STSM in Fundación MEDINA – Report

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Purpose of the STSM

There were two main objectives of the STSM:

1. Getting to know the research techniques used in Fundación MEDINA
    - To extend knowledge about the research methods concerning screening of bioactive compounds from endophytic fungi
    - To see how microorganisms are cultivated to be used to explore the bioactive compounds
    - To see how the fermentations obtained are extracted using innovative methods
    - To see how screening of extracts is carried out to select those with components of interest
    - To participate in the characterization of the compounds by using various analytical and spectrometric technologies
2. Investigate the bioactivities of three endophytic fungi extracts and to try to identify the main components of the extracts
    - I had 3 endophytic fungi (*Acephala applanata (A)*, *Phialocephala fortinii (R)* and *Humicolopsis cephalosporioides (S16)*) extracts originally isolated from Finnish Forests with me in Fundación MEDINA that had been found to contain interesting bioactivities in previous tests. The aim was to test the extracts with the methods available in Fundación MEDINA to learn more valuable information of the research techniques used and to obtain as much information about the extracts as possible.

Description of the work carried out during the STSM

During the stay I was able to become familiar with daily work carried out the departments of Fundación MEDINA. I stayed several days in each of them and worked with research techniques used in every laboratory.

Fungi laboratory

I was able to see the methods used to cultivate different endophytic fungi from materials obtained from nature and to try some of them. I isolated endophytic fungi from growths in solid agar-medium and directly from the surface of the plant. I could also participate in preparing growth medium in use at the Fundación MEDINA and transferred growths from solid media to liquid for larger scale production of interesting fungal samples.

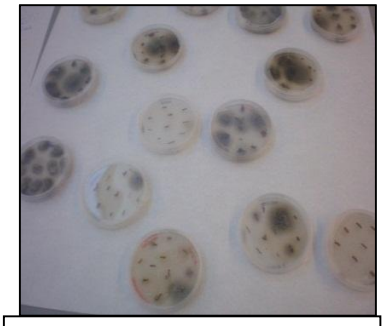

Fig.1 Endophytic fungi cultivation from plant material

Fig.2 *Actinomycetes* bacteria in solid growth medium

Prokaryotes laboratory

I was introduced to Duetz -technique for microfermentation and to the principles and equipment used in co-cultivation. I got to help in the antimicrobial testing of some marine extracts and to detect contamination from bacterial growths in solid medium. The cultivation and large scale biofermantation procedures were also introduced.

Sample preparation

I was able to see and follow the procedures used in Fundación MEDINA for sample preparation and extraction and to witness the sample fractionation process using solid phase extraction resin.

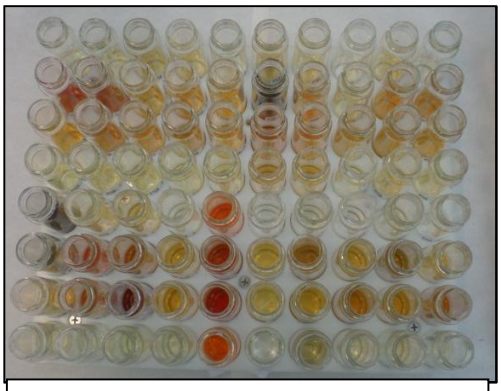

Fig. 3 Samples are first prepared into EPA-vials before transferring to 96-well plates

Screening department – Microbes

I tested antimicrobial activity of the endophytic fungi extracts I had brought with me. With this aim, I dissolved some of the lyophilized fungi extracts in sterile water so that the concentration was 10 mg /ml. I then prepared 10 different dilutions of the extracts with each containing 50 % of the concentration of the previous one. The samples were tested in twofold series of dilutions for their activity against both *Acinetobacter baumannii* (gram-negative bacteria) and Methicillin Resistant *Staphylococcus aureus* (gram-positive bacteria). The controls used were rifampicin (25 µg/ml) – amphotericin B (62,5 µg/ml) for gram-negative bacteria and vancomycin for gram-positive bacteria. Each microplate well contained 10 µl of the extract or the control solution and 90 µl of the bacterial inocula used (5×10^5 cfu / ml for the gram-negative bacteria and $1{,}1\times10^6$ cfu / ml for the gram-positive bacteria). The plate was shaken and the absorbance was measured at the starting time (T_0). Plates were then sealed into plastic bags and incubated at 37ºC for overnight. Next morning, absorbance was then measured again for final time (T_f). Previously the assay plate was slightly shaken.

I was also briefly introduced to the reazurin → resorufin –assay [1].

Screening department – Cell lines

In the cell line screening department the cytotoxicity and activity against Parkinson´s decease [2] was detected from the extracts I had brought with me.

Cytotoxicity screening was done by using a hepato-carcinoma cell line (HepG2). I used the same sample dilutions as in bacterial screening department. HepG2 cells were first pipetted into two clear microplates. Numbers of cells per culture well were 1,25 M cells/ml for the 24 h assay 1 M cells/ml for the 72 h assay. Cells were incubated overnight at 37 °C, 5 % CO_2. Then the medium was removed and replaced by 195 µl of fresh medium. Five µl of each sample dilution were pipetted into the plates in twofold series along with control samples. As a positive control MMS (Miracle Mineral Supplement, 28 % of $NaCl_2$ in distilled water) was added and water was used as a negative control. Also Doxorubicin was added as a control series of 8 dilutions with 250 µM as the strongest dilution. The plates were incubated at 37 °C, 5 % CO_2. The 24 h plate was read the next day and the 72 h plate two days later. The medium was replaced before the measurement and 100 µl of PBS was added. The plates were then emptied again from the medium and 100 µl of MTT – Thiazol Blue was added (0,5mg/ml) after which the plates were incubated at 37 °C, 5 % CO_2 for 3 hours. Then the plates were again emptied from the medium and 100 µl of DMSO was added. The plates were then measured with Victor Multilabel equipment for their absorbance at 540 nm. If the cells are healthy they appear blue instead of clear which indicates that the cells are dead and the samples are cytotoxic.

For the screening for activity against Parkinson's decease a cell line SHSYSY was used. Cells were first pipetted into black microplates. Numbers of cells per culture well were 4 M cells/ml and 1 M cells/ml. Cells were incubated for 42 h at 37 °C, 5 % CO_2. Then the medium was removed and replaced by 195 µl of fresh medium. Five µl of each sample dilution were pipetted into the plates in twofold series

along with 20 % DMSO as a control sample after which the plates were incubated at 37 °C, 5 % CO_2 for 3 hours. Then the medium was removed and replaced by 195 µl of fresh medium with 5 µl of 20 µM Rotenone. The plates were incubated overnight at 37 °C, 5 % CO_2.The plates were then again emptied and Hoechst 33342, YO-PRO-1 and Propidium Iodide (PI) dyes were used simultaneously to provide information about the permeability of membranes. The green-fluorescent dye YO-PRO-1 enters the apoptotic cells, where-as the red-fluorescent PI cannot. PI can only enter if cells are already dead. Blue-fluorescent Hoechst 33342 stains brightly the condensed chromatin of apoptotic cells compared to the live cells. Into the plate with 4 M cells/ml we added only 100 µl of 5 µM YO-PRO-1 and Propidium Iodide solution and into the plate with 1 M cells/ml also Hoechst was added. Plates were then incubated at 37 °C, 5 % CO_2 for 40 minutes after which they were emptied from medium and 100 µl of PBS was added for rinsing. Plates were emptied from the PBS and another dose of 100 µl of PBS was added. Measurement was the carried out with $FLIPR_{TETRA}$ by using the 491 nm/509 nm excitation/emission wavelengths for YO_PRO-1, 535 nm/617 nm for PI, and 350 nm/461 nm for Hoechst 33342.

Chemistry laboratory

In the chemistry department the purpose was to identify the main components of the extracts indicating activity. We used Agilent 1200 RR-Bruker maXis LC/MS ESI-TOF for High resolution mass spectroscopy measurements and Bruker Avance III 500 MHz NMR equipped with a micro-cryoprobe for the structural determination of the compounds. I dissolved some of the lyophilized fungi extracts into deionized water so that the concentration was 1 mg/ml and later 10 mg /ml. Because we had used boiling water as the extraction solvent for the fungal mycelium, also primary metabolites such as arginine, mannitol and adenosine were found from the extracts. In addition, also several unknown components were observed in the extracts. To identify these, the next step is to separate fractions with preparative-HPLC and to test the bioactivity of the fractions followed by further chemical analysis. For the NMR more purified samples would have been needed in order for the structural determination.

I was also introduced to sample fractionating techniques with using HPLC and to the general compound identifying process done in the laboratory.

Description of the main results obtained and future collaboration with host institute

Out of the three extracts I had with me none showed antimicrobial activity against neither of the bacterial strains. In the cell line screening department it was wound that the extracts did not show cytotoxicity and one of the fungal extracts indicated minor activity in the test against Parkinson's decease. This active extract was *Acephala applanata (A)* and it was agreed that the test would be carried again with higher concentrations of the extracts to confirm the activity in Fundación MEDINA as there was not enough time for me to rerun the test.

During the work carried out in chemistry department we were able to find some interesting unidentified peaks from the LC-HRMS grams. If in future tests after fractionation these fractions turn out to be bioactive I can send them to Fundación MEDINA and they will try to identify the compounds responsible for the activities found.

References:

1 Monteiro MC, de la Cruz M, Cantizani J, Moreno C, Tormo JR, Mellado E, De Lucas JR, Asensio F, Valiante V, Brakhage AA, Latgé JP, Genilloud O, Vicente F (2012) A new

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2 de Pedro N, Cautain B, Melguizo A, Vicente F, Genilloud O, Peláez, F, Tormo, JR (2013) Mitochondrial complex I inhibitors, acetogenins, induce HepG2 cell death through the induction of the complete apoptotic mitochondrial pathway.J Bioenerg Biomembr 45:153 – 164