.

A rotary evaporator was used to evaporate the methanol from the water/methanol phase. More water was poured into this fraction to reach a final volume of 200 mL before transferring this phase back into the funnel. Afterward, 3 washes with chloroform were performed on the water/methanol fraction, as done with hexane. Then, the same procedure was performed with 3 washes of ethyl acetate done on the water/methanol fraction. Therefore, we ended up with 3 different fractions: hexane, chloroform, and ethyl acetate. These fractions are listed by an increasing order of their polarities. The fractions were then dried and dissolved in DMSO.

2. Column Chromatography

Flash column chromatography was used to separate the components of the ethyl acetate fraction based on their polarity, which leads them to migrate at various speeds through the column as solvents were being added. The ethyl acetate fraction was loaded into the adsorbent column which is made from silica gel. The eluent (organic solvent) was drained in the column, so the components of the ethyl acetate were partitioned between the fixed silica and the mobile eluent. At this step, we obtained different sub-fractions A, B, C, D, E, F, G, and H.

3. Thin-Layer Chromatography (TLC)

TLC was conducted on the ethyl acetate fraction to determine the best solvent system before proceeding with column chromatography and on the sub-fractions that were received from the column chromatography to group them in tubes. [90% dichloromethane (DCM)/10% methanol + 0.1% formic acid] was used as a solvent system. The plate was withdrawn after the solvent had reached 1 cm from the top edge.

Under UV, the components were visible as dots with varying retention factors. Based on the TLC results, the different collection tubes were combined; eight sub-fractions were obtained and tested for their cytotoxic activity on the A549 cancer cell line via MTT assay.

D. Statistical Analysis

Statistical significance and p values of the data were determined with the unpaired Student's t-test. Differences were considered statistically *Significant ($P < 0.05$), **Highly significant ($P < 0.001$) and ***Extremely significant ($P < 0.0001$) with respect to control. IC50 values were computed using GraphPad Prism 8.0.1 software with the following equation: log (inhibitor) vs. normalized response – variable slope.

CHAPTER IV

RESULTS

A. Evaluation of the Cytotoxicity of the Crude Bacterial Extract of *Streptomyces* MCH11 Cultured in Medium C on A549 Cells

MTT assay was performed to determine the cytotoxic effect of the crude bacterial extract *Streptomyces* MCH11 cultured in medium C on A549 cells after 24 and 48 hours. The extract was prepared in DMSO to a concentration of 10 mg/ml. Cells were treated with increasing concentrations as shown in Figure 6. The percentage of cell viability for each concentration treatment was computed relative to that of its corresponding control having the same DMSO concentration. The viability of A549 cells treated with the extract was significantly decreased in a time- and dose-dependent manner (Figure 6).

The significant decrease in the cell viability at both time points started to appear after treatment with 0.03125 mg/ml of the extract. However, treatment with 0.0625 mg/ml of the extract decreased the cell viability with extreme significance where it reached 49% and 26% for 24 and 48 hours respectively. This extreme decrease in viability was also observed after treatment with higher concentrations of the extract. For the concentration 0.125 mg/ml, viability reached 28% and 17% at 24 and 48 hours respectively; while for the concentration 0.25 mg/ml, it reached 19% at 24 hours and 20% at 48 hours. The IC50 values were computed as 0.04909 and 0.03355 mg/ml at 24 and 48 hours respectively.

Thus, we concluded that the crude bacterial extract *Streptomyces* MCH11 cultured in medium C significantly inhibited the viability of A549 cells *in vitro*.

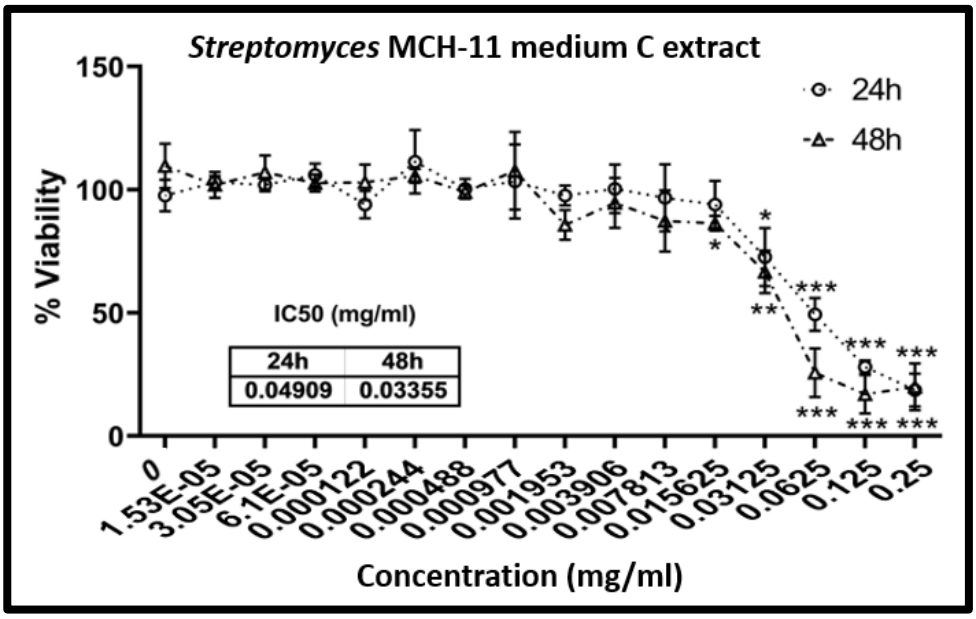


Figure 6 Evaluation of the cytotoxicity of the crude bacterial extract *Streptomyces* MCH11 cultured in medium C on A549 cells. The percentage of cell viability was calculated as the percentage of optical density (OD) ratio (treated versus control DMSO). Values represent the average of three independent experiments with standard deviations. IC50 was calculated via GraphPad Prism 8 software. *Significant when ($P<0.05$), **Highly significant ($P<0.001$), and ***Extremely significant ($P< 0.0001$) with respect to control.

B. Induction of Apoptotic Cell Death upon Treatment of A549 Cells with the Crude Bacterial Extract of *Streptomyces* MCH11 Cultured in Medium C

To further investigate the cell death induced by the crude bacterial extract *Streptomyces* MCH11 cultured in medium C, Annexin V/PI was performed on A549 cells treated with (0.01, 0.03, and 0.06 mg/ml) of the extract for 24 and 48 hours.

A549 cells were distributed in four quadrants as early apoptotic cell in lower right quadrant, late apoptotic cells in upper right quadrant, alive cells in lower left quadrant, and necrotic cells in upper left quadrant (Figure 7A). As shown in (Figure 7B), a dose-dependent increase in the percentage of apoptosis (sum of early and late) was observed at 24- and 48-hours post-treatment. The percentage of viable, apoptotic,

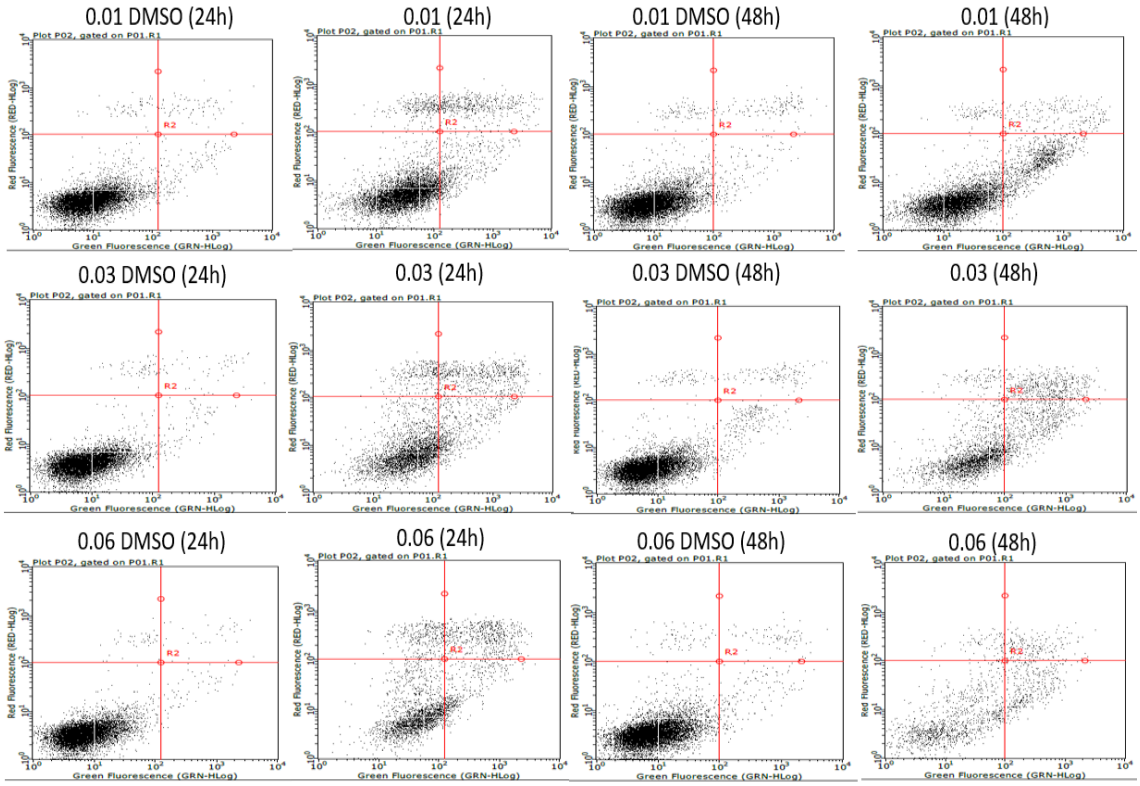
or necrotic cells for each treatment concentration was computed relative to that of its corresponding control having the same DMSO concentration.

At 24 hours, the treatment with 0.01, 0.03, and 0.06 mg/ml of the crude extract significantly raised the percentage of apoptotic cells from the baseline of 4% to 11%, 30%, and 52 % respectively. Treatment with these concentrations also significantly decreased live cells from 96% to 87%, 67%, and 44% respectively. The percentage of necrotic cells did not show any significant difference between treatment and control.

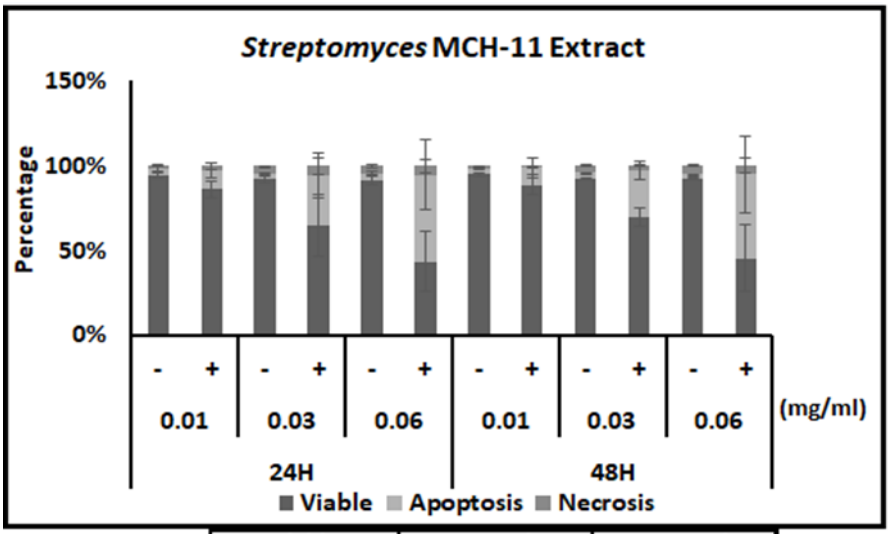
It is also notable that after 48 hours of treatment with 0.03 mg/ml of the crude extract, the percentage of live cells decreased in a highly significant manner from 96% to 70% accompanied with a highly significant increase in the percentage of apoptotic cells from 3% to 27%. The percentage of necrotic cells significantly decreased compared to control after treatment with this concentration. In addition, the percentage of live cells significantly decreased from 97% to 46% accompanied with a significant increase in the percentage of apoptotic cells from 2% to 49% after 48 hours of treatment with 0.06 mg/ml of the extract.

Therefore, the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C significantly induces the apoptosis of A549 cells *in vitro.*

A)



B)



	Viable		Necrosis		Apoptosis	
	24h	48h	24h	48h	24h	48h
0.01	*	*N.S.*	*N.S*	*	*	*N.S*
0.03	*	**	*N.S*	*	*	**
0.06	*	*	*N.S*	*N.S*	*	*

Figure 7 Annexin V/PI double staining analysis of apoptosis in A549 cells treated with the crude extract *Streptomyces* MCH11 cultured in medium C. (A) A549 cells were

treated with 0.01, 0.03, and 0.06 mg/ml of extract or with the corresponding concentrations of DMSO at 24 and 48 hours. Cells that are shown in the upper right quadrant are in the late stage of apoptosis, whereas cells in the bottom right quadrant are in the early stage of apoptosis. The bottom left quadrant shows viable cells, while the upper left quadrant represents necrotic cells. (B) Shows the percentage of apoptotic, necrotic, and alive cells. Cells in early and late apoptosis were combined as apoptotic cells. Values represent the average of three independent experiments. Non-significant (*N.S*), *Significant ($P<0.05$), **Highly significant ($P<0.001$), and ***Extremely significant ($P<0.0001$) with respect to the control.

1. Cleavage of PARP-1 upon Exposure of A549 Cells to the Crude Bacterial Extract of Streptomyces MCH11 Cultured in Medium C

Since apoptosis was triggered by the extract, we next investigated the cleavage of PARP-1 in A549 cells treated with 0.01, 0.03, and 0.06 mg/ml of the extract at 24 and 48 hours in order to confirm that cell death was apoptotic.

Western blot results shown in (Figure 8) revealed an increase in the expression of cleaved PARP-1 by around 1.6-folds after 24 and 48 hours of A549 cells treatment with 0.03 mg/ml of the extract, whereas the treatment with the concentration of 0.06 mg/ml showed a significant increase in the expression of cleaved PARP-1 by around 3.9 and 3.6 folds at 24 and 48 hours respectively. Therefore, the expression of cleaved PARP-1 is increased upon treatment with the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C in a dose-dependent manner. PARP-1 cleavage confirms the induction of apoptosis that we previously observed.

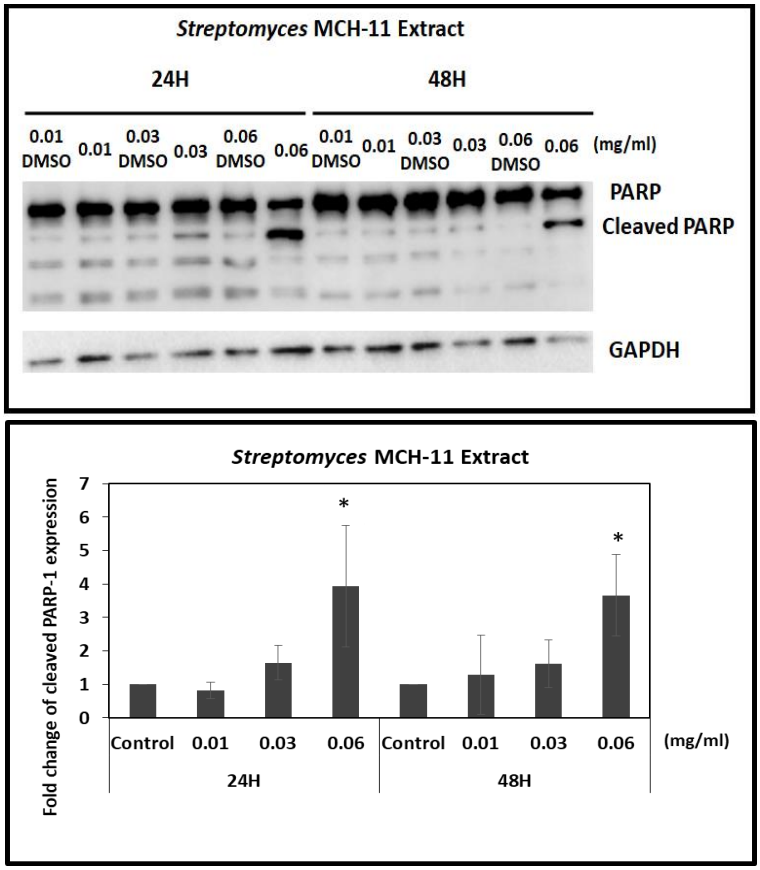


Figure 8 The expression of cleaved-PARP-1 in A549 cells upon treatment with the crude extract *Streptomyces* MCH11 cultured in medium C. Cells were treated with (0.01, 0.03, and 0.06 mg/ml) of the extract for 24 and 48 hours. The corresponding control was A549 cells treated with the same DMSO concentration. The intensity of bands was quantified via ImageJ software and normalized to GAPDH bands. The graph represents the average of two independent experiments with standard deviations. *Significant difference ($P< 0.05$) with respect to the control.

2. Activation of Caspases upon Exposure of A549 Cells to the Crude Bacterial Extract of Streptomyces MCH11 Cultured in Medium C

The mechanism of programmed cell death involves the activation of caspases by their cleavage to two subunits. Accordingly, we studied the apoptotic pathways activated in A549 cells upon treatment with 0.01, 0.03, and 0.06 mg/ml of the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C at 24 and 48 hours.

Results of western blot shown in (Figure 9) revealed that cells exhibited activation of caspase-9 upon treatment with 0.06 mg/ml of the extract at 48 hours. At this

concentration of treatment, a significant cleavage of caspase-9 was observed with 2.5-folds increase compared to the control.

Similarly, the activation of the initiator caspase recruited to death receptors, caspase-8, was also investigated. We observed a significant decrease in the expression of pro-caspase 8 upon treatment with 0.03 mg/ml of the extract at 48 hours (Figure 10). The decrease in pro-caspase 8 expression could either indicate its cleavage or its decreased synthesis. Unfortunately, the antibody available could only detect pro-caspase 8, but not the corresponding cleaved band, although this could be possible at both concentrations. Therefore, more experiments are underway to confirm this.

Taken together, we conclude that the crude bacterial extract *Streptomyces* MCH11 cultured in medium C induces the apoptosis of A549 cells *in vitro* via activating the intrinsic apoptotic pathway. The involvement of the extrinsic pathway is also possible but needs to be confirmed.

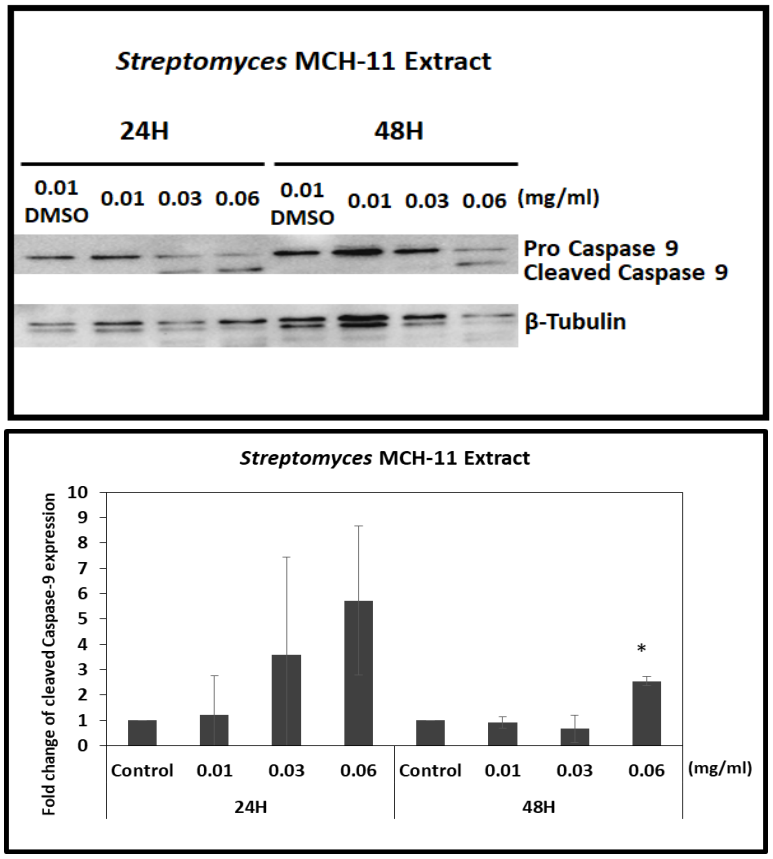


Figure 9 The cleavage of caspase-9 in A549 cells treated with the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C. Cells were treated with (0.01, 0.03, and 0.06 mg/ml) of the extract for 24 and 48 hours. The control was cells treated with 0.1% DMSO. The bands were quantified using ImageJ software and normalized to β-tubulin bands. The graph represents the average of two independent experiments with standard deviations. *Significant difference ($P< 0.05$) with respect to the control.

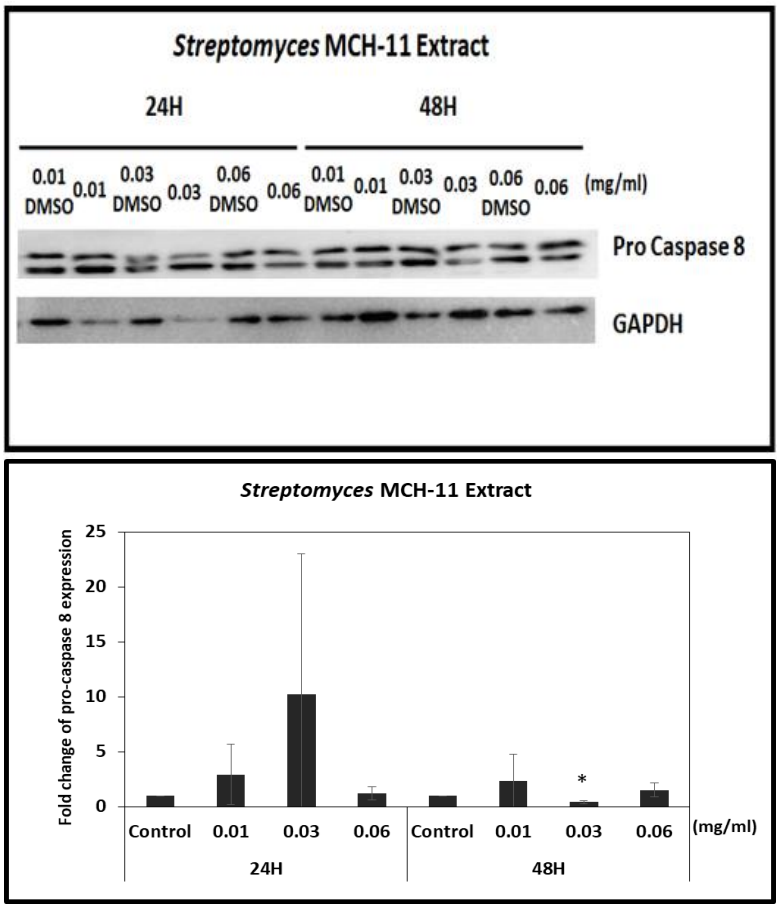


Figure 10 Pro-caspase 8 expression in A549 cells treated with the crude bacterial extract *Streptomyces* MCH11 cultured in medium C. Cells were treated with (0.01, 0.03, and 0.06 mg/ml) of the extract for 24 and 48 hours. The corresponding controls were cells treated with the same DMSO concentration. The bands were quantified using ImageJ software and normalized to GAPDH. The graph represents the average of two independent experiments with standard deviations. *Significant difference ($P < 0.05$) with respect to the control.

C. Assessment of Migration and β-catenin Expression in A549 Cells upon Treatment with the Crude Bacterial Extract of *Streptomyces* MCH11 Cultured in Medium C

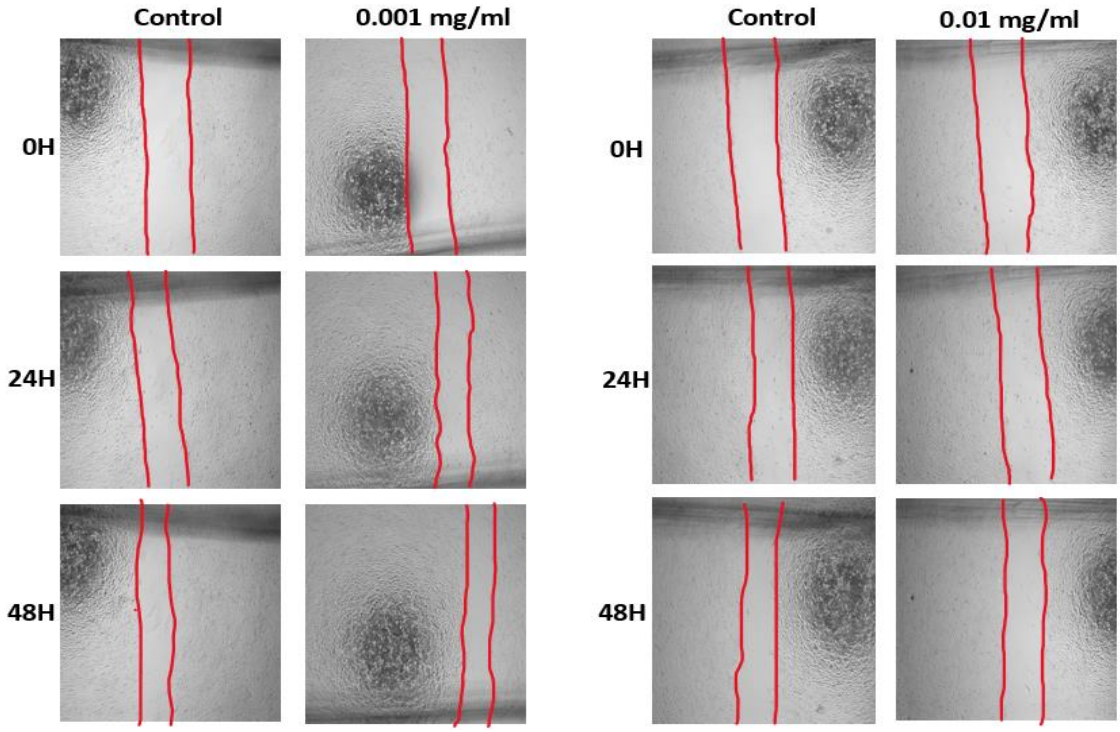
1. Effect of Streptomyces MCH11 extract cultured in medium C on cell migration in A549 cells

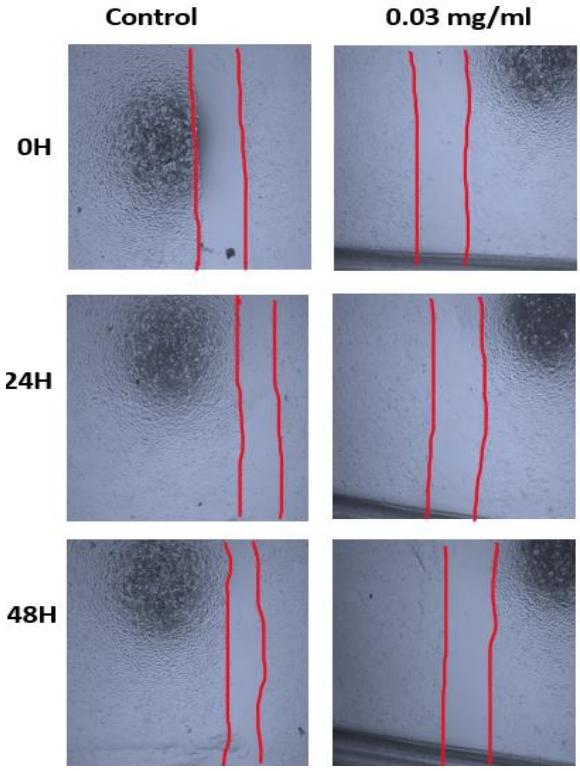
In order to assess the effect of the crude extract on the migration of A549 cells, a wound closure assay was performed. A549 cells were treated with 0.001, 0.01, and 0.03 mg/ml of the extract for 24 and 48 hours. The percentage of closure for each treatment concentration was computed relative to that of its corresponding control having the same DMSO concentration.

As shown in Figure 11A and 11B, the treatment with 0.03 mg/ml of the extract reduced the motility of A549 cells preventing them from migrating across the wound to "heal" it. This treatment significantly decreased wound closure from 13% to 4 % in cells treated with 0.03 mg/ml at 48 hours, compared to control DMSO treated cells. Whereas, lower doses like 0.001 and 0.01 mg/ml could not inhibit the cell migration. 0.01 mg/ml of the extract significantly induced cell motility at 24 hours.

According to the above results, the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C inhibited the migration of A549 cells after treatment with a concentration of 0.03 mg/ml at 48 hours but not lower.

A)


Control
0.001 mg/ml
Control
0.01 mg/ml
0H
24H
48H
0H
24H
48H


Control
0.03 mg/ml
0H
24H
48H

B)

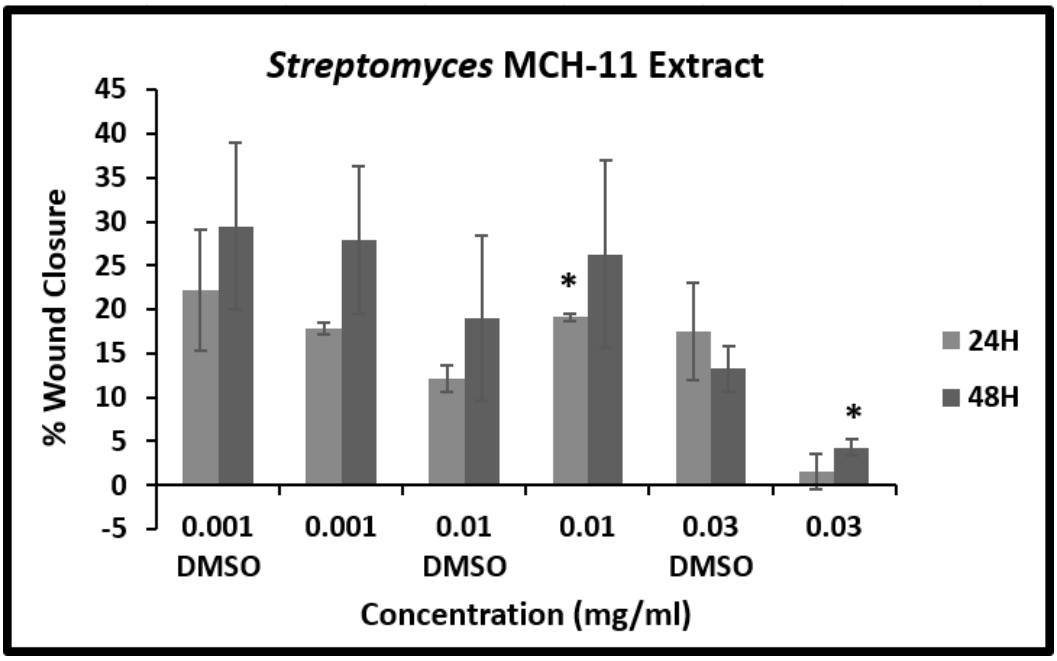


Figure 11 The impact of the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C on the migration of A549 cells. A) Representative images, captured by phase-contrast microscopy (magnification 4X), demonstrate A549 cells motility upon treatment with 0.001, 0.01, and 0.03 mg/ml of the extract or with the corresponding control having the same DMSO concentration at 24 and 48 hours. B) The bar chart shows the percentage of wound closure. The graph represents the average of two independent experiments with standard deviations. *Significant difference (P< 0.05) with respect to the control.

2. Evaluation of β-catenin Expression Upon Exposure of A549 Cells to the Crude Bacterial Extract of Streptomyces MCH11 Cultured in Medium C

Since the inactivation of Wnt/ β-catenin was shown to inhibit NSCLC metastasis (Han et al., 2021), we decided next to correlate the inhibition of A549 cellular motility with the protein expression of β-catenin by western blotting. As expected, in preliminary results, we observed a downregulation in β-catenin expression at the concentration of 0.03 mg/ml that induced inhibition in migration (Figure 12). At 48 hours, the expression of β-catenin slightly decreased from 1 fold in the vehicle control (0.03% DMSO) to 0.72 fold in cells treated with 0.03 mg/ml of the crude extract.

Thus, our preliminary results suggest that the previously observed inhibition of migration of A549 cells upon treatment with 0.03 mg/ml of the extract correlated with the downregulation β-catenin protein expression.

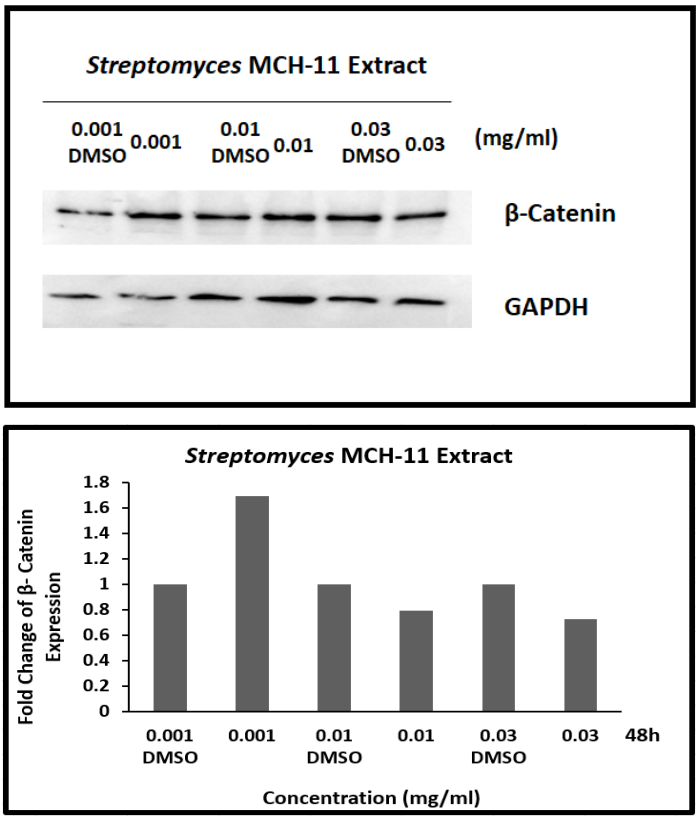


Figure 12 The expression of β-catenin in A549 cells upon treatment with the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C. Cells were treated with different concentrations (0.001, 0.01, and 0.03 mg/ml) for 48 hours. The control was A549 treated with 0.001, 0.01, and 0.03 mg/ml of DMSO. The intensity of bands was quantified via ImageJ software and normalized to GAPDH bands. The graph represents the values of one experiment that need to be confirmed.

D. Evaluation of the Cytotoxicity of Chloroform, Hexane, and Ethyl Acetate Fractions of the Crude Bacterial Extract of *Streptomyces* MCH11 Cultured in Medium C on A549 Cells

MTT assay was conducted to determine the cytotoxic effect of the chloroform, hexane, and ethyl acetate fractions of the crude bacterial extract of *Streptomyces*

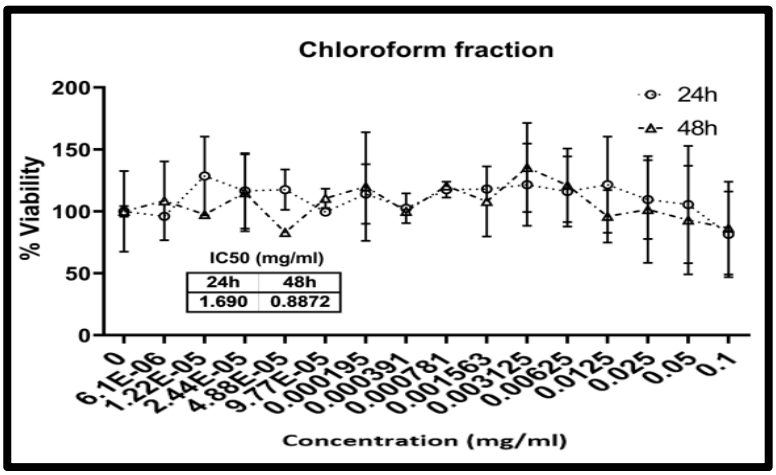
MCH11 cultured in medium C on A549 cells after 24 and 48 hours. All the fractions were dissolved in DMSO. The highest concentration of chloroform fraction was 0.1 mg/ml with 10 mg/ml initial concentration, while the highest concentration for ethyl acetate and hexane was 1 mg/ml with 100 mg/ml initial concentrations. These fractions correspond to 0.1% DMSO, which is tolerated well by cells in culture. The chloroform fraction did not show any significant cytotoxicity on A549 cells at 24 or 48 hours up to a concentration of 0.1 mg/ml; with the Image J-calculated projected IC50 values of 1.690 and 0.8872 mg/ml at 24 and 48 hours, respectively (Figure 13. A).

The viability of A549 cells treated with the hexane fraction decreased in a dose-dependent manner (Figure 13. B). At 24 hours, this fraction significantly decreased the viability at a concentration of 0.25 mg/ml where it reached 37%. However, the viability decreased with extreme significance upon treatment with the concentrations of 0.5 and 1 mg/ml where it reached 29% and 16%, respectively at 24 hours, and it reached 21% and 16%, respectively, at 48 hours. The calculated IC50 values were 0.1656 and 0.2059 mg/ml at 24 and 48 hours, respectively.

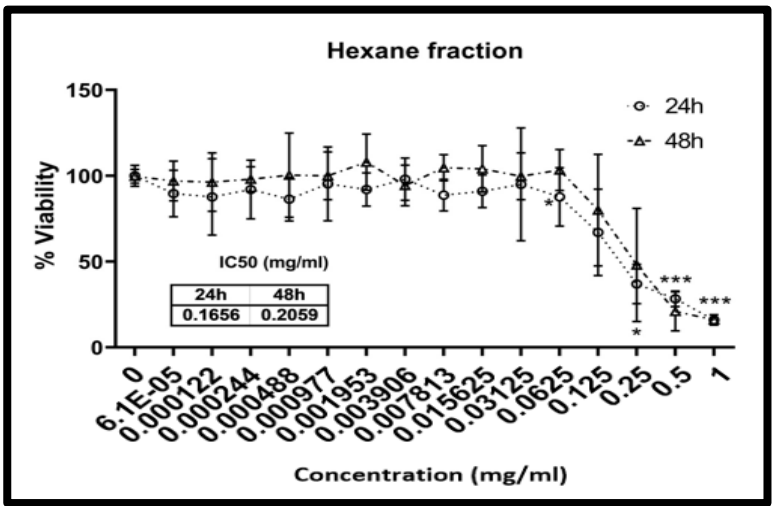
The ethyl acetate fraction showed the highest cytotoxic effect among the three fractions (Figure 13. C). At 24 hours, the cell viability started to decrease significantly upon treatment with a concentration of 0.0625 mg/ml where it reached 57%. However, a highly and extremely significant decrease in cell viability was observed after treatment with concentrations of 0.125, 0.25, 0.5, and 1 mg/ml reaching 27%, 12%, 16%, and 16%, respectively, at 24 hours, and 19%, 12%, 13 %, and 15%, respectively, at 48 hours. The IC50 values for the ethyl acetate fraction were determined to be 0.06994 and 0.06636 mg/ml for the time points 24 and 48 hours, respectively. Thus, the IC50 values of this fraction were the lowest compared to the hexane and the chloroform

fractions. This indicated that the ethyl acetate fraction had the most potent cytotoxic effect on A549 cells and was subjected to sub-fractionation.

A)



B)



C)

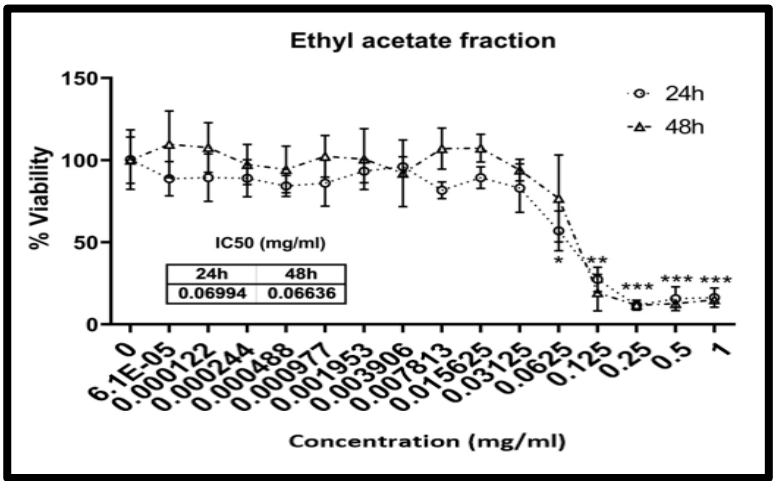


Figure 13 The assessment of the cytotoxic effect of the chloroform, hexane, and ethyl acetate fractions of the crude bacterial extract *Streptomyces* MCH11 cultured in medium C on A549 cells. Cells were treated with different concentrations of the chloroform fraction (A), the hexane fraction (B), or the ethyl acetate fraction (C) at 24 and 48 hours. The percentage of cell viability was calculated as the percentage of OD ratio (treated versus control). Values represent the average of three independent experiments with standard deviations. IC50 was computed using GraphPad Prism 8 software. *Significant (P< 0.05), **Highly significant (P<0.001) and ***Extremely significant (P< 0.0001) with respect to the control.

E. Evaluation of the Cytotoxicity of the Eight Sub-fractions (A, B, C, D, E, F, G, and H) of the Ethyl Acetate Fraction of the Bacterial Extract of *Streptomyces* MCH11 Cultured in Medium C on A549 Cells

The MTT assay was performed to detect the cytotoxic effect of the 8 sub-fractions (A, B, C, D, E, F, G, and H) of the ethyl acetate fraction of the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C on A549 cells as described in Methods.

A549 cells were treated with different concentrations of the sub-fractions (1, 2.5, 5, 7.5, 10, 15, 20, and 30 μg/ml) for 24 and 48 hours. Our results showed that neither sub-fraction A nor C demonstrated any cytotoxic effect on A549 cells at the two-time points (Figure 14. A and 14. C).

Sub-fraction B significantly decreased the viability of A549 cells at 48 hours after treatment with the concentrations of 15 and 20 μg/ml, where it reached 87% and 83% respectively. This decrease in viability was highly significant after treatment with the concentration of 30 μg/ml where it reached 74%. Using GraphPad Prism 8 software, the IC50 value was projected to be 44.75 μg/ml at 48 hours (Figure 14. B).

Sub-fraction D significantly reduced the viability of A549 cells at 48 hours upon treatment with the concentration of 30 μg/ml to reach 81% (Figure 14. D). The IC50 values were projected to be 67.93 and 69.01 μg/ml at 24 and 48 hours.

Regarding the sub-fraction E, the treatments with 20 and 30 μg/ml decreased the viability in a significant and highly significant manner to reach 72% and 74% respectively at 24 hours (Figure 14. E). At 48 hours, treatment with 15 μg/ml significantly reduced the viability to 85%. IC50 values were projected to be 71.21 and 76.26 μg/ml at 24 and 48 hours respectively.

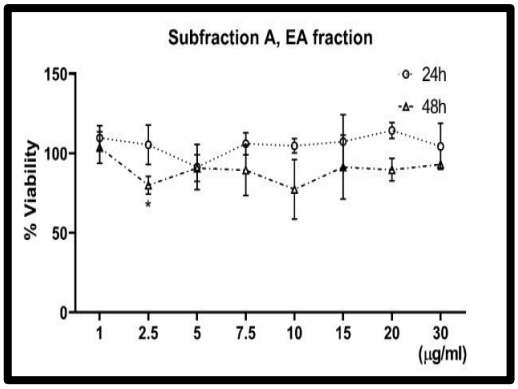
Sub-fraction F significantly reduced the viability upon treatment with the concentration of 30 μg/ml where it reached 78% at 24 hours. At 48 hours, a significant decrease in the viability was observed after treatment with a concentration of 20 μg/ml where it reached 73%. The IC50 values were projected to be 93.50 and 65.41 μg/ml at 24 and 48 hours, respectively (Figure 14. F).

Sub-fraction G significantly decreased the viability after treatment with the concentrations of 20 and 30 μg/ml to reach 82% and 81%, respectively, at 48 hours (Figure 14. G). The IC50 values were projected to be 78.56 and 145.0 μg/ml at 24 and 48 hours, respectively.

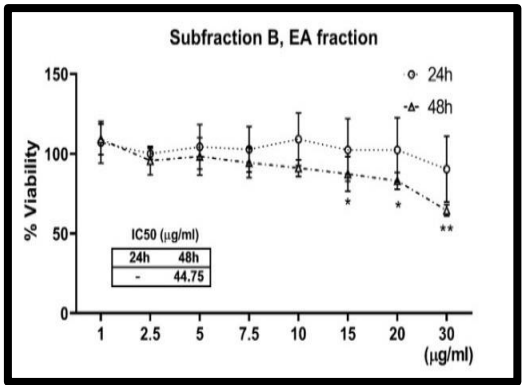
Similarly, sub-fraction H significantly reduced the viability to 85% at 48 hours after treatment with a concentration of 30 μg/ml (Figure 14. H). The IC50 values were projected to be 163.4, and 139.4 μg/ml at 24 and 48 hours, respectively.

To conclude, sub-fraction B appeared to be the most potent sub-fraction among the eight tested sub-fractions where the highest three concentrations of treatment showed a consistent decrease in the viability of A549 cells at 48 hours. Sub-fraction B also had the lowest projected IC50 value at 48 hours. Thus, we hypothesize that it is the sub-fraction that likely contains the bioactive compound(s).

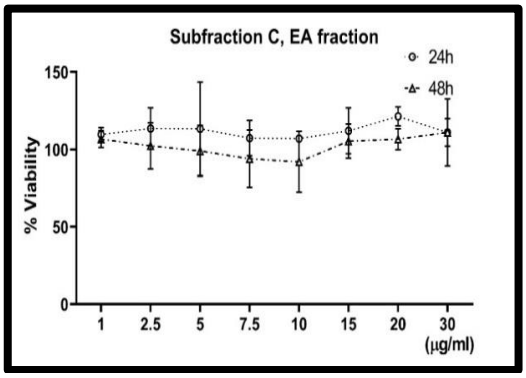
A)


Subfraction A, EA fraction
24h
48h
% Viability
150
100
50
0
1
2.5
5
7.5
10
15
20
30
(µg/ml)

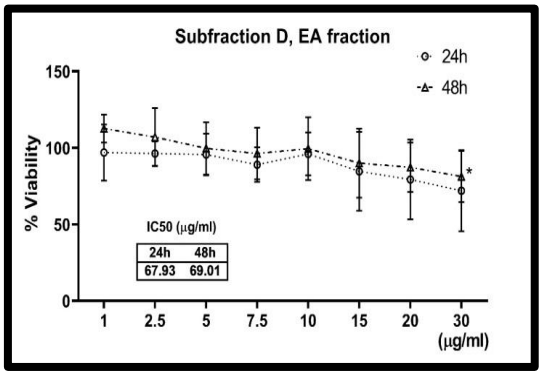
B)


Subfraction B, EA fraction
24h
48h
% Viability
IC50 (µg/ml)
24h 48h
- 44.75
(µg/ml)

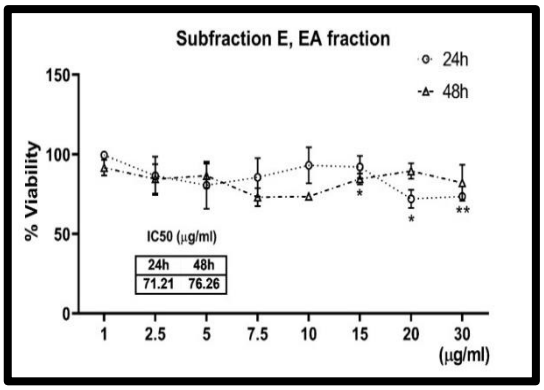
C)


Subfraction C, EA fraction
24h
48h
% Viability
(µg/ml)

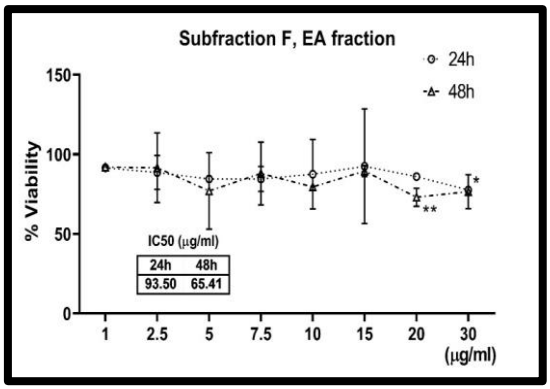
D)


Subfraction D, EA fraction
24h
48h
% Viability
IC50 (µg/ml)
24h 48h
67.93 69.01
(µg/ml)

E)


Subfraction E, EA fraction
24h
48h
% Viability
IC50 (µg/ml)
24h 48h
71.21 76.26
(µg/ml)

F)


Subfraction F, EA fraction
24h
48h
% Viability
IC50 (µg/ml)
24h 48h
93.50 65.41
(µg/ml)

G) **H)**

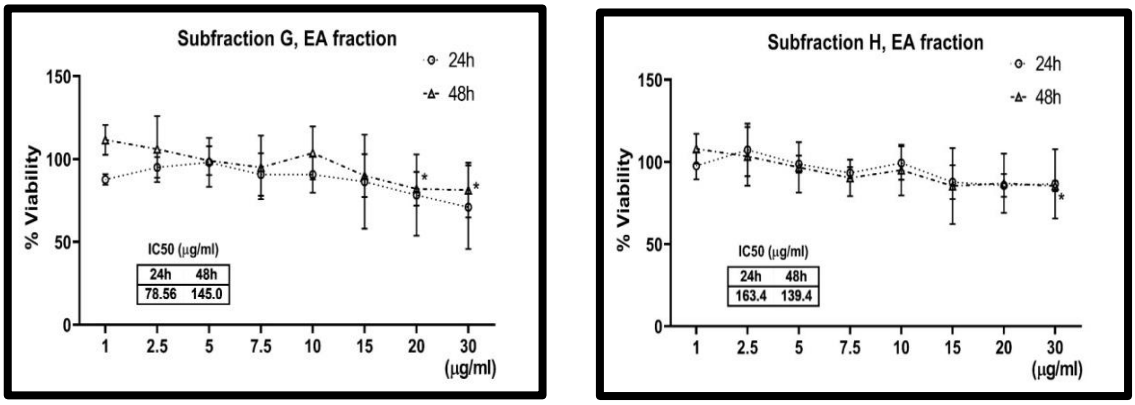


Figure 14 The evaluation of the cytotoxicity of the sub-fractions (A, B, C, D, E, F, G, and H) of the ethyl acetate fraction of the crude bacterial extract of *Streptomyces* MCH11 cultured in medium C on A549 cells. Cells were treated with different concentrations of the sub-fractions (1, 2.5, 5, 7.5, 10, 15, 20, and 30 µg/ml) at 24 and 48 hours and the corresponding control was treated with the same DMSO concentration. (A) →(F) show the results for sub-fraction A through sub-fraction F, respectively. Cell viability was calculated as the percentage of OD ratio (treated versus control). Values represent the average of three independent experiments with standard deviations for all sub-fractions except E and F whose graphs represent the average of two independent experiments. IC50 was computed using GraphPad Prism 8 software. *Significant ($P < 0.05$), **Highly significant ($P<0.001$) and ***Extremely significant ($P < 0.0001$) with respect to the control.

CHAPTER V

DISCUSSION

In the present work, the newly isolated Streptomyces species from the soil of Machghara, a Lebanese village in West Bekaa, by the group of Dr. Antoine Abou Fayad at the American University of Beirut was evaluated as a potential source of bioactive compounds. The species was provisionally named *Streptomyces* MCH11. In the current study, we have demonstrated the cancer-suppressive activity of *Streptomyces* MCH11 crude extract cultured in medium C on A549 non-small cell lung cancer cells *in vitro*. That was illustrated by the induction of the intrinsic apoptotic pathway and possibly of the extrinsic apoptotic pathway and by the inhibition of migration potential of A549 cells. Our results also showed that the cytotoxic effects shown were mostly present in the ethyl acetate fraction and appeared to concentrate in its sub-fraction B.

This work highlights that the Lebanese soil in the Machgara region could be a good source of Streptomyces strains having anticancer activity. In previous studies, soil was also the source of other Streptomyces species that have an anticancer potential as well, such as the soil isolated from the Mirzapur region of Rajshahi city area in Bangladesh, and from Malaysia (Khatun, Haque, Anisuzzaman, & Islam, 2021b; Tan et al., 2015). Previous work done in our laboratory determined that the crude extract from *Streptomyces* MCH11 cultured in medium C provided the optimum cytotoxic potential on A549 cells. The composition of medium C (tween 60, glycine, $CaCO_3$, $MgSO_4.7H_2O$, K_2HPO_4, and $FeSO_4$) and its neutral pH 7 were very close to that of a medium used in a previous study with the extract of *Streptomyces artemisiae* MCCB 248 against NCI-H460 lung cancer cells (Dhaneesha et al., 2017). The composition of

this medium is thought to account for the changes in the bacterial metabolites; the work by Scofield et al. suggests a potential change in the bacterial metabolism in association with increased nutrient input (Scofield, Jacques, Guimarães, & Farjalla, 2015).

Cytotoxicity studies have played an essential role in the discovery of several anticancer drugs. The *Streptomyces* MCH11 crude extract cultured in medium C showed significant cytotoxicity against non-small cell lung cancer A549 cell line with IC50 values of 0.04909 mg/ml (24 hours) and 0.03355 mg/ml (48 hours). The extract also triggered apoptosis in A549 cells in a dose-dependent manner as shown by Annexin V/PI assay. This technique has been widely used to demonstrate apoptosis (Kumar, Saneja, & Panda, 2021); Annexin V detects phosphatidylserine, which translocates to the extracellular portion of the membrane in early apoptosis, while propidium iodide (PI) reveals DNA fragmentation in late apoptosis. Many mechanisms of cell death are triggered by anticancer drugs derived from Streptomyces including apoptosis (Olano, Méndez, & Salas, 2009b). This is considered one of the more desirable mechanisms to control cancerous cells (Hsiao et al., 2014). Several previous studies applied extracts isolated from the Streptomyces genus against non-small lung cancer cells and exerted significant cytotoxicity and induced cell death. For example, *Streptomyces* sp. strain 196 extract affected A549 cells' morphology and induced apoptosis (P. Kumar et al., 2021). The induction of apoptosis by our bacterial extract was also observed in other studies; with a flavonoid (ERINLG-4) isolated from *Streptomyces* sp. (Balachandran et al., 2014), with Neoantimycin F (NAT-F) isolated from *Streptomyces conglobatus* (L. Liu et al., 2019), with the ethyl acetate extract isolated from *Streptomyces artemisiae* MCCB 248 (Dhaneesha et al., 2017), and with

the ethyl acetate extract isolated from the *Streptomyces* FEAI-1 (Khatun, Haque, Anisuzzaman, & Islam, 2021).

Apoptosis is triggered through the extrinsic death receptor-mediated or the intrinsic mitochondrial-dependent signaling pathways through a p53-dependent or independent manner (Wang & Scadden, 2015). Caspases are a family of cysteine proteases that play critical roles in both intrinsic and extrinsic signaling pathways of apoptosis (Degterev, Boyce, & Yuan, 2003). In the intrinsic pathway, the initiator caspase-9 promotes a cascade activation of effector caspases-3,-6, and-7 which eventually leads to apoptosis (Kuribayashi et al., 2006). While the extrinsic pathway activates caspase-8 which eventually initiates apoptosis by cleaving other caspases (Walczak & Krammer, 2000). In our results, we observed a significant cleavage of pro-caspase 9 in A549 cells treated with 0.06 mg/ml of the extract at 48 hours, and that was accompanied with a significant cleavage of PARP-1. We reasoned that the expression of cleaved caspase-9 indicated that downstream caspases such as caspase-3 were activated to cleave the PARP-1 protein, leading to DNA fragmentation, which is also considered a hallmark of apoptosis (Kaufmann, Desnoyers, Ottaviano, Davidson, & Poirier, 1993; C Soldani & Scovassi, 2002). In a previous study, polyether compounds produced by *Streptomyces cacaoi* also induced the intrinsic apoptotic pathway by cleaving caspase-3, caspase-9, and PARP-1 (Khan et al., 2019). Similarly, a flavonoid, ERINLG-4, isolated from *Streptomyces* sp. induced apoptosis by activating caspase-9 and caspase-3 in A549 cells (Balachandran et al., 2014).

In our study, we found that in addition to caspase-9 cleavage after treatment with 0.06 mg/ml of the extract, pro-caspase 8 expression was significantly downregulated in A549 cells treated with 0.03 mg/ml of the extract at 48 hours. The

decrease in the pro-caspase 8 expression could either indicate its cleavage or its lower expression. Technically, our antibody could only detect pro-caspase 8, so we were not able to demonstrate on the blot the pro-caspase 8 cleavage and the corresponding cleaved band, although it could be possible that this happened at both concentrations that we studied. In this regard, more experiments are underway to confirm this. Therefore, we reasoned that the extract triggered apoptosis of A549 cells through the intrinsic or mitochondria-mediated apoptotic pathway induced by cleaved caspase-9. The extrinsic or receptor-dependent pathway induced by cleaved caspase-8 might also be involved and cannot be excluded until further experiments are performed. Both apoptotic pathways were shown to be involved in other studies. For instance, the extract *Allium atroviolaceum* Bulb induced apoptosis through both the intrinsic and extrinsic pathways by inducing the cleavage of caspase-8, -9, and -3, along with downregulation of Bcl-2 in MCF7 breast cancer cells (Khazaei et al., 2017). In the case of the marine bacterial extract P3-86B(2), both intrinsic and extrinsic pathways of apoptosis were also involved in its mechanism of action (Sagar et al., 2013). Similarly, the marine antimicrobial peptide MSP4 induced apoptosis of the osteosarcoma cell line MG63 through both a Fas/FasL- and mitochondria-mediated pathway (Kuo et al., 2018). The intrinsic and the extrinsic pathways of apoptosis were shown to be interconnected. Death receptor stimulation and activation of caspase-8 may cleave Bid protein which leads to initiating the mitochondrial pathway (Roy & Nicholson, 2000). On the other hand, mitochondrial triggered caspase-6 may feed back to the receptor pathway and cleave caspase-8 (Slee, Adrain, & Martin, 1999).

Interestingly, our work has also shown that the *Streptomyces* MCH11 crude extract cultured in medium C suppressed A549 cancer cell motility after treatment with

a dose of 0.03 mg/ml. This was associated with downregulation of the protein expression of β-catenin. These results indicate that our extract might have the ability to inhibit the metastatic potential of NSCLC cells. This effect was also demonstrated with Streptomyces-derived extracts or compounds in other studies; for instance, broth of a cultured *Streptomyces* sp. MK929-43F1 produced a compound called migrastatin that repressed the metastatic potential of EC17 human esophageal cancer cells (Nakae et al., 2000). In addition, *Streptomyces* sp. strain 196 extract inhibited cell migration of A549 lung cancer cells (P. Kumar et al., 2021). Furthermore, metastasis inhibition was observed in A549 cells treated with migracins isolated from *Streptomyces* sp (Arai et al., 2013).

Three fractions of *Streptomyces* MCH11 crude extract were obtained by the liquid-liquid partition technique. These fractions have different polarities, the least polar fraction is hexane with 0.1 polar index, chloroform has 4.1 polar index, while ethyl acetate was the fraction with the highest polarity with 4.4 polar index. The hexane fraction being the least polar fraction exerted a moderate effect on A549 cells' viability and had an IC50 of 0.1656 and 0.2059 mg/ml at 24 and 48 hours, respectively. The chloroform fraction did not show any significant cytotoxic impact on A549 cells and its IC50 values were the highest among the three fractions (1.690, and 0.8872 mg/ml at 24 and 48 hours, respectively). This might be due to the fact that this fraction is not a compound-rich fraction. The ethyl acetate fraction had the most prominent cytotoxicity on A549 cells with the lowest values of IC50 among all fractions (0.0699 and 0.0663 mg/ml at 24 and 48 hours, respectively). This could be due to its richness in bioactive compounds. In fact, in several previous studies, the cytotoxicity of Streptomyces extracts against non-small lung cancer cell lines was obtained with the ethyl acetate

fractions of the extracts. For instance, the crude ethyl acetate extract of the bacterial strain FEAI-1, belonging to the *Streptomyces* species, elicited an apoptotic effect on H1299 non-small lung cancer cells (Khatun et al., 2021a). Moreover, the ethyl acetate extract of *Streptomyces artemisiae* MCCB 248 showed cytotoxicity on H460 human non-small lung cancer cells (Dhaneesha et al., 2017). Subsequently, the ethyl acetate fraction of our extract was fractionated, and eight sub-fractions were obtained. After analyzing the cytotoxicity results, we found that the sub-fraction B had a profound cytotoxic anti-proliferative effect on A549; it suppressed A549 cell proliferation to reach 74% at 48 hours and its IC50 value was projected at 44.75 μg/ml at 48 hours. The IC50 value of sub-fraction B was the lowest among all eight sub-fractions. Thus, sub-fraction B was hypothesized to possibly contain the bioactive compound or compounds which might be responsible for the anticancer activity on A549 cells.

Many anticancer compounds from actinomycetes were previously identified and they belonged to several structural classes such as anthracyclines, enediynes, indolocarbazoles, isoprenoides, macrolides, non-ribosomal peptides, (Olano, C., Méndez, C., & Salas, J. A. 2009b). Many compounds were also identified from the Streptomyces genus including neoantimycin F (L. Liu et al., 2019), a flavonoid ERINLG-4 (Balachandran et al., 2014), milbemycin (Li et al., 2020), and migracins (Arai et al., 2013). Our future work will be focused on the isolation and the characterization of the bioactive compound or compounds present in the bioactive sub-fraction of the *Streptomyces* MCH11 extract.

CHAPTER VI

CONCLUSION AND PERSPECTIVES

In the current study, we have shown that Lebanese soil could be a good source of anti-cancer drug discovery. We have shown the cancer-suppressive activity of *Streptomyces* MCH11 extract isolated from Machghara's soil on A549 non-small cell lung cancer cells *in vitro* as illustrated by the induction of the intrinsic apoptotic pathway and possibly the extrinsic apoptotic pathway and by the inhibition of cell migration. Our results also highlight that the anti-cancer effect shown partitioned to the ethyl acetate fraction. We hypothesize that sub-fraction B is the bioactive sub-fraction from which the bioactive compound(s) will be isolated and identified in the future. Once characterized, this compound(s) might provide a potential alternative treatment for NSCLC or could be possibly integrated with conventional anti-cancer treatments to aid in the eradication of lung cancer.

In the future, we aim to:

- Determine the molecular structure of the bioactive compound or compounds by Liquid Chromatography-Mass Spectrometry (LC-MS) and 1H NMR.
- Confirm the downregulation in the protein expression of β-catenin by western blot.
- Confirm the cleavage of pro-caspase 8 with the appropriate antibody and hence the involvement of the extrinsic pathway of apoptosis.
- Study the intrinsic apoptotic pathway by investigating the expression of several pro-apoptotic proteins and anti-apoptotic proteins of the Bcl-2 family; and the extrinsic apoptotic pathway by studying the expression of candidates like death receptors.
- Examine the role of p53 in the cell death induced by the extract or compound.

- Examine the accumulation of ceramide in A549 cells in response to treatment with the extract or compound by immunocytochemistry or LC-MS. Ceramide was described in many studies as a signaling sphingolipid mediating mitochondrial apoptosis (Hage-Sleiman, Esmerian, Kobeissy, & Dbaibo, 2013).

- Examine the effect of the isolated compound against a myriad of human cancer cell lines and on human normal cells.

- Increase the concentration of sub-fractions to be able to see the effect of IC50 values rather than estimation of the software.

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