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Novel unbiased DARTS approach to find protein targets for endophyte extracts in a selected number of cancer cell lines

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Abstract: Background: DARTS "Drug Affinity Responsive Target Stability" is a novel methodology for identifying and studying protein-ligand interactions. This technique depends on the reduction in the protease susceptibility of the target protein upon drug binding.

Objectives: The aim of this study to prove the effectiveness of the method to identify the molecular target proteins of some bioactive endophyte extracts. The basic idea of this research is studying the possibility of the use of Darts technique for the detection of cellular targets for natural extracts before doing chemically analyzed to determine the type of vehicle which they are located.

Methodology: To achieve the aim of study, some natural extracts of fungi endophyte were selected for the study, three of which are able to stop the growth of three kinds of cancer cells leukemia and lung cancer and cervical cancer, as demonstrated by tests performed in this study.

Results: Then Darts technique was used to identify the cellular targets of these extracts successfully. Possible, as already identified a number of proteins that interact with these extracts within the cell. It was possible for first time by using DARTS followed by MALDI-TOF analysis and peptid mass fingerprinting to identify a number of cellular target molecules for bioactive extracts of endophytes.

Conclusion: This will help reduce the number of extracts that go into the process of drug development because the extracts which is not suitable to be excluded from the start without the need to chemically analyzed to determine composition, which helps to reduce the cost and the time period required by the process of drug development in the future. This opens up a new area for the use of this technique in the process of scanning cytometry initial natural extracts without having to analyze it chemically which reduces the effort and expense. Finally, A number of very interesting proteins could be identified that might stimulate the researchers in a future study to the chemical composition of these extracts to exactly identify the active component in each of them.

Keywords: DARTS, protein targets, endophyte extracts, cancer, cancer cell lines.

تطبيق منهجية DARTS الجديدة لإيجاد أهداف البروتين لمستخلصات الخلايا الداخلية في عدد محدد من خطوط الخلايا السرطانية

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الملخص: هدفت الدراسة الحالية إلى دراسة جدوى طريقة DARTS لتحديد ودراسة التفاعلات بين ربيطات البروتين، وإثبات فعالية الطريقة لتحديد البروتينات المستهدفة الجزيئية لبعض مقتطفات الخلايا الحيوية النشطة بيولوجيا. الفكرة الأساسية لهذا البحث هي دراسة إمكانية استخدام تقنية DARTS للكشف عن الأهداف الخلوية للمستخلصات الطبيعية قبل القيام بالتحليل الكيميائي لتحديد نوع الحاضنة التي توجد فيها. ولتحقيق الهدف من الدراسة، تم اختيار بعض العينات الطبيعية من الفطريات للدراسة، ثلاثة منها قادرة على وقف نمو ثلاثة أنواع من خلايا سرطان الدم وسرطان الرئة وسرطان عنق الرحم، كما يتضح من الاختبارات التي أجريت في هذه الدراسة.

وبينت لنتائج أن استخدام تقنية DARTS لتحديد الأهداف الخلوية لهذه المقتطفات تم بنجاح. كما تم التعرف بالفعل على عدد من البروتينات التي تتفاعل مع هذه العينات داخل الخلية. كان من الممكن لأول مرة باستخدام DARTS متبوعًا بتحليل MALDI-TOF وبصمات الأصابع peptidmass لتحديد عدد من الجزيئات المستهدفة الخلوية للمستخلصات الحيوية النشطة من العينات الفطرية.

الخلاصة: تساعد هذه الدراسة في تقليل عدد المستخلصات التي تدخل في عملية تطوير الدواء لأن المستخلصات غير المناسبة يتم استبعادها من البداية دون الحاجة إلى تحليل كيميائي لتحديد التركيب، مما يساعد على تقليل التكلفة والوقت الفترة التي تتطلبها عملية تطوير الدواء في المستقبل. هذا يفتح مجالًا جديدًا لاستخدام هذه التقنية في عملية مسح المستخلصات الطبيعية الأولية الخلوية دون الحاجة إلى تحليلها كيميائيًا مما يقلل من الجهد والتكاليف. أخيرًا، يمكن تحديد عدد من البروتينات المثيرة للاهتمام التي قد تحفز الباحثين في دراسة مستقبلية على التركيب الكيميائي لهذه العينات لتحديد المكون النشط في كل منها تمامًا.

الكلمات المفتاحية: DARTS، البروتين، السرطان، الخلايا السرطانية.

1- Introduction

Historically, many scattered facts mentioned the efforts of humankinds to harness the healing properties of natural compounds. However, what we know for certain is that ancient peoples made extensive use of plant, animal and mineral sources for this purpose (Stojanoski, 1999).

The Ebers papyrus, written in Egypt in the 16th century, lists the extensive pharmacopeia of that civilization. Included in this are: beer, turpentine, myrrh, juniper berries, poppy, lead, salt and crushed precious stones. Also included were products derived from animals, including lizard's blood, swine teeth, goose grease, ass hooves and the excreta from various animals. The effects of many of these drugs on patients of antiquity can only be imagined (Glesinger,1954).

From ancient China comes evidence of that culture's extensive efforts to heal through the use of natural products. The Pen Tsao, or Great Herbal, comprised forty volumes describing several thousands of prescriptions. Interestingly, the eastern herb Artemisia annua (wormwood), used in China since antiquity to treat fevers, is the source of the modern drug qinghaosu, which shows great promise as a modern anti-malarial compound (Wiart, 2007).

Antiquity to the modern era the ancients considered disease a consequence of demonic possession, or the wrath of god. Thus, in ancient times, the treatment of illness with natural products was invariably accompanied by religious rituals deemed essential to the healing process (Dimitrova,1999).

Over time, as a more sophisticated view of illness evolved, an increasingly scientific approach to the isolation of drugs from natural products was taken. In the early 19th century, morphine was isolated from the opium poppy (Papaver somniferous) and the anti-malarial compound quinine from the bark of the cinchona tree (Cinchona officinalis) (Dervendzi,1992).

In 1897, Felix Hoffman, a research chemist synthesized acetylsalicylic acid. On February 1, 1899, Aspirin® was registered as a trademark. On March 6th of the same year, this drug was registered with the Imperial Patent Office in Berlin. Aspirin quickly become popular worldwide, and remains an important drug today. Interestingly, it was not until 1971 that Sir John Vane discovered the mechanism of action of aspirin, a feat that earned him the 1981 Nobel Prize for Medicine.

The modern era these, and additional advances in the fields of chemistry and physiology, lead to the birth of modern pharmacology in the latter half of the 19th century. Thus, Materia Medical evolved into the experimental science of pharmacology, which is devoted to understanding the physiological action of these molecules (Madigan, 2005). The 20th century has witnessed the discovery of a steady stream of important new drugs that have immeasurably improved the human condition. Not very long ago, vast numbers of humans perished prematurely or suffered an existence filled with pain due to the effects of infection or disorders that are now successfully treated.

The potential prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great endophyte simply means: the location of an organism. “endo” means “inside” “phyte” means “plants”. Endophyte refers to organisms that live within plants. Fungi and bacteria are the most common organisms associated with the term endophyte.

Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships, ranging from symbiotic to slightly pathogenic. Because of what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances of potential use to modern medicine, agriculture, and industry. Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation, culture, purification (Wilson, 1995).

Endophytic microbes occupy within plant and usually contribute to plant health. Some groups of entophytic microorganisms have been believed to be mutualists that protect plants against biotic stress (Tadych and White, 2009). Co-evolution may exist between endophytes and their host in resist to environmental stresses. During the last two decades endophytes have been targeted as valuable sources of new bioactive compounds. Endophytic fungi can be found within the leaves and stems of plants, with remarkable diversity. Systemic endophytic fungi are able to produce physiologically active alkaloids in host plant tissues.

The bioactive natural products from endophytes are promising resources for medicine, agriculture and industry. Different kinds of Alkaloids are contributed to plant by endophytes. Some of these alkaloids increase plants resistance to environmental stress, and some are growth-promoting compounds. Amines and amides are very common metabolite product from endophytes and have shown to be toxic to insect but not to mammals. Indole-3-acetic acid is a well-known phytohormone, which also can be produced by endophytes. Other bioactive compounds, such as steroids, terpenoids and diterpenes also are generated

by endophytes. Besides the compounds mentioned above, endophytes produce extracellular hydrolyses in order to establish a resistance mechanism against plant invasion. The enzymes include cellulases, lipases, proteinase and esterase. The actions of those enzymes support the hypothesis of co-evolution between endophytes and their hosts (Tan and Zou, 2001).

2- Research objectives

The aim of the current study is to use a new technology known as Drug Affinity Responsive Target Stability (DARTS) to identify the direct binding targets of biologically active extracts isolated from endophytes. The technique is based on the principle that when a small molecule compound binds to a protein, the interaction stabilizes the target protein's structure so that it becomes protease resistant. The method was originally described by Lomenick and proved to be a fast and robust method to determine the binding partner of small purified molecules in crude cells extracts. In a novel application we will employ DARTS-Method to identify the molecular target(s) of some biologically active endophytic extracts in selected cancer cell line. By using this novel target-to-drug approach, the study is intended to identify new targets in cancer cells that are impaired by new bioactive secondary metabolites from endophytes. These results could lead to drug candidates or new strategies for cancer chemotherapy.

3- Literature Review

The key challenge of drug development to determine the mechanism by which drugs and chemicals affect a biological system (Stock well, 2004). On the other hand, identifying the primary targets of these drugs determine the selection of compounds suitable for drugs development, and also increase their therapeutic potency. Historically such kind of investigation has taken years if not decades, because of the absence of a systematic method to investigate how drugs exert their biological activities.

However, recently, several approaches have been developed to accelerate such studies including, affinity and biochemical purifications, RNA expression profiling, gene expressing profiling, proteins arrays, yeast three-hybrid analysis, chemical genetics and proteomics technologies. One of the most interesting examples is using of synthetic lethal analysis in yeast to determine proteins and pathways affected by cytotoxic drugs (Stockwell, 2004). In traditional yeast synthetic lethal screens, one looks for combinations of non-lethal gene deletions or mutations that together cause cell death, but individually has no lethal effect. When using this approach to identify cellular drug targets, one has to generate a set of synthetic lethal profiles by deleting a specific gene and testing the effects of deleting all other genes (individually) in combinations with the test genes. Therefore, the resulting gene-gene synthetic lethal combination profile is a molecular fingerprint for each gene deletion. By comparing the synthetic lethal profile of gene deletion to the synthetic lethal profile of a compound, one can determine whether they affect similar cellular processes. However, the main disadvantage of this approach is that it cannot be applied generally,

because many compounds of interest act on proteins that are not expressed in yeast cells, and also many knock-outs of interesting pathways are enviable.

Therefore, despite the large number of target identification techniques used to date, affinity chromatography remains the most widely used method for drug target identification (Lomenick, 2011).The typical project begins with structure–activity relationship studies in which various functional groups of the small molecule of interest are modified or removed to determine which one(s) are dispensable for drug activity. These nonessential site(s) are then used as points of attachment for an affinity tag (e.g., biotin). Then, similar to immunoprecipitation of specific proteins using an antibody and conjugated beads, the drug-linked beads are incubated with protein extracts, followed by extensive washing to remove nonspecifically bound proteins. Finally, the tightly bound proteins are eluted with excess free drug or under highly denaturing conditions. Most often the eluted proteins are subsequently analyzed by SDS– PAGE and protein bands are identified by mass spectrometry (Duong, 2011).

The primary limitation of affinity chromatography is the need to chemically modify the small molecules of interest, to enable an immune-precipitation step. Indeed, many small molecules cannot be chemically modified without affecting bioactivity, and presumably binding, or cannot be easily obtained or synthesized in quantities large enough to permit structure-activity relationship and subsequent studies. However, in comparison to other newly developed target identification methods that rely on various phenotypic or molecular signatures to narrow down potential targets, the advantage of affinity-based methods is that they rely solely on binding of the drug to its target protein, rather than specific cellular or biochemical readouts that are only useful for a fraction of compounds (Lomenick, 2010).

To overcome the problem of chemical modifications, and at the same time, to use the advantage of affinity chromatography, Lomenick and co-workers developed a novel proteomics approach to identify the molecular target of bioactive drugs without needing any chemical modifications of the drug of interest. This new method, DARTS (Drug Affinity Response Target Stability), takes advantage of a reduction in the protease susceptibility of the target protein upon drug binding (Figure 2-1). In addition, it is universally applicable because it requires no modification of the drug and is independent of the mechanism of drug action (Lomenick, 2009).

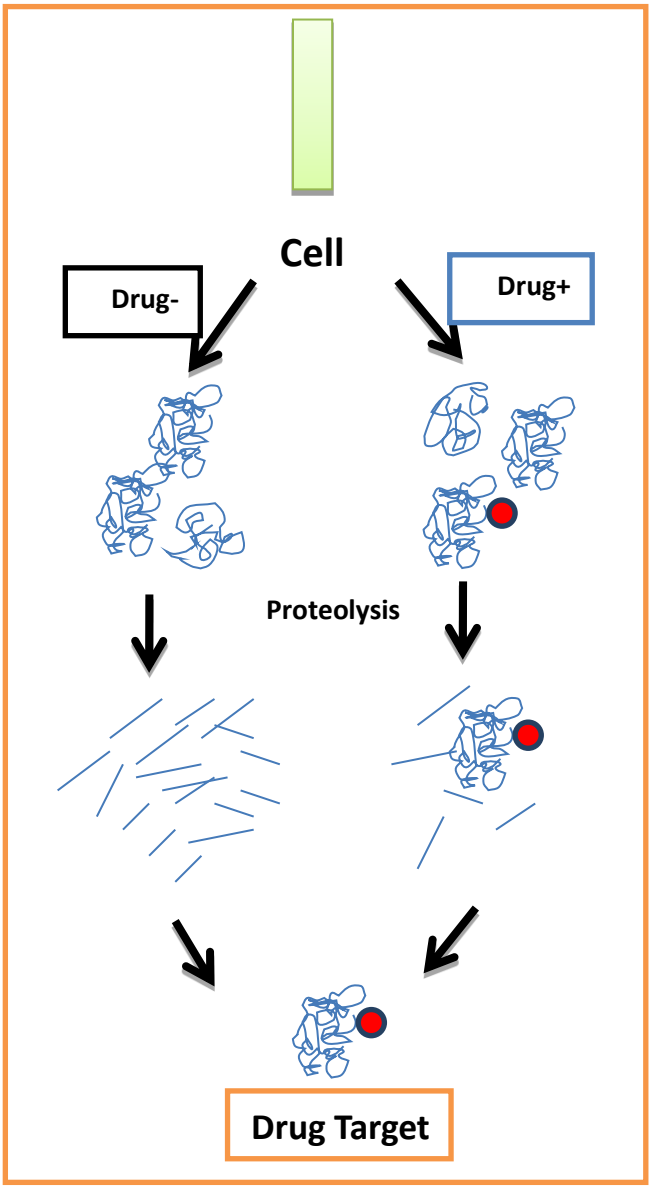

Figure (2-1) the principle of DARTS method (Lomenick, 2009).

The key advantage of DARTS is that it does not require labeled ligands and instead uses unmodified small molecules for binding; it is not limited by chemistry and can potentially be used to identify binding targets for any small molecule. Additionally, unlike cell-based methods, DARTS is completely independent of any effects of the drug on the system, and is therefore compatible with any mechanism of action, making it useful for any small molecule of interest.

In this study the ability of DARTS to identify molecular targets of bioactive drugs will be tested and modified to be able to identify molecular targets of bioactive extracts (not a purified substance) from endophytes. We identify bioactive extracts were expected that can be further developed for cancer chemotherapy. The novelty of the suggested approach is that we first look for the targets in the active

extracts and then try to identify the ligands of interesting targets. This procedure is reverse to strategies followed up so far.

4- Method and Procedures

For the current study, the researcher developed a special experimental protocol to implement all lab work and do the extracts successfully. The numbers, figures and calculations have been conducted according to the results achieved following each test. The study involved no statistical analysis except the calculations needed for each test that were completed using the special assays and lab devices.

4-1 Extraction of endophyte

Three endophyte fungal methanol extracts have been tested against the proliferation of different cancer cell lines (jurkat cell, a549 cell and Hella229). The three extracts used in this work are symbolized with sample numbers 82, 266 and 267. Sample number 82 was extracted from Alternaria alternate that was growing on Vinca rosea leaf. Alternaria alternate is a fungus that has been recorded to cause leaf spot and other diseases on over 380 host species of plant. It is an opportunistic pathogen on numerous hosts causing leaf spots, rots and blights on many plant parts. It can also cause upper respiratory tract infections and asthma in humans with compromised immunity. Sample number 266 was extracted from Spegazziniana sundara that was growing on Randia duemetorum. Randia dumetorum is a large deciduous thorny shrub belonging to family Rubiaceae. It is also known as a Catunaregam spinosa. It occurs in almost throughout India up to 4,000 feet attitude. Methanol extracts of fruit of Randia dumetorum known to have Anti-inflammatory effect (Patel, Pathak Nimish, 2011). The last sample has the number 267 and was extracted from Bartalinia sp. That was growing on Bridelia retusa. It is a species of Bridelia found in Bangladesh, Nepal, southern India, and Sri Lanka. The bark of the roots has supposed anti-rheumatic properties, and is used as a medicine in traditional medicine. It is also reported that some extracts of the plants exhibit antibacterial (Mishra, 2013) as well as anti-fungal activities (Jayasingh, 2003). All information about the sample details are summarized in table 4-1.

Table (4.1) Biological origin and concentration factor of the tested extracts.

Extract number	Fungus	Plant Host	Concentration factor
Sample 82	Alternaria alternate	Vinca rosea	200
Sample 266	Spegazzinnia sundara	Randia duemetorum	200
Sample 267	Bartalinia sp.	Bridelia retusa	200

4-2 Cell Culture and Culture conditions

The cell lines including Jurkat cells (acute T cell leukemia), A459 cells (lung carcinoma), and HeLa229 cells (cervix carcinoma). Information about the tested cancer cell lines are given in table 4-2.

Table (4.2) tested cancer cell lines.

Cell line	Organism	Tissue	Disease	ATCC-No.
Jurkat	Homo sapiens	T lymphocyte	Acute T cell leukemia	TIB-152™
A459	Homo sapiens	Lung	Carcinoma	CCL-185™
HeLa229	Homo sapiens	Cervix	Adenocarcinoma	CCL-2.1™

Cell lines were obtained from the American Type Culture Collection (ATCC), and the German Collection of Microorganisms and Cell Cultures (DMSZ). All cell lines were cultivated under conditions recommended by their respective depositors. The cultivation media were supplemented with 10% fetal bovine serum (FBS), and 1% antibiotics solution (Penicillin and streptomycin). The cultivation was performed at 37°C, 5% CO_2 and 99% humidity. The sub-cultivation was done each three days. Cell culture reagents were purchased from Life Technologies Inc (GIBCO BRL). The procedures can be seen in the all reference (Freshney, 1993).

4-3 Growth inhibition and MTT-Assay

The MTT assay measure the mitochondrial function and is most often used to detect loss of cell survival cell viability due to a drug.

The ease, sensitivity, rapidly, and low cost have made MTT method as one of the most widely used assay for measuring cytotoxicity effects of compounds.

Tetrazolium salt MTT ……mitochondrial activity……▶ formazan crysalts.

Growth inhibition was measured in 96-well plates. Aliquots (120 µL) of cells (10^4 cellsmL^{-1}) were incubated with serial dilution of the endophyte extracts (each 60 µL). After 5 days, cell viability was measured by the MTT assay .Brifely, 20 µL of MTT solution (5 mg/ml in Phosphate Buffer Saline (PBS)) were added to each well, and cells were incubated for 2 hours at above mentioned growth conditions. The supernant was then discarded and the formed crystals were dissolved in Isopropanol (100 µL/well). The plate was mixed for 10 minutes then the absorbance was measured at 595nm (Storeng, 1989).

4-4 Protein extraction from cultivated cells

70% confluent cells were scraped and were collected by centrifugation (1500 rpm, 5 min, and 4°C). The supernatant was discarded. Then, cell pellets were washed with PBS, and centrifuged again (1500 rpm, 5 min, and 4°C). After removing the PBS cells were lysed on ice with 0.5 ml to 1 ml MPER lysis buffer for 60 min. The lysate was then centrifuged at mix speed (14000 rpm) for 10 min. Finally, the supernatant was collected and was stored until using at -20 or -80°C (Bradford, 1976).

4-5 Bradford assay

Protein concentration was determined by using Bradford assay. The Bradford dye-binding assay is a colorimetric assay for measuring total protein concentration. It involves the binding of Coomassie Brilliant blue to protein (Bradford, 1976).

- Preparation of BSA standards

A set of standards is created from a BSA, whose concentration is known (1, 0.8, 0.6, 0.4, 0.2 and 0 mg/ml). The Bradford values obtained for the standard are then used to construct a standard curve to which the unknown values obtained were compared to determine their concentrations.

- Determination of protein concentration of cell lysate:

10 µL of each protein sample as well as the standards were mixed with 200 µL of Bradford solution in a well of 96-wellplate, and were incubated for 5 min at room temperature. The absorbance was measured at 595 nm in a plate reader. The test was done in triplicates. The average absorbance of each standard was plotted against its concentration. The produced linear curve was used to determine the protein concentration of the unknown samples (Noble, 2009).

- Drug Affinity Responsive Target Stability (DARTS) Method:

Drug Affinity Responsive Target Stability is a general methodology for identifying and studying protein-ligand interactions. The technique is based on the principle that when a small molecule compound binds to a protein, the interaction stabilizes the target protein's structure such that it becomes protease resistant. The approach is simple and advantageous because it can be performed using crude cell lysates and other complex protein mixtures (without requiring purified proteins), and uses native, unmodified small molecules. The protocol used in this study to identifying the molecular target of endophytes extracts can be summarized as follow:

Cells (10 - 15 ml 3 days old culture) were collected by centrifugation (1500 rpm, and 4°C for 5 min). The supernatant was discarded, and the cell pellet was washed one time by Phosphate buffer saline (PBS). After removing the PBS, cells were lysed with 0.5 to 1 ml MPER buffer for 15 min with shaking on ice. After 15 min cells were centrifuged for 15 min at 14000 rpm and 4 °C. The supernatant was collected, and a protein measurement was carried out using Bradford method as described above. The protein lysate was then incubated with various concentrations of endophytes extracts on ice for 2 hours. A control (undigested) was incubated with the same amount of solvent (methanol), and control (digested without compound (without drug) was also incubated only with the solvent. Then 10 µl of pronase solution were added to every 20 µl of lysate (add 1µgpronase for every 100 µg of protein in the lysate). The samples were digested with pronase for 30 min at 37°C or room temperatures. After digestion has been completed the different sample were separated with SDS-PAGE, followed by gel staining and MALDI-TOF analysis to identify protein(s) of interest.

4-6 Protein sample separation by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoreses): SDS-PAGE:

SDS-PAGE, with full name of sodium Dodecyl sulfate polyacrylamide gel electrophoreses, is the most widely used technique to separate proteins from complicated samples being present in an electric field, proteins migrate towards the negative anode inside the poly-acryl amide gel under denaturing conditions. In SDS-PAGE, the detergent SDS determine that the electrophoretic mobility of a single kind of protein is only affected by its molecular weight in the porous acryl amide gel.

The components of all buffers and reagents needed for performing SDS-PAGE are given in tables 1 to 4. The performing of gel casting and electrophoresis can be summarized as follow:

1- The glass plate sandwich was assembled. Prepare the separation gel solution was Prepared as described in table 1.
2- APS and TEMED last were added, was mixed carefully to avoid formation of bubbles.
3- Important Note. Polymerization begins as soon as APS is added to the mixture, so all subsequent actions must be performed promptly.
4- The gel solution was poured between the glass plates with a pipette; leave about 1/4 of the space free for the stacking gel. Carefully cover the top of the resolving gel with 50% Isopropanol, 0.1% SDS solution or water, and wait until the resolving gel polymerizes (about 30 min). A clear line will appear between the gel surface and the solution on top when polymerization is complete.
5- The water was discarded, Isopropanol or SDS solution. Wash gently with double-distilled water.
6- T/h0 e stacking gel solution was poured as described in table 2 (add APS and TEMED last) carefully with a pipette to avoid formation of bubbles.
7- Combs were inserted. the gel was allowed to polymerize for at least 60 min.
8- Combs were removed carefully. Put the gel was put into the electrophoresis tank, fill the tank (bottom and top reservoirs) with fresh 1X running buffer (table 4), and make sure that the gel wells are covered with the buffer.
9- Protein ladder/marker was loaded and the samples (add sample buffer (table 3) to the sample before loading into the gel 1:1).
10- An appropriate voltage was set and current depending on how many gels you run (25 mA/gel for 0.75 mm gels).
11- The electrophoresis was stopped run when the dye front reaches the bottom of the gel.
12- The gel sandwich was disassembled and was proceed with gel staining or Western blot procedures (Laemmli, 1970).

Table (4- 3) preparation separation gel (12%)

N	Substance/Buffer	Volume
1	1.5 Tris-HCL (separation buffer)PH 8,8	2ml

N	Substance/Buffer	Volume
2	Acryl amide 30%	3.2 ml
3	Distilled water	2.8 ml
4	10% SDS	80µl
5	Ammonium per sulfate	100 µl
6	TEMED	20 µl

Table (4-4) preparation stacking gel (7%)

N	Substance/Buffer	Volume
1	1.5 Tris-HCL (stacking buffer) pH 6,8	1ml
2	Acryl amide 30%	1 ml
3	Distilled water	3 ml
4	10% SDS	80µl
5	Ammonium per sulfate	100 µl
6	TEMED	20 µl

Table (4-5) preparation of sample buffer (SDS Reducing Buffer)

N	Substance/Buffer	Volume
1	Deionized water	3.55ml
2	Tris–HCl pH 8.8	1.25 ml
3	Glycerol	2.5 ml
4	10% SDS	2 ml
5	Bromophenol blue	0.2 ml
6	β-mercaptoetanol	50 µl to 950 µl

Table (4-6) preparation of Running Buffer.

N	Substance	Amount
1	Tris base	30.3 g
2	Glycine	144.0 g
3	SDS	10.0 g
4	Deionized water	add up to 1 liter

4-7 Gel Staining

After electrophoresis gels were stained in Coomassie Brilliant Blue dye to visualize the protein bands (Laemmli, 1970).

- Coomassie Brilliant Blue staining:

One gram of Coomassie Brilliant Blue (Bio-Rad) was dissolved in 1 liter of a solution containing (50%) methanol, (10%) glacial acetic acid, and (40%) H_2O. The solution was stirred for 3-4 hours and then filtered through Whatman filter paper, and finally stored at room temperature.

- Gel de-staining:

Gels were detained by gentle stirring in a solution containing 30% methanol, 10% acetic acid and 60% H2O until the background became transparent.

- Detection of tubulin by western blotting

After separation of protein by SDS-PAGE as described by 4.2.6.the proteins were then transferred onto an immune-Blot PVDF-membrane (Bio-Rad) in buffer [Tris (25 mm), glycine192mm), methanol (20%), SDS (0.05%)] in a semidry system (Bio-Rad) for 30 min (15 V) at room temperature. Residual binding site son the membrane were blocked by incubating the membrane inTris-buffered saline [TBS; Tris-HCl (20 mm, pH7.4), NaCl (0.15m،(containing nonfat dry milk (5%) and Tween-20 (0.1%)), at room temperature for 2 h. Membranes were incubated with suitable primary antibody (e.g. mouse monoclonal anti α-tubulin antibody (Sigma), diluted (1:100) at 4°C for 6 h. The blots were then washed three times in TBS and were incubated for 1 hour with anti-mouse horseradish peroxidase-conjugated (HRP conjugated) antibodies (Sigma; diluted (1:100) in blocking buffer). After washing with TBS, the protein bands were analyzed by enhanced chemiluminescence by using the Super signal West Pico Chemiluminescent Substrate System (Mahmood, 2013).

- Protein Identification by MALDI-TOF MS

Coomassie stained gel spots were excised manually, washed (3X, ddH_2O) and distained (50% ethanol/50 mM ammonium bicarbonate (NH_4HCO_3), 75% acetonitrile (CH_3CN) before drying. Gel pieces were then rehydrated and digested with trypsin (60 minutes, 10 µl of ice-cold trypsin solution (20 ng) sequencing grade modified porcine trypsin (Promega, USA) in 25 mM NH_4HCO_3 pH 8.0 at 4 ^{o}C) and then continue digestion for 16-24 h, at 37^{o}C. The hydrolysates were next transferred to a 0.5 mL tube and extraction of the digested peptides was performed in two successive steps by addition of 70% acetonitrile containing 0.1% trifluoroacetic acid. The samples were then concentrated to approximately 10 µl by vacuum centrifugation (Eppendorf, USA) and stored at -80 ^{o}C until use. The mixture of tryptic peptides (0.5 µL) derived from each protein was spotted onto a MALDI target (384 anchor chip MTP 600 µm Anchor chip; Bruker Daltonik, Germany) together with matrix (10 mg α-cyano-4-hydroxycinnamic acid (CHCA)/1 mL of 30% CH_3CN and 0.1% aqueous TFA, 0.5 µL) and left to dry before MS analysis. Spectra were acquired by MALDI-TOF MS (Ultra Flex Trem, Bruker Daltonics, Germany) in the positive mode with a target voltage of 25 kV and a pulsed ion extraction of 20 kV. The reflector voltage was set to 21 kV and the detector voltage to 17 kV. Peptide mass fingerprints (PMF) were calibrated using a peptide calibration mix standard II (mono-isotopic masses: bradykinin (1-7), m/z 757.399; angiotensin I, m/z 1296.685; angiotensin II, m/z 1046.54; rennin-substrate, m/z 1758.93; ACTH clip (1-17), m/z 2093.086; and

somatostatin, m/z 3147.471). The PMF's were processed using Flex Analysis™ software (version 2.4, Bruker Daltonics, Germany) and the SNAP algorithms were used for peak detection (S/N, 3; maximum number of peaks, 100; quality factor threshold, 30). MS spectra were recorded automatically across the mass range m/z 700-3000 and spectra were typically the sum of 400 laser shots. MS data were interpreted by using Bio Tools v3.2 (Bruker Daltonics, Germany), together with the Mascot search algorithm (version 2.0.04 updated 07/03/2012; Matrix Science Ltd., UK). Mascot search algorithm parameters were as follows: fixed cysteine modification with propionamide, variable modification due to methionine oxidation, one missed cleavage site in case of incomplete trypsin hydrolysis, and a mass tolerance of 100 ppm). Identified proteins were accepted as correct if they showed a Mascot score higher than 57. Not all spots of interest could be identified because some proteins were of low abundance and did not yield sufficiently intense mass fingerprints; other spots were mixtures of multiple proteins.

5- Results

5-1 The biological activity of the Endophyte extracts:

All the tested extracts showed a high growth inhibition effects on the tested cancer cell lines at low dilution level. However, by increasing the dilution level of the extracts the sensitivity of each cell line to the treatment varied considerably (Figure 5.1).

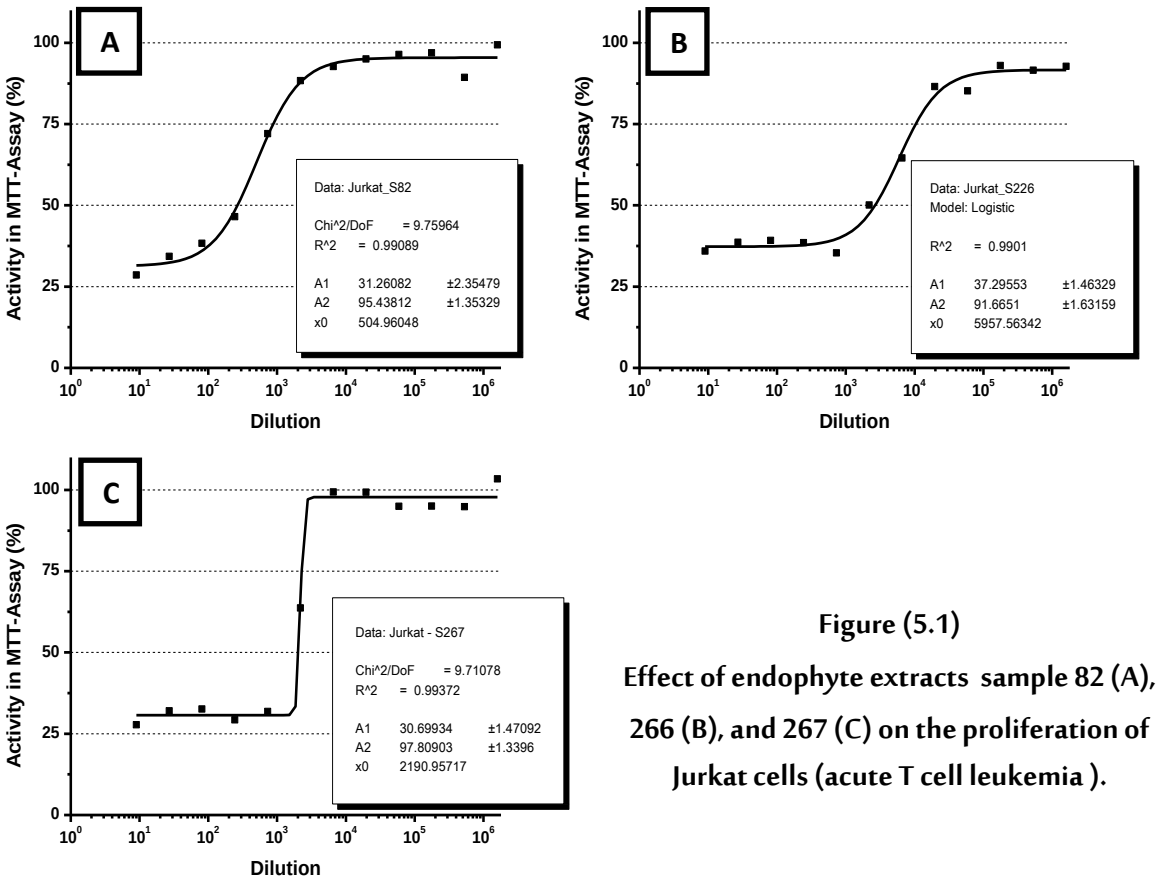

Figure (5.1)

Effect of endophyte extracts sample 82 (A), 266 (B), and 267 (C) on the proliferation of Jurkat cells (acute T cell leukemia).

Sample 266 showed the strongest growth inhibition effect on the jurkat cells. It was able to inhibit the cell growth by 50% even at dilution factor of 5957 as shown in figure 5.1.B. In contrast, the same level of growth inhibition was already reached at dilution factor of 504 and 2190 for sample 82 (Fig. 5.1A) and sample 267 (Fig. 5.1C), respectively.

In addition, the result showed that the sensitivity of lung carcinoma cell line A549 to the drug treatments was in general lower than that of the Jurkat cells (T cell leukemia) (Figure 5.2). In comparison to the Jurkat cells, here showed sample 267 the strongest growth inhibition effect. It was able to inhibit the growth of A549 cells by 50% at dilution factor of 3353 (Figure 5.2.C). In contrast the same level of growth inhibition for sample 266 was already reached at dilution factor of 1252 (Figure 5.2.B). Sample 82 showed the lowest growth inhibition effect on A549 cells. It inhibited the cell growth by 50% already at dilution factor of 41 (Figure 5.2. A), at higher dilution level no growth inhibition effect could be detected.

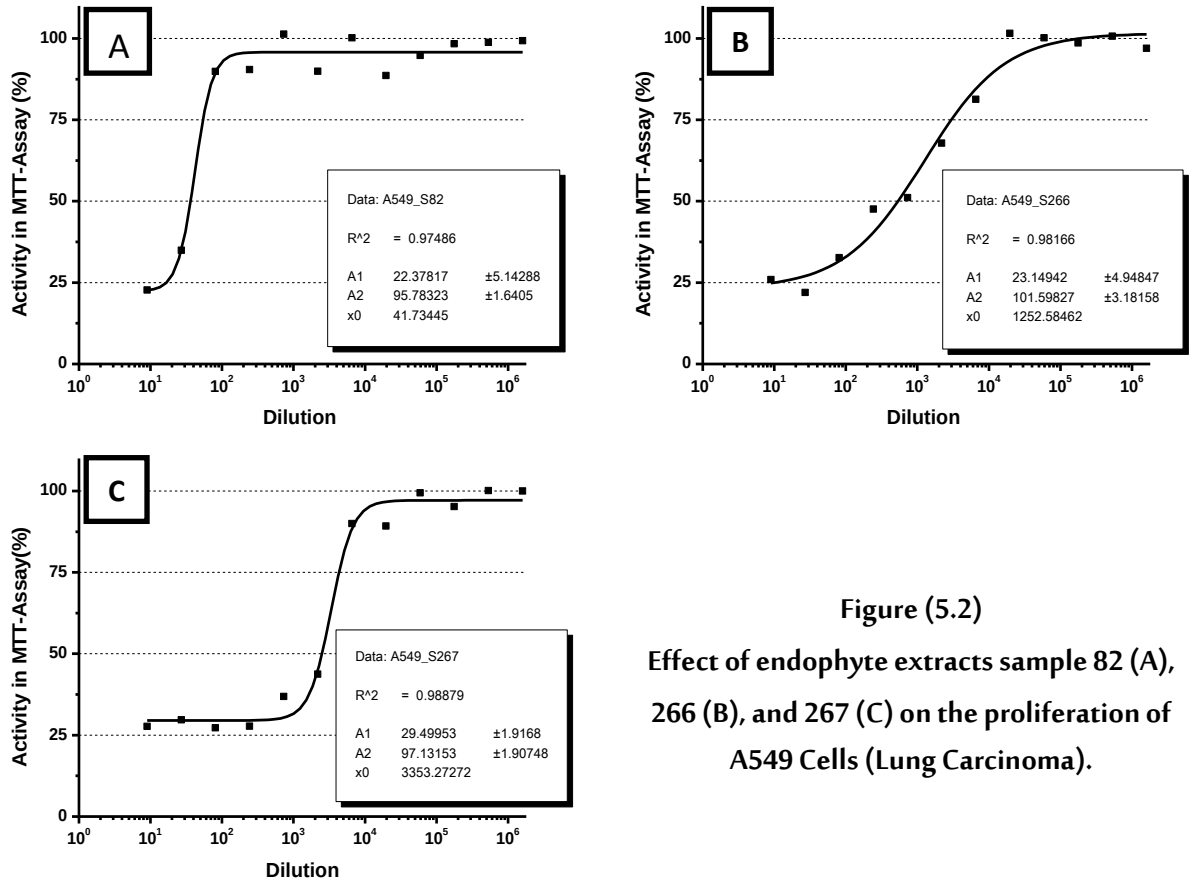

Figure (5.2)
Effect of endophyte extracts sample 82 (A), 266 (B), and 267 (C) on the proliferation of A549 Cells (Lung Carcinoma).

The growth inhibition effects of the fungal extracts were further investigated on an additional cell line (Hela229 cells, cervix carcinoma). 50% growth inhibition of HeLa229 was measured after treatment the cells with samples 82 and 266 already at dilution factors of 39 and 63, respectively. At higher dilution levels, the two drugs showed no effect on the call growth as indicated in MTT-assay (Figure 5.3. A and B). HeLa299 cells showed a higher sensitivity to sample 267, here the 50% growth inhibition was reached at dilution factor of 756 (Figure 5.3.C).

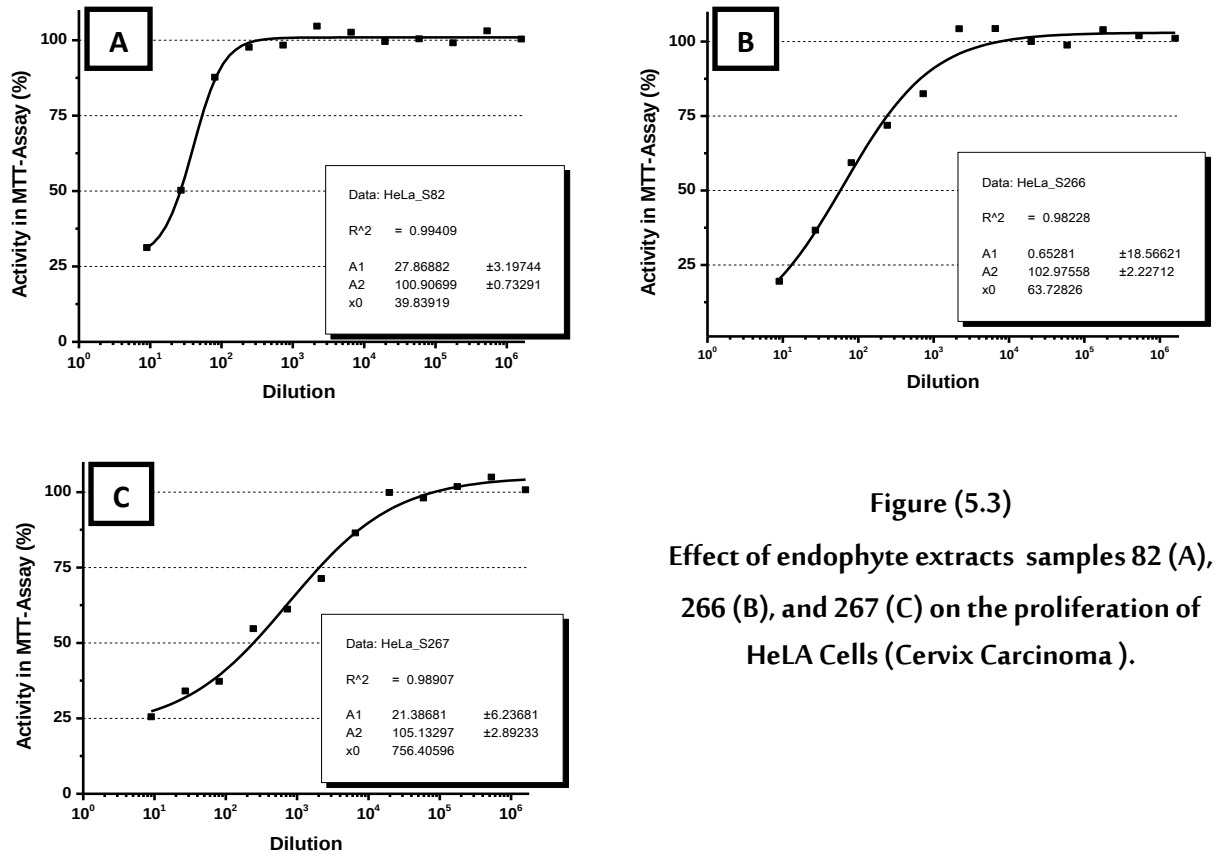

Figure (5.3)

Effect of endophyte extracts samples 82 (A), 266 (B), and 267 (C) on the proliferation of HeLA Cells (Cervix Carcinoma).

In summary the three endophyte extracts showed different growth inhibition potential on the three tested cancer cell lines. Jurkat cells seemed to be the most sensitive cells to the different treatments in comparison to the two other cell lines. Therefore, this cell line has been selected for Target identification study (see below).

5-2 Proof of principle of DARTS method using the anti-tubulin drug vincristine.

After confirming the growth inhibition effects of the endophte extracts as described under 5.1., it was aimed to use DARTS method was used to identify their molecular targets in the Jurkat cell line. To do that, it was necessarily firstly to prove the principle of the method using a drug with known molecular target. The anti-tubulin drug vincristine had been chosen to proof the principle of the DARTS method in Jurkat cells.

Vincristine is a vinca alkaloid from the Catharanthus roseus (Madagascar periwinkle), formerly Vinca rosea and hence its name. Vincristine bind to tubulin at the vinca-domain of the α-subunit of the tubulin dimer (Sackett DL, 1995) and plays a key role in the treatment of childhood acute lymphoblastic leukemia.

In order to investigate binding of vincristine to tubulin a drug affinity responsive target stability (DARTS) assay had been performed. DARTS is based on the principle that a protein is in a dynamic

equilibrium with alternative conformations. Ligand binding, mediated by hydrophobic, hydrogen bonding and/or electrostatic interactions, then favors a specific conformation so that upon saturation with a specific ligand, the equilibrium shifts towards the ligand bound conformation. This leads to a thermodynamically more stable state in which resistance to protease degradation is markedly increased (Lomenick, 2010). To test this in the context of vincristine-tubulin interaction, lysates from jurkat cells were incubated with vincristine (2 and 6 µg vinblastine/ 20 µg protein of jurkat cell lysate) or Methanol (negative control). Samples were digested with pronase and subjected to SDS-PAGE analysis as described in the method section. The produced gel was stained with coomassie brilliant blue. According to DARTS Principe, the bands of the target protein (i.e. tubulin) incubated with the drug (i.e. vincristine) should be more intense than those of the target protein incubated with methanol. As shown in Figure 5.4.A vincristine exerts a clear protection of a band around 55 KDa that corresponds to tubulin. This band is clear to see in lane 2 and 6 (vincristine treated, digested) and completely absented in lane 4 (methanol control, digested). Lanes 3 and 7 represent the protein bands of the undigested treated samples, lane 3 represents the undigested control sample, and lane 1 represents the protein ladder. To proof whether the protected band in lane 2 and 6 is tubulin the SDS gel is distained and subjected to Western Blotting using specific anti-tubulin antibodies. Anti-GAPDH antibodies had been used as control. The results of the western blotting indicate the assuming that tubulin is the tubulin is the molecular target of vincristine. As shown in figure 5.4. B (I) the tubulin band was to see in all undigested samples (lanes 3, 5, and 7). It was absent in the digested control (lane 4) and present in the digested treated sample (lane 2 and 6). For the control antibodies Anti-GAPDH, bands have been detected in all lanes except lane 4 (Figure 5.4.B (II)). It is possible that this protein was also digested after pronase treatment, which can clearly explain the absence of the band in the digested methanol control. These results indicate that tubulin is a molecular target of vincristine and it indicate also that the method might be suitable to identify the molecular targets of endophyte extracts (see below).

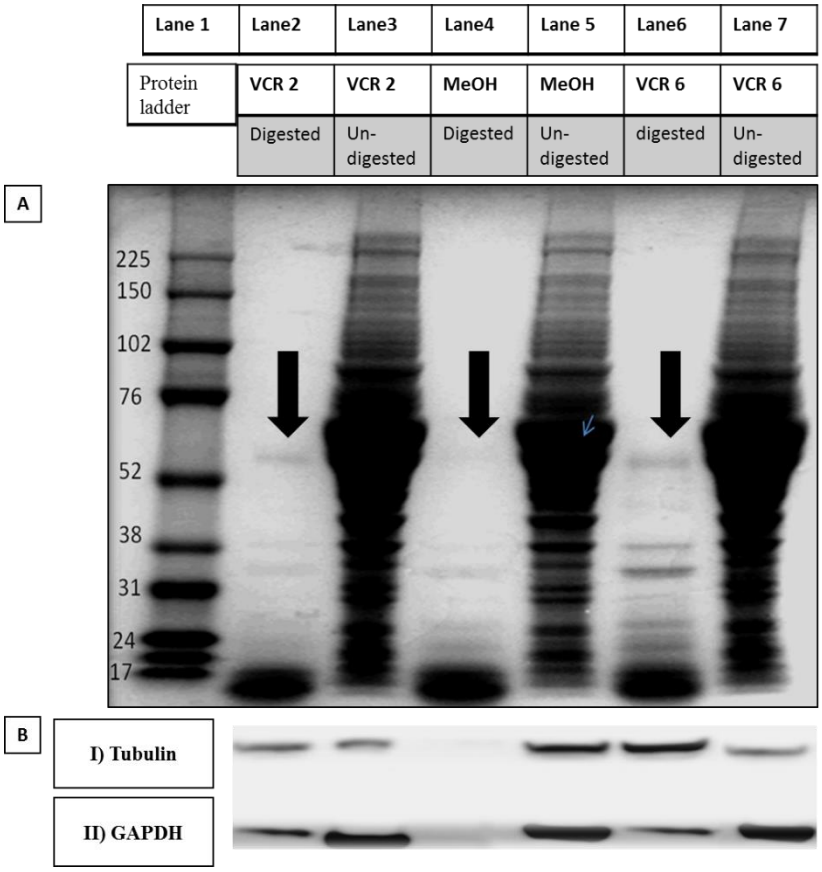

Figure 5.4: DART experiment to proof the molecular target of vincristine (VCR). A) SDS-PAGE analysis, B) Western Blotting analysis. Lysates from Jurkat cells were treated with methanol (MeOH) or incubated with different concentration of vincristine (VCR) and digested with Pronase. Samples were loaded on a SDS-PAGE gel and developed by Western Blot (see Materials and methods). Lane 1: protein standard ladder, Lane 2: Digested VCR treated cell (2 µg/20 µg protein of cell lysate), Lane 3: Undigested VCR treated cell (2 µg/20 µg protein of cell lysate); Lane 4: Digested methanol control, Lane 5: undigested methanol control, Lane 6: Digested VCR treated cell (6 µg/20 µg protein of cell lysate), Lane 7: Undigested *VCR treated cell (6 µg/20 µg protein of cell lysate). Abbreviations: VCR = vincristine, and MeOH = Methanol.*

5-3 Using of DARTS and MALDI-TOF analysis to identify the molecular target of the bioactive endophytes' extracts

After confirming the bioactivities of the endophyte extracts as described under 5.1., and also after proofing the DART method to identify the molecular target of a Drug of known mechanism of action as described under 5.2, the last step in this study was to use the DARTS technique to identify the molecular targets of 3 bio-active endophytes extracts. To test this lysate from jurkat cells were incubated individually with suitable amount of the three endophytes extracts (6 µl extracts/ 20 µg protein of jurkat cell lysate) or Methanol (negative control, 6 µl methanol/ 20 µg protein of jurkat cell lysate). After incubation time (see method section), samples were digested with pronase and subjected to SDS-PAGE analysis as described in

the method section. The resulting gel was stained with Coomassie brilliant blue to visualize the protein bands. As shown in figure 5.5. All undigested samples (lanes 3, 5, 7 (treated), and 9 (control)) show the normal distribution of the protein bands of the whole cell lysate, however the digested sample show different band distribution. In the digested control sample (lane 8) there are a number of bands between 38 and 17 KD (kilo Dalton), these bands were also observed in all the treated digested samples (lanes2, 4, and 6). Lane 8 (digested control sample) showed also a band in the molecular weight range between 52 KD and 67 KD, this band was also observed in digested treated samples 266 and 267 (lanes 2, and 4 respectively) and was absent in digested treated sample 82 (lane 6). Digested Sample 82 (treated) showed a band in the molecular weight range between 67 and 102 KD (lane 6), this band was also observed in digested treated sample 276 (lane 4), but was absent in lane 2 (sample 266) and lane 8 (control). Digested sample 267 (lane 4) showed many bands in the molecular weight range between 38 KD and 67 KD, some of them are not available in the digested control sample (lane 8). None of the test sample showed a single band.

To identify possible molecular targets for the bioactive endophytes extracts, Bands of interest (see arrows figure 5.5) have been cut from the gel, destined, digested with trypsin and analyzed using MALDI-TOF MS (UltraFlexTrem, Bruker Daltonics, Germany) in the positive mode with a target voltage of 25 kV and a pulsed ion extraction of 20 kV as described in the method section.

MS spectra were recorded automatically across the mass range m/z 700-3000 and spectra were typically the sum of 400 laser shots. MS data were interpreted by using Bio Tools v3.2 (Bruker Daltonics, Germany), together with the Mascot search algorithm (version 2.0.04 updated 07/03/2012; Matrix Science Ltd., UK). Identified proteins were accepted as correct if they showed a Mascot score higher than 57.

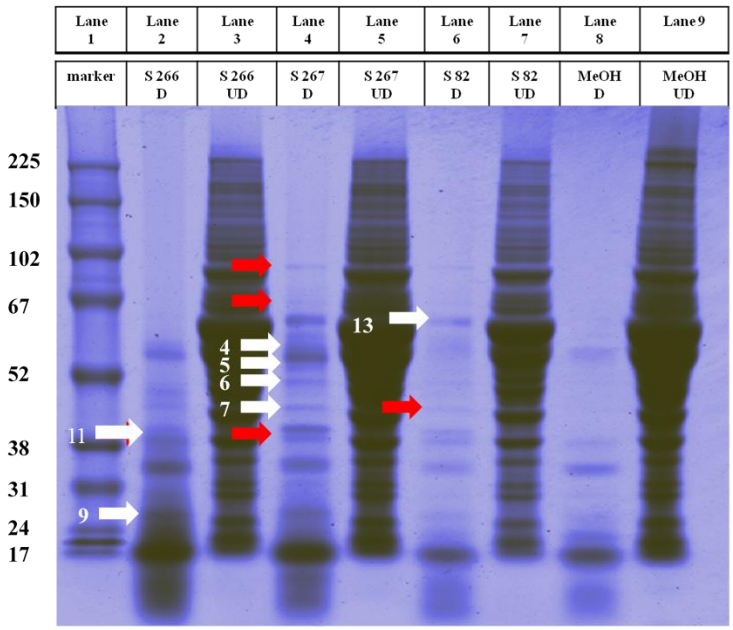

Figure 5.5: DARTS experiment to identify the molecular targets of sample 82, 266 and 267.Lysates from Jurkat cells were treated with methanol (MeOH) or incubated with the endophytes extracts and digested with Pronase. Samples were loaded on a SDS-PAGE gel and developed coomassie blue staining. Lane 1: protein standard ladder, Lane 2: Digested sample 266 treated. Lane 3: Undigested sample 266 treated Lane 4: Digested sample 267 treated, Lane 5: Undigested sample 267 treated, Lane 6: Digested sample 82 treated, Lane 7: Undigested sample 82 treated, Lane 8: Digested methanol control, Lane 9: Undigested methanol control. Abbreviations: S = sample, MeOH = Methanol, D = digested, UD =, undigested. White arrows = identified proteins, red arrows = none identified proteins.

Not all bands of interest could be identified because some proteins were of low abundance and did not yield sufficiently intense mass fingerprints; other bands were mixtures of multiple proteins. seven proteins could be identified (white arraows in figure 5.5) and 5 proteins proteins that could not (red arrows in figure 5.5), these proteins are summarized in table 5.1.

Table (5.1) the identified protein after MALDI-analysis and peptide mass fingerprinting.

Spot No	Accession No	MASCOT ID	Protein Name	Pi	MW**	Score	The importance of protein
4	B3KS09	B3KS09_ HUMAN	cDNA FLJ35234 fis	8.19	50276	72	I could not find any information about the nature and function of this protein.
5	P52179	MYOM1_ HUMAN	Myomesin-1	6.51	187509	70	it is a component of cell cytoskeleton.
6	Q92783	STAM1_ HUMAN	Signal transducing adapter molecule 1	4.70	59142	62	It is a protein that in humans is encoded by the STAM gene. This gene was identified by the rapid tyrosine-phosphorylation of its product in response to cytokine stimulation.
7	Q9BXF9	TEKT3_ HUMAN	Tektin-3	6.93	56601	67	Tektins are microtubule-associated cytoskeletal proteins.
9	Q8NEZ3	WDR19_ HUMAN	WD repeat-containing protein 19	5.94	151483	65	cellular functions, including transmembrane signaling mRNA modification,
11	Q96N23	CL055_ HUMAN	Uncharacterized protein C12orf55	7.84	92263	61	I could not found any information about this protein in the literature.
13	Q13489	BIRC3_ HUMAN	Baculoviral IAP repeat-containing protein 3	5.71	68328	61	proteins that inhibit apoptosis.

*Protein Identification **Molecular Weight

Although some protein bands could not be identified, but many very interesting proteins were identified as possible targets for the bioactive endophyte extracts.

For sample 82, a protein named Baculoviral IAP repeat-containing protein 3 was identified as a possible target protein of this extract (band 13, lane 6, figure 5.5). Baculoviral IAP repeat-containing protein 3 belongs to IAP family of proteins that inhibit apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2, probably by interfering with activation of ICE-like proteases. The protein inhibits apoptosis induced by serum deprivation but does not affect apoptosis resulting from exposure to menadione, a potent inducer of free radicals. It contains 3 baculovirus IAP repeats and a ring finger domain (Liston, 1996).

For sample 266 a protein named WD repeat-containing protein 19 was identified as a possible cellular target of the extract (band 9, lane 2, Figure 5.5). This protein belongs to the WD repeat protein family. WD-repeat proteins are a large group of structurally related proteins that participate in a wide range of cellular functions, including transmembrane signaling, mRNA modification, vesicle formation, and vesicular trafficking. The WDR19 gene comprises 36 exons and is located on chromosome 4p15-4p11. The predicted protein contains six WD repeats, a clathrinid heavy-chain repeat, and three transmembrane domains. Sequence analysis reveals that the WDR19 gene is conserved from Caenorhabditis elegans to human. WDR19 is expressed in normal and neoplastic prostate epithelium as demonstrated by RNA in situ hybridization and is regulated by androgenic hormones. WDR19 transcripts exhibit alternative splicing in which two is forms appear to be prostate restricted, a property that could be exploited for designing diagnostic or therapeutic strategies for prostate carcinoma. Therefore, this protein has been reported to be a tissue marker for prostate cancer (Biaoyang Lin,2008). which makes the endophyte extract sample 266 and its components very interesting to study the function of this protein in a future work. An additional protein named uncharacterized protein C12orf55 could be also identified this extract (lane 2, figure 5.5), however I could not find any information about this protein in the literature.

For sample 267 four proteins could be identified. These proteins seem to be protected after treatment the cell extract with sample 267. The first protein was cDNA FLJ35234 fis. I could not found any information about the nature and function of this protein. The second protein was Myomesin-1. It is a protein that in humans is encoded by the MYOM1 Gene, and it is a component of cell cytoskeleton (Pinotsis, 2012).The 3rd protein is tektin-3. Tektins are microtubule-associated cytoskeletal proteins that are expressed primarily in the male germ cell-lineage in centrioles and basal bodies and within ciliary and flagellar doublet microtubules (Roy, 2004) . Other functions are also known for tektin proteins. It has been reported that Tektin 2 (e.g.) associates with the spindle poles throughout mitosis, organizes the spindle midzone microtubules during anaphase, and assembles into the midbody matrix surrounding the compacted midzone microtubules during cytokinesis . These results suggest that sample 267 might

interact with cytoskeletal elments of treated cells. The last protein in this group is signal transducing adapter molecule 1. It is a protein that in humans is encoded by the STAM gene. This gene was identified by the rapid tyrosine-phosphorylation of its product in response to cytokine stimulation. The encoded protein contains an SH3 domain and the immunoreceptor tyrosine-based activation motif (ITAM). This protein associates with JAK3 and JAK2 kinases via its ITAM region, and is phosphorylated by the JAK kinases upon cytokine stimulation, which suggests the function of this protein is as an adaptor molecule involved in the downstream signaling of cytokine receptors. HGS/HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) has been found to bind and counteract the function of this protein (Takeshita, 1997).

In summary: it was possible for first time by using DARTS followed by MALDI-TOF analysis and peptidmass fingerprinting to identify a number of cellular target molecules for bioactive extracts of endophytes. A number of very interesting proteins could be identified that might stimulate the researchers in a future study to the chemical composition of these extracts to exactly identify the active component in each.

6- Discussion

Developing new methods for drug target identification is an area of intense interest, and both experimental and computational approaches have been developed (Terstappen, 2007). In the past many methods and techniques have been developed, but many limitations remain. Traditionally, affinity chromatography has been used for the process of target identification. However, the most limitation of this method was the modification of the chemical structure of the drug to enable an affinity purification step, which lead in many cases to lose the biological activity of the drug or at least to minimize it. In addition to affinity chromatography, many new methods for drug target identification have been developed, ranging from biochemistry to genetics, proteomics, and imaging (Ong, 2009). However, affinity chromatography remains the most widely used method for drug target identification. The typical project begins with structure–activity relationship studies in which various functional groups of the small molecule of interest are modified or removed to determine which one(s) are dispensable for drug activity (Lomenick, Olsen and Huang, 2011). These nonessential site(s) are then used as points of attachment for an affinity tag (e.g., biotin). Then, similar to immunoprecipitation of specific proteins using an antibody and conjugated beads, the drug-linked beads are incubated with protein extracts, followed by extensive washing to remove nonspecifically bound proteins (Sato et al., 2010). Finally, the tightly bound proteins are eluted with excess free drug or under highly denaturing conditions. Most often the eluted proteins are subsequently analyzed by SDS– PAGE and protein bands are identified by mass spectrometry.

Like affinity chromatography, DARTS relies on the affinity between a drug molecule and its protein target and thereby is able to pinpoint direct binding partner(s) of the drug. The key advantage of

DARTS, however, is that it does not require labeled ligands and instead uses "native" (i.e., unmodified) small molecules for binding, it is not limited by chemistry and can potentially be used to identify binding targets for any small molecule (Lomenick et al., 2009). Additionally, unlike cell-based methods, DARTS is completely independent of any effects of the drug on the system, and is therefore compatible with any mechanism of action, making it useful for any small molecule of interest. Moreover, DARTS can be performed by using any cell or tissue type from any organism and is thus not limited by the availability and coverage of knockout (or knockdown) libraries and genome arrays for model organisms (Roti and Stegmaier, 2012).

DARTS proves to be a fast and robust method to determine direct binding of a small molecule (or metabolite) without requiring large amounts of pure protein and is even amenable to using whole-cell lysates.

7- Conclusion

The key question of this study was to test whether it is possible to use DARTS for screening of biologically active extracts of some fungal endophytes. The expected difficulties here was that these extracts containing usually more than one substance, which might disrupt the concept of DARTS (depends on binding of small purified drug molecule, and not substance mixture, with its target protein). In MMT-assay I screened the biological activities of number of fungal endophyte extracts that have been provided by Dr. T. S. Suryanarayana (Vivekananda institute of tropical mycology – India). Three of these extracts showed robust growth inhibition effects on number of cancer cell lines as shown in results section. These extracts were applied then to DARTS followed by MALDI-TOF analysis and peptide mass fingerprinting to identify their cellular target. As shown in results section, it was possible using this technique to identify the protein targets of the extracts in Jurkat cell line. A number of interesting proteins could be identified. This result will open a new horizon for DARTS applications on the field of drug screening. Because it becomes possible now and before starting the chemical analysis of the biologically active extract to get an idea about the possible proteins target included in these extracts, which finally will help the investigator to decide to start a chemical analysis or not. These will save a lot of time and efforts. However, the success of the method has also some limitations. The target protein could either not be sufficiently abundant in the cell to be visibly stained, or even if it is abundant enough to see, its enrichment in one sample over another could be masked because the protein co-migrates with many other proteins of the same molecular weight on the gel. If just one of these co-migrating proteins is much more abundant than your target protein, the fact that it is present at the same amount in both samples will hide the fact that the target protein is highly enriched in one sample. Moreover, the presence of several low or moderately abundant proteins could just as easily mask the differential abundance of the co-migrating target protein. Therefore, additional modifications are still needed to increase the sensitivity of the method.

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