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Саратовский государственный университет
(СГУ)**

**Heinrich-Heine-Universität Düsseldorf**

# Isolation and identification of some secondary metabolites from associated apple plant fungus *Aspergillus tubingensis*

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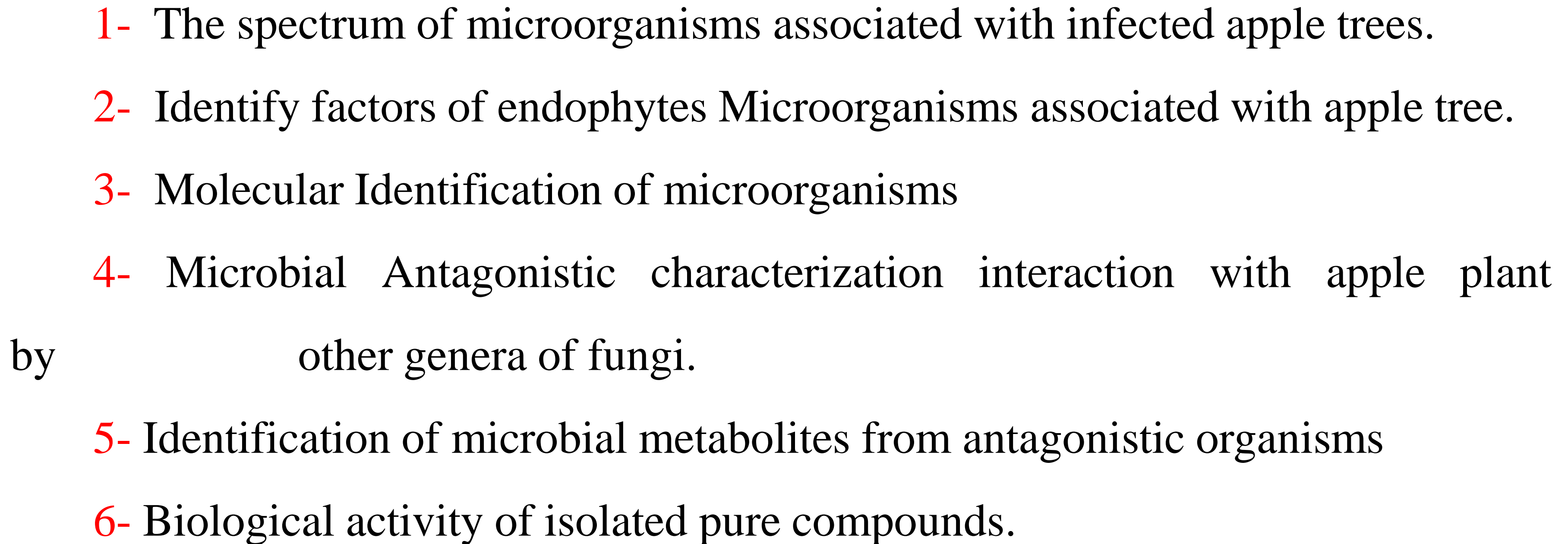
# Scope of the present study



1- The spectrum of microorganisms associated with infected apple trees.

2- Identify factors of endophytes Microorganisms associated with apple tree.

3- Molecular Identification of microorganisms

4- Microbial Antagonistic characterization interaction with apple plant by other genera of fungi.

5- Identification of microbial metabolites from antagonistic organisms

6- Biological activity of isolated pure compounds.

# Introduction

Secondary metabolites in most fungi are chemically diverse and are, among others, comprised of unusual nucleosides, terpenes, peptides, alkaloids, nonribosomal peptides and polyketides **Liu, Z. M *et al* 2003**. Many studies have reported on the isolation of marine sponge-associated *Aspergillus* spp. as producers of bioactive metabolites **Höller, U *et al* 2000**. which have cytotoxic activity against a panel of tumor cell lines. And described the biological active metabolites produced by the fungus *A. versicolor* associated with the South China Sea sponge **Holoxea sp. and Cohen *et al*. 2014.**

The fungal cell wall is comprised of a mix of cross-linked fibres (mainly the polysaccharides glucan and chitin) and matrix components, primarily proteins and mannans. In filamentous fungi, growth and cell wall assembly occur mainly at hyphal apices, where the carbohydrate polymers are synthesized by membrane-associated enzymes, some of which are transported within vesicles to their site of activity. The polymers are then cross-linked and modified by extracellular proteins.

The requirement of a functional cell wall for survival, growth, development and pathogenicity of fungal species makes it an attractive target for antifungals, especially due to the fact that some of the constituents of the fungal cell wall are not present in potential hosts **Osherov, N and Yarden, O. 2010.** Examples of fungal cell wall biosynthesis inhibitors include the peptide nucleoside antibiotics like polyoxins, used to inhibit chitin synthases **Beauvais, A. and Latge, J.P. 2001.**

In this study, Among the secreted extract components, six dimeric naphtho-g-pyrones, named

**1- Fonsecin** **2- Pyranonigrin A**

**3- TMC 256 A1** **4- Tensidol A**

**5- Tensidol B** **6- Asperazine**

All of these compounds were isolated from apple associated endophytic fungus *Aspergillus tubingensis* (AN103).

# MATERIALS AND METHODS

## Isolation of microorganisms from apple shoots:-

### Collection of plant samples:

Samples of apple plant (*Malus domestica*) were collected from different locations in Saratov city and different apple types, Samples consisted of three groups: group I, Uwealth **Уэлсь;** group II, Golden delicious **Голден делешясь;** group III, Perkytofka **Берктовка,** the samples were collected in clean plastic bags. Plant material surface, their aphids and all other debris were first removed and transferred to laboratory until the isolation procedures for microorganisms was conducted.

### Surface sterilization of the plant material:

The method most frequently utilized to detect and quantify endophytes involves isolation from surface-sterilized host plant tissues. Healthy plant material was first cleaned by washing several times mechanically under running tap water and then cut into small segments. Isolation procedures carried out under aseptic conditions. Surface sterilization was performed by sequentially soaking the plant material within 70% ($C_2H_5OH$) for 5 min, followed by immersion in NaOCl (Sodium hypochlorite), For 30 Min., They were then rinsed 2-3 times in sterile NaCL (0.90%) to clear them of microorganisms, and to detect internal microflora.

## Isolation of microorganisms from external surface parts

After proper drying, in case of healthy plants the surface sterilized plant material i.e stems 8-10 cm long, are cut vertically into small segments to expose the surface and then inoculated on the NA medium for bacteria and potato dextrose agar (PDA) media for fungi. But in case of infected plant materials, to isolate microorganisms we applying 10 sterile swabs to take inoculums from 10 plant material then added to sterile 10 test tubes containing 1 ml NaCl (0.90%), mix swab well in these amount of saline, 0.1 ml from mixture was streaked on the NA medium for bacteria and (PDA) for fungi, and diluted to $1:10^{-2}$ , $1:1^{-4}$ and the plates were incubated at 28°C for 48-72 hrs. Bacterial and Fungal colonies that appeared frequently and looked morphologically different were randomly selected and purified. Each isolate was stored in slants and keeping in refrigerator at 4 ºC for next use.

## Isolation of microorganisms from internal tissues

To eliminate external contamination, each stem segment was sterilized within 70% ($C_2H_5OH$) for 5 min, followed by immersion in NaOCl, For 30 Min. The samples were then washed three times in sterile NaCl (0.90%), 0.1 g from each plant material segment added to 0.1 ml sterile saline solution (0.90%), and transferred

aseptically into a sterile mortar and grinding with a sterile pestle, 0.1 ml from mixture was streaked on the NA medium for bacteria and potato dextrose agar (PDA) media for fungi, and diluted to $1:10^{-2}$ and $1:1^{-4}$ and the plates were incubated at 28°C for 48-72 hrs.

## Molecular Identification of Associated apple plant fungus *A. tubingensis:*

Fungal strains were also identified using a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (**ITS**) region. ITS 1 (with base sequences: TCCGTAGGTGAACCTGCGG) and ITS 4 (with base sequences: TCCTCCGCTTATTGATATGC) This was carried out at the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf, Germany.

## Antagonistic activity *in vitro*:

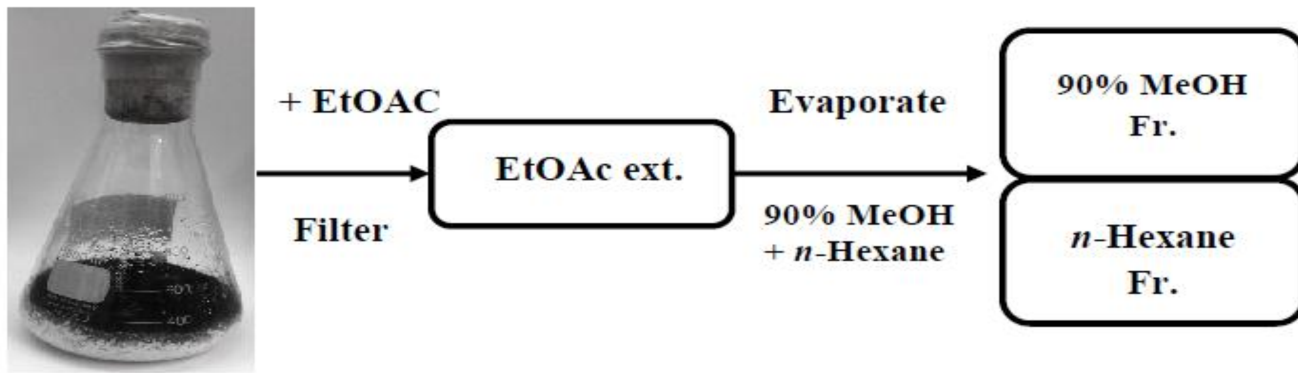
The assay for antagonism was performed on PDA on Petri dishes by the dual culture method (Fokkema, 1978). The mycelial plugs (5 mm diameter) of pathogens and fungal antagonists were placed on the same dish in opposite position from each other. To test for antagonistic bacteria, on Petri dish containing PDA medium. The dishes were incubated at 28 °C for 3-5days. The experiment was repeated twice with three replications of each treatment.

## Cultivation and isolation of secondary metabolites:

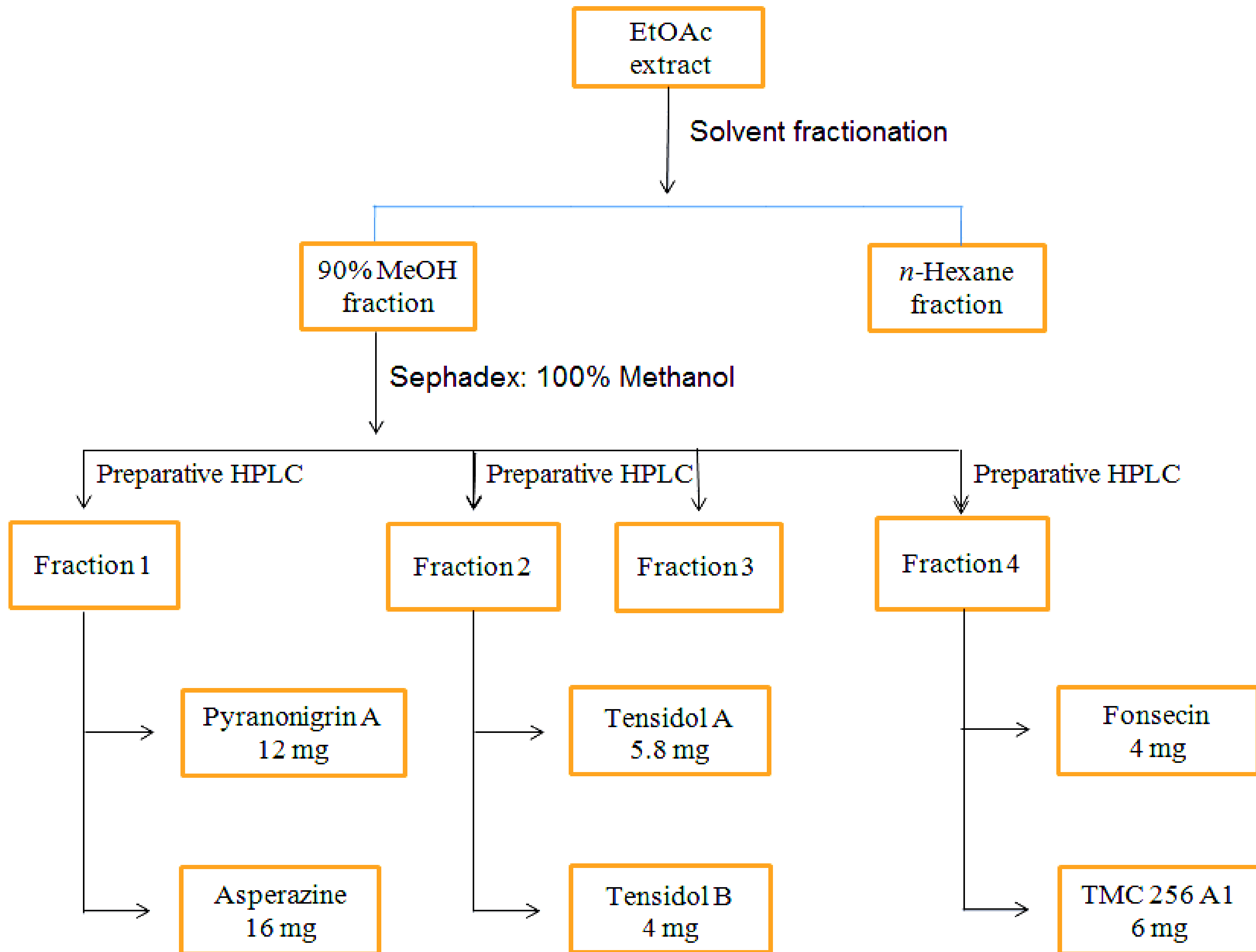
Mass growth of pure fungi for isolation and identification of secondary metabolites was carried out by transferring fresh fungal culture into Erlenmeyer flasks (1L each) containing 100 g rice for solid cultures. The cultures were then incubated at room temperature (no shaking) between 21 and 30 days.

## Extraction of solid rice cultures

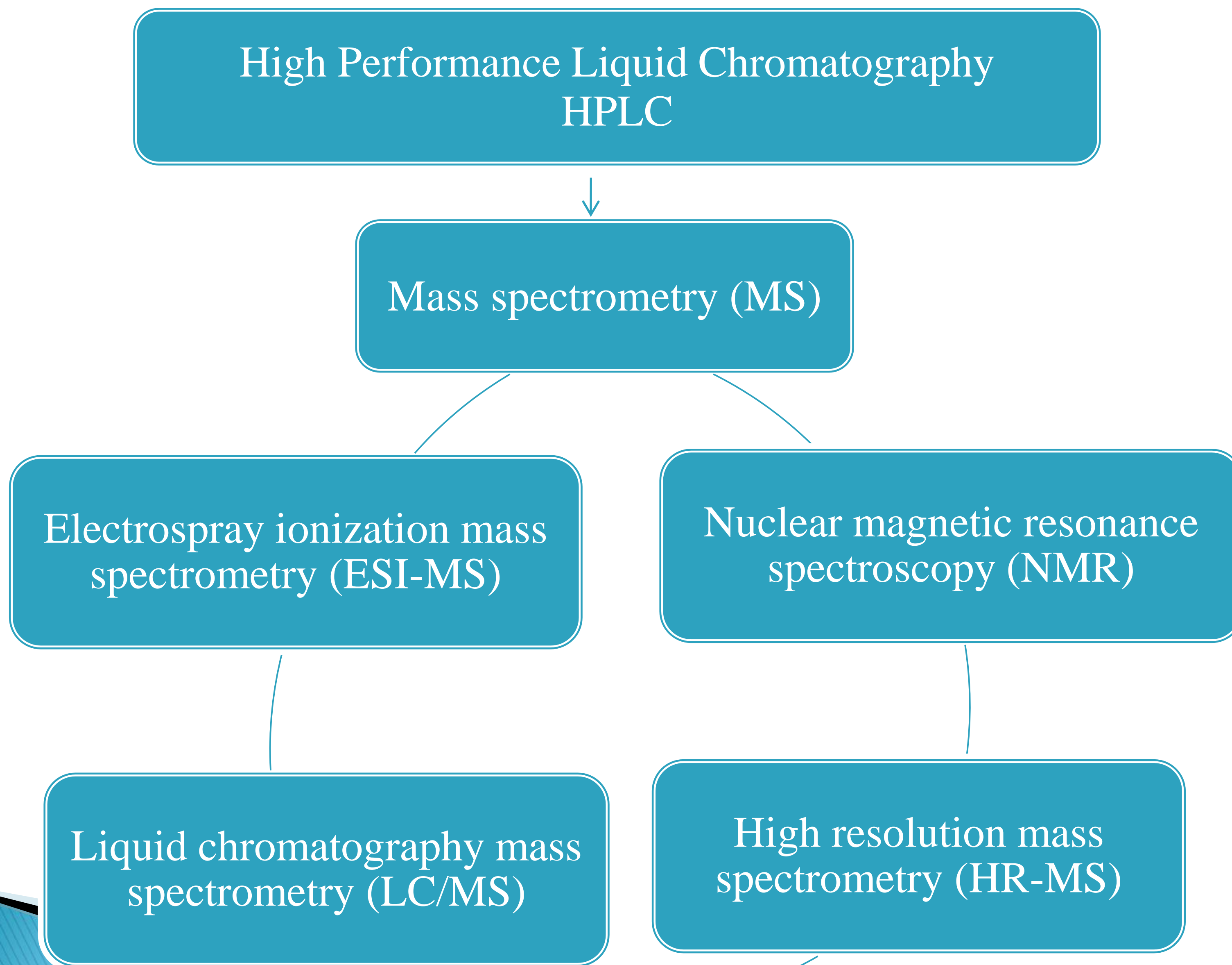
250 ml EtOAc were added to the cultures and left overnight. Culture media were then cut in pieces to allow complete extraction and left for 3–5 days. Then filtration was done followed by repeated extraction with EtOAc and MeOH till exhaustion. The combined EtOAc phases were washed with distilled water and then taken to dryness.



## Isolation and purification of secondary metabolites:



# Structure elucidation of the isolated secondary metabolites



# Occurrence of microorganisms (%) associated with apple shoots of different varieties

| | Microbial species | Беркутовское | | Уэлси | | Голден Делишес | |
|---|---|---|---|---|---|---|---|
| | | External | Internal | External | Internal | External | Internal |
| Bacteria | *Aureobacterium barkeri* | 0 | 0 | 6,7 | 0 | 0 | 0 |
| | *Bacillus amyloliquefaciens* | 0 | 0 | 26,7 | 40,0 | 6,7 | 13,4 |
| | *B. Farraginis* and *B. lentus* | 0 | 6,7 | 0 | 0 | 0 | 0 |
| | *B. megaterium* | 0 | 26,7 | 0 | 0 | 6,7 | 0 |
| | *B. methylotrophicus* | 0 | 0 | 36,7 | 36,7 | 0 | 6,7 |
| | *B. neidei* | 33,4 | 0 | 0 | 0 | 0 | 0 |
| | *B. pumillus* | 20,0 | 0 | 0 | 0 | 26,7 | 13,4 |
| | *B. simplex* | 13,4 | 13,4 | 0 | 0 | 0 | 0 |
| | *B. subtilis* | 63,4 | 46,7 | 6,7 | 33,4 | 33,4 | 33,4 |
| | *Brevibacterium halotolerans* | 0 | 0 | 3,4 | 0 | 6,7 | 0 |
| | *Deinobacter grandis* | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Listeria welshmeri* | 13,4 | 0 | 0 | 0 | 0 | 0 |
| | *Microbacterium lacticum* | 0 | 0 | 0 | 0 | 26,7 | 0 |
| | *Pantoea agglomerans* | 26,7 | 6,7 | 40,0 | 0 | 20,0 | 13,4 |
| | *Serratia ficaria* | 0 | 0 | 0 | 3,4 | 0 | 0 |
| | *Stenotrophomonas maltophilia* | 0 | 0 | 0 | 6,7 | 0 | 0 |
| Fungi | *Alternaria alternata.* | 73,4 | 0 | 90,0 | 6,7 | 93,4 | 0 |
| | *Aspergillus tubingensis* | 76.8 | 14.2 | 92 | 11.5 | 89.4 | 15 |
| | *Cladosporium cladosporioides* | 6,7 | 0 | 0 | 0 | 20,0 | 0 |
| | *Fusarium tricinctum* | 46,7 | 3,4 | 73,4 | 6,7 | 73,4 | 0 |
| | *Penicillium sp.* | 13,4 | 10,0 | 6,7 | 10,0 | 0 | 26,7 |

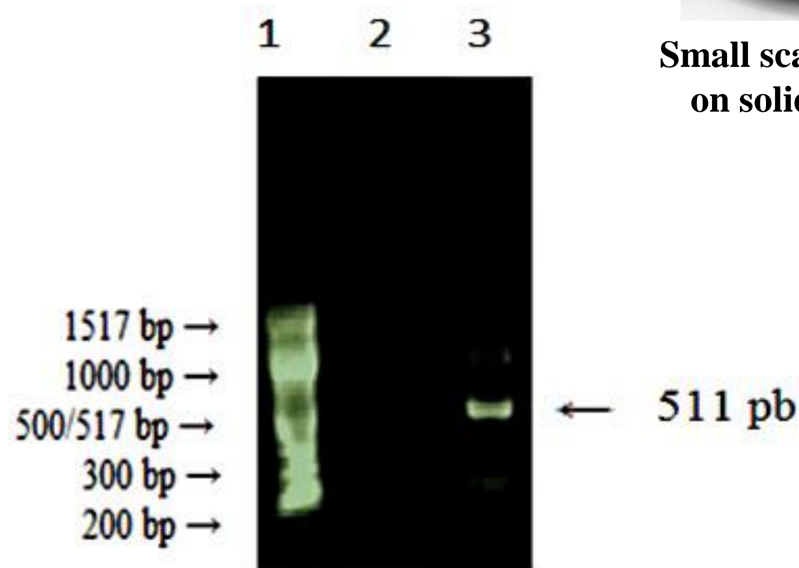
## Purification and Identification of fungal culture

The ITS gene of *A. tubingensis* was amplified with primers ITS1-ITS4. The size of amplified fragment was 511bp. The results of the DNA sequence analysis is similarity (100%) to the *A. tubingensis*

Plant material

Pure fungal strain on malt agar plate

Small scale fermentation on solid rice medium




Agarose gel electrophoresis and the presence of amplified PCR products from fungal strains, Lane 1 DNA marker. Lane 2: control Lane 3: amplified gene from *A. tubigensis*.

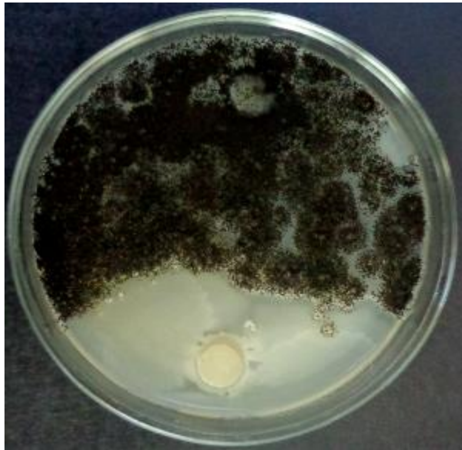
## Fungal taxonomy:

| | |
|---|---|
| Domain: | Eukaryota |
| Kingdom: | Fungi |
| Phylum: | Ascomycota |
| Subphylum: | Pezizomycotina |
| Class: | Eurotiomycetes |
| Order: | Eurotiales |
| Family: | Trichocomaceae |
| Genus: | *Aspergillus* |
| Species: | - *Aspergillus niger*<br>- *Aspergillus niger var. tubingensis* |

## *In vitro* screening of isolates for antagonism:

Fungal isolate was screened *in vitro* against *Brevibacterium halotolerans* (A) and *Bacillus methylotrophicus* (B). By applying a dual culture technique, one 5-mm diameter of fungi agar plug was placed on the edge of PDA medium in a Petri dish with 11 cm diameter. The inhibitory effect on fungal growth was evaluated. All *in vitro* antagonism assays were made in triplicate.

(A)



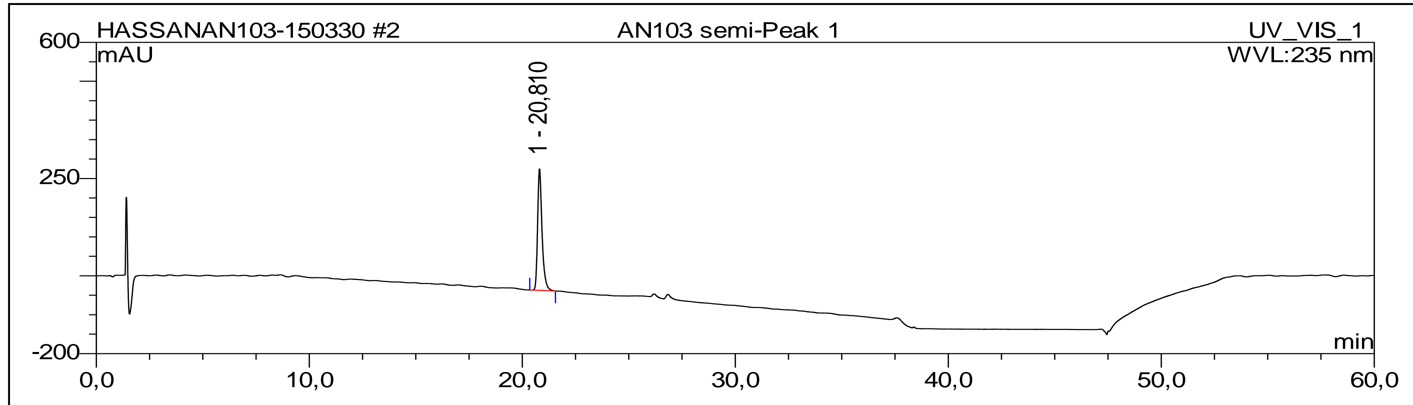
(B)

# Identification of isolated compounds:

## Compound 1, Fonsecin

1- HPLC chromatogram
2- HPLC spectra
3- LC-MS

1

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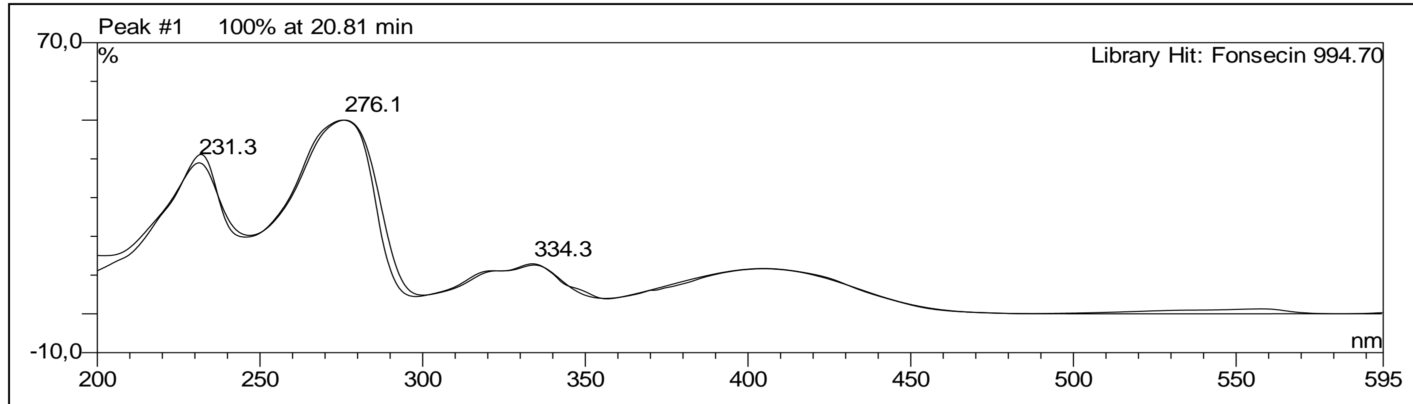
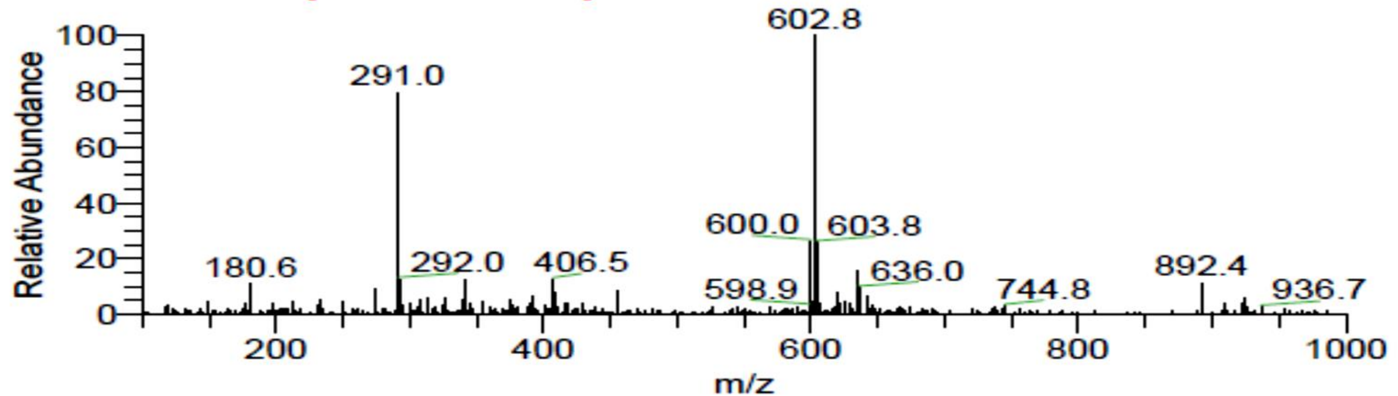
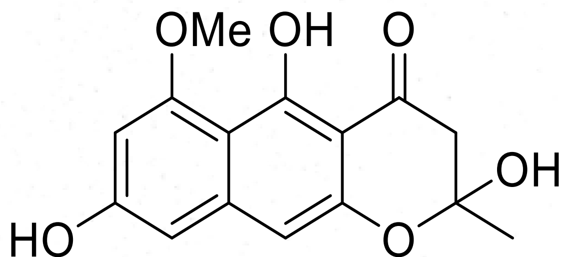



3



# NMR result of Fonsecin and its chemical structure

AN103-Semi-Peak1



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| INSTRUM | spect | |
| PROBHD | 5 mm PABBO BB- | |
| PULPROG | zg30 | |
| TD | 65536 | |
| SOLVENT | CDCl3 | |
| NS | 16 | |
| DS | 2 | |
| SWH | 6188.119 | Hz |
| FIDRES | 0.094423 | Hz |
| AQ | 5.2953587 | sec |
| RG | 322 | |
| DW | 80.800 | usec |
| DE | 6.50 | usec |
| TE | 298.0 | K |
| D1 | 1.00000000 | sec |
| TD0 | 1 | |

======== CHANNEL f1 ========

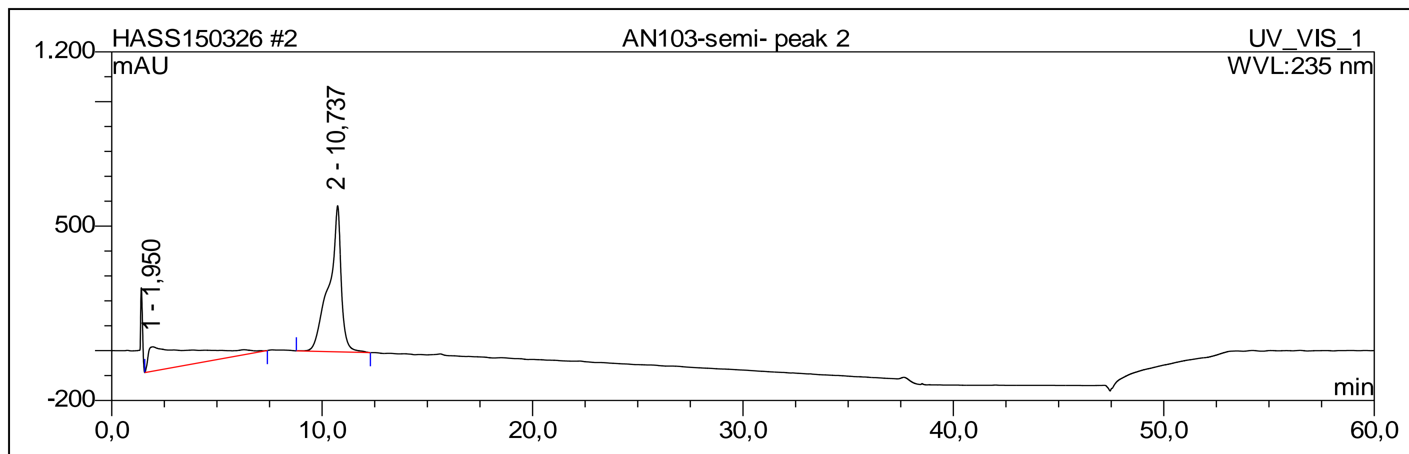
| | | |
|---|---|---|
| NUC1 | 1H | |
| P1 | 9.00 | usec |
| PL1 | -2.00 | dB |
| PL1W | 17.95463371 | W |
| SFO1 | 300.1318534 | MHz |
| SI | 32768 | |
| SF | 300.1300000 | MHz |
| WDW | EM | |
| SSB | 0 | |
| LB | 0.30 | Hz |
| GB | 0 | |
| PC | 1.00 | |

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 −1 −2 ppm

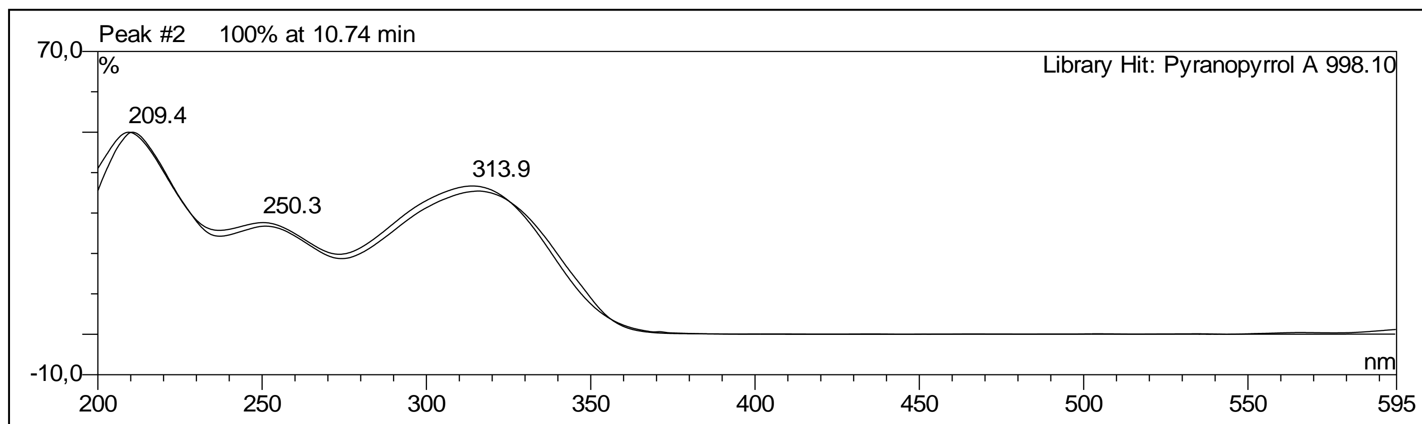
9.04 0.94 6.06 6.20 1.09 0.85 2.25 0.57 2.64 70.35

# Compound 2
## Pyranonigrin A
## $C_{10}H_9NO_5$

1

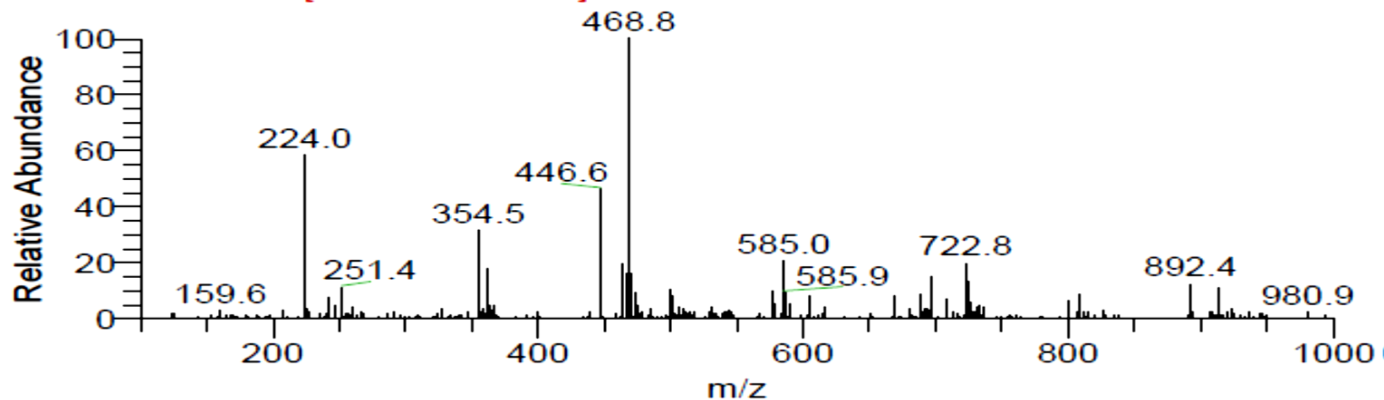



2



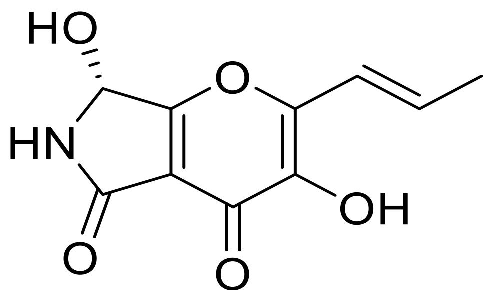

1- HPLC chromatogram
2- HPLC spectra
3- LC-MS

3



# NMR result of Pyranonigrin A and its chemical structure

AN103-Semi-Peak2



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| PULPROG | zg30 |
| TD | 65536 |
| SOLVENT | DMSO |
| NS | 16 |
| DS | 2 |
| SWH | 6188.119 Hz |
| FIDRES | 0.094423 Hz |
| AQ | 5.2953587 sec |
| RG | 256 |
| DW | 80.800 usec |
| DE | 6.50 usec |
| TE | 298.0 K |
| D1 | 1.00000000 sec |
| TD0 | 1 |

======== CHANNEL f1 ========

| | |
|---|---|
| NUC1 | 1H |
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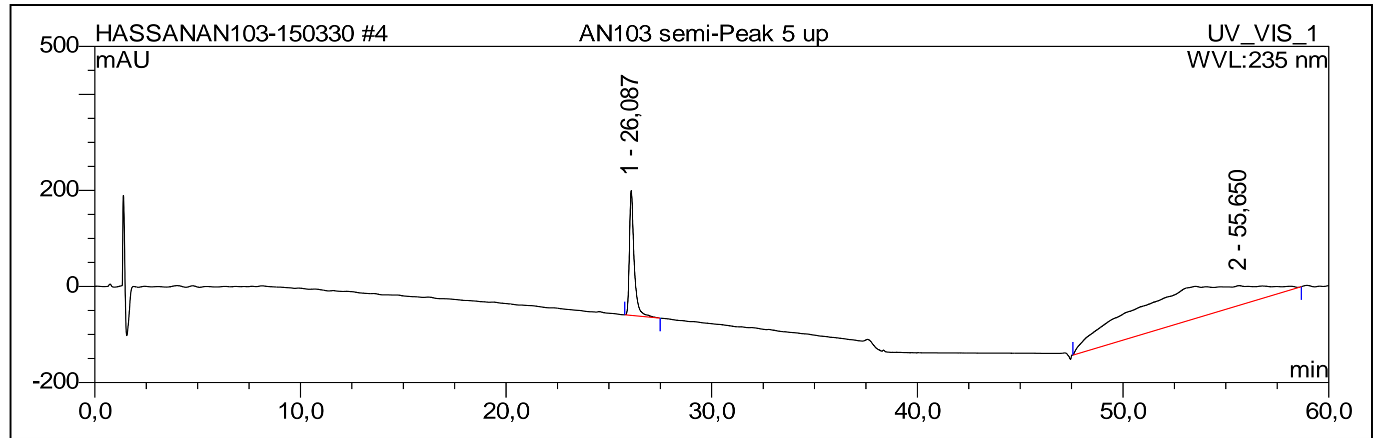
| PL1 | -2.00 dB |
| PL1W | 17.95463371 W |
| SFO1 | 300.1318534 MHz |
| SI | 32768 |
| SF | 300.1300000 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 0.30 Hz |
| GB | 0 |
| PC | 1.00 |

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 −1 −2 ppm

3.22 3.20 3.37 6.49 3.83 23.47 56.43

**Compound 3**
**TMC 256 A1**
**$C_7H_{14}O_3$**

1

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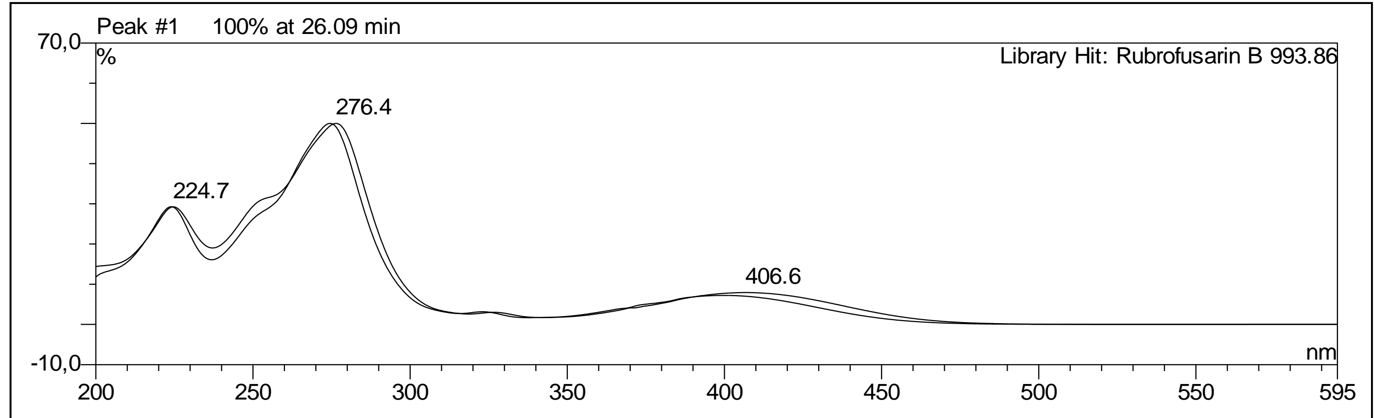
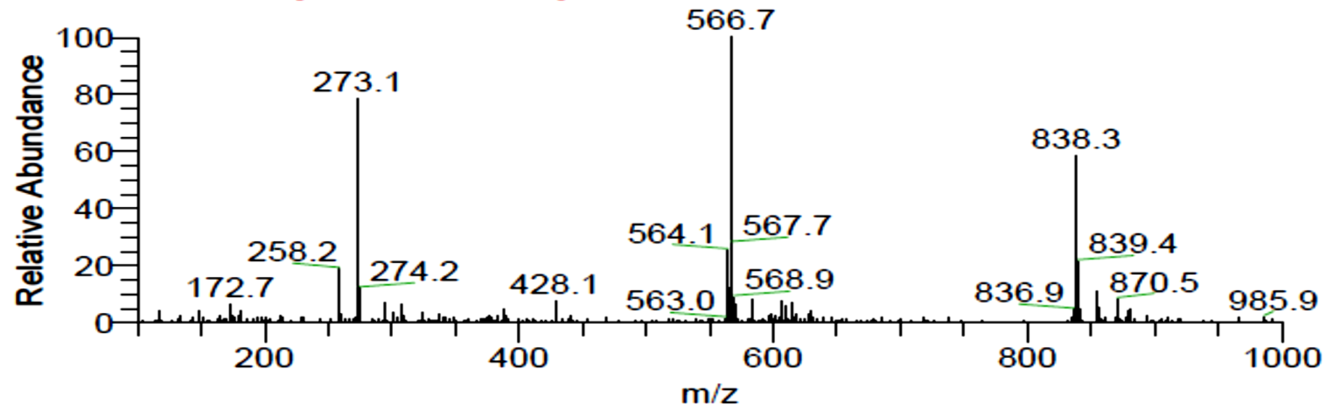


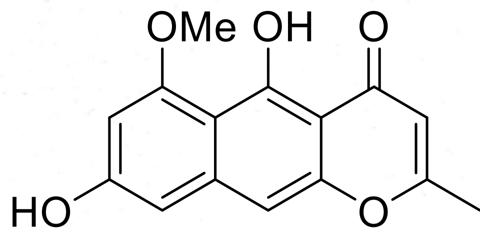

3




1- HPLC chromatogram
2- HPLC spectra
3- LC-MS

# NMR result of TMC 256 A1 and its chemical structure

AN103-Semi-Peak5



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| PULPROG | zg30 |
| TD | 65536 |
| SOLVENT | DMSO |
| NS | 16 |
| DS | 2 |
| SWH | 6188.119 Hz |
| FIDRES | 0.094423 Hz |
| AQ | 5.2953587 sec |
| RG | 322 |
| DW | 80.800 usec |
| DE | 6.50 usec |
| TE | 298.0 K |
| D1 | 1.00000000 sec |
| TD0 | 1 |

======== CHANNEL f1 ========

| | |
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| PL1 | -2.00 dB |
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| SFO1 | 300.1318534 MHz |
| SI | 32768 |
| SF | 300.1300000 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 0.30 Hz |
| GB | 0 |
| PC | 1.00 |

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 −1 −2 ppm

0.59 0.58 0.60 0.62 0.64 0.66 1.47 2.31 25.41 65.07 2.05

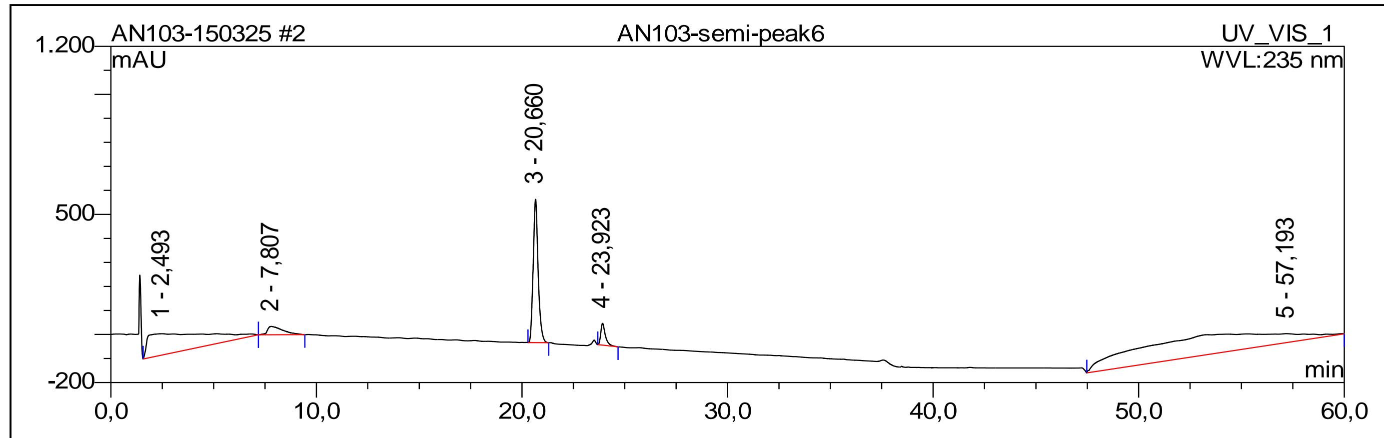
**Compound 4**

**Tensidol A**

**$C_{13}H_{11}NO_3$**

1- HPLC chromatogram
2- HPLC spectra
3- LC-MS

1

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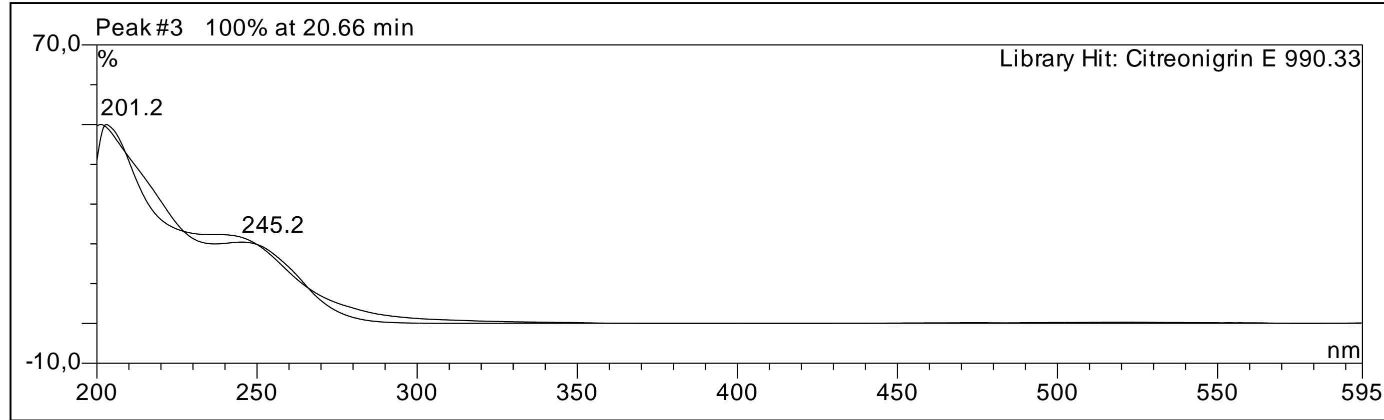
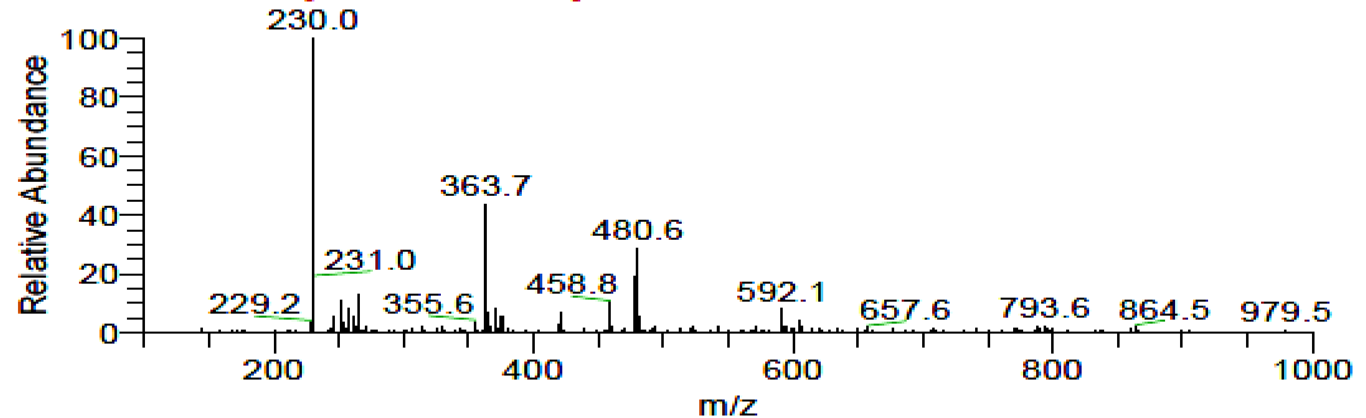
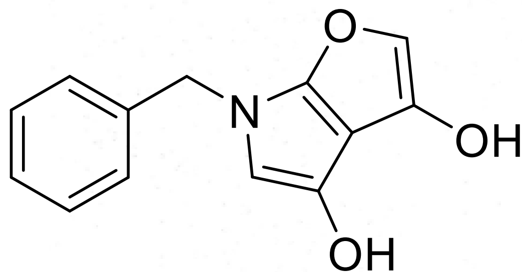



3



# NMR result of Tensidol A and its chemical structure

AN103-semi-Peak6



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| PULPROG | zg30 | |
| TD | 65536 | |
| SOLVENT | CDCl3 | |
| NS | 16 | |
| DS | 2 | |
| SWH | 6188.119 | Hz |
| FIDRES | 0.094423 | Hz |
| AQ | 5.2953587 | sec |
| RG | 406 | |
| DW | 80.800 | usec |
| DE | 6.50 | usec |
| TE | 298.0 | K |
| D1 | 1.00000000 | sec |
| TD0 | 1 | |

======== CHANNEL f1 ========

| | | |
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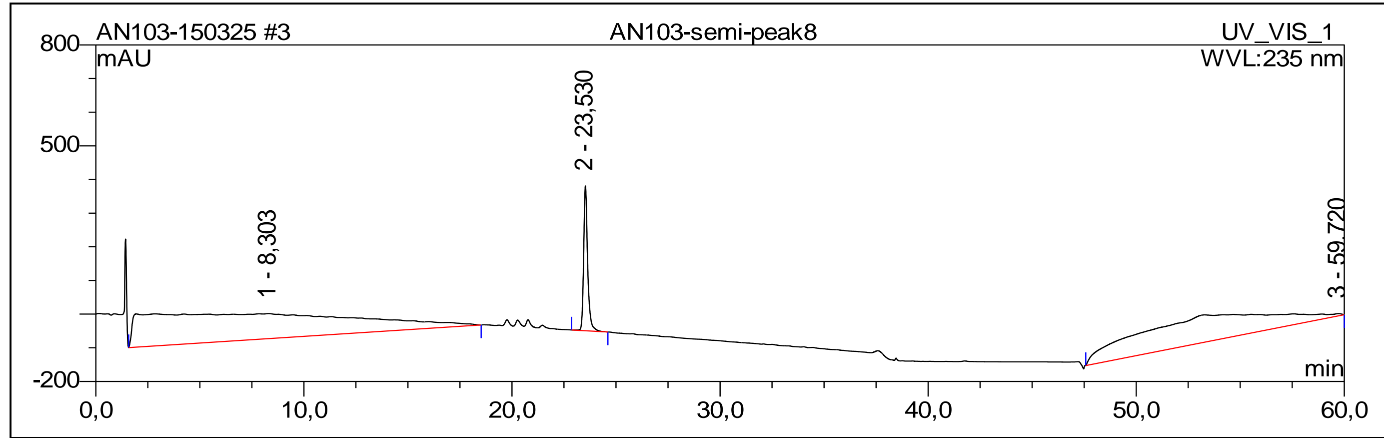
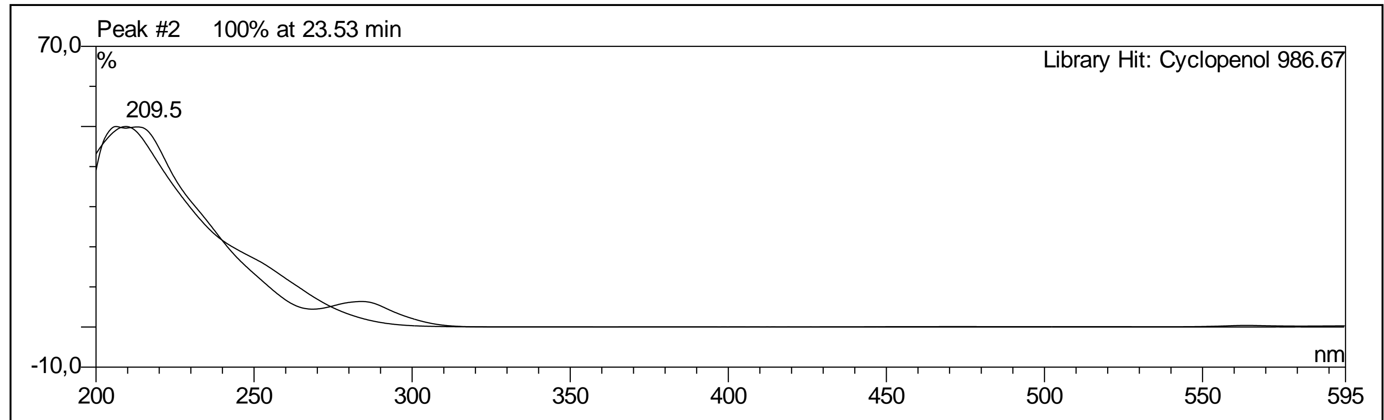
| NUC1 | 1H | |
| P1 | 9.00 | usec |
| PL1 | -2.00 | dB |
| PL1W | 17.95463371 | W |
| SFO1 | 300.1318534 | MHz |
| SI | 32768 | |
| SF | 300.1300000 | MHz |
| WDW | EM | |
| SSB | 0 | |
| LB | 0.30 | Hz |
| GB | 0 | |
| PC | 1.00 | |

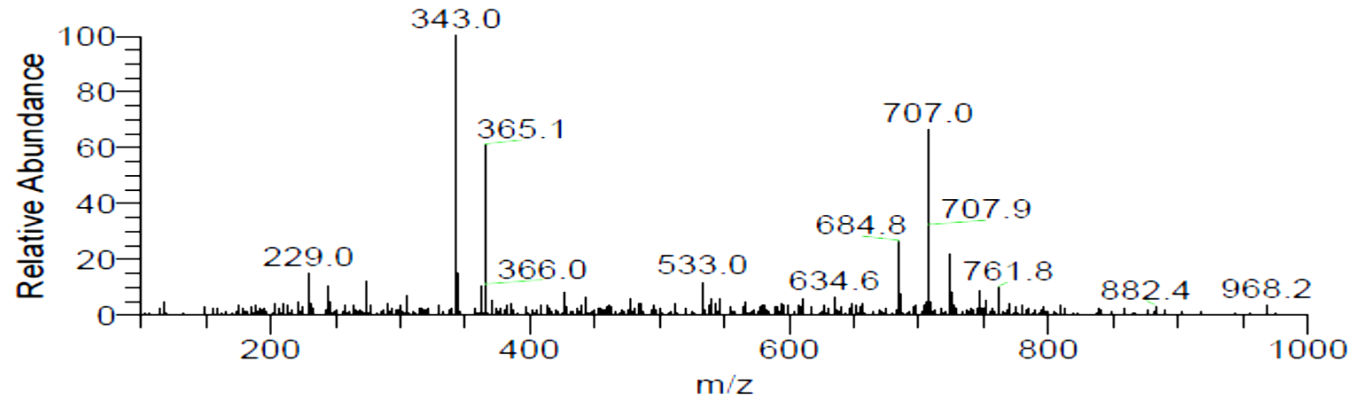
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 −1 −2 ppm

0.65 0.53 2.69 0.73 2.97 49.08 2.88 0.76 2.58 7.95 2.68 24.08 1.94 0.49

# Compound 5
## Tensidol B
## $C_{18}H_{17}NO_6$

1 

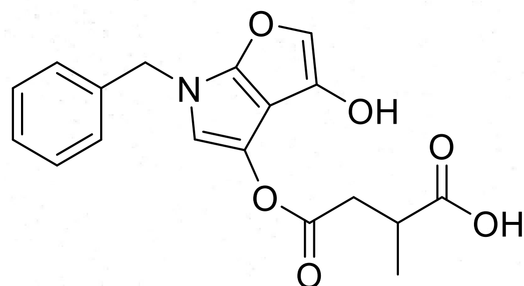

2 


3 


1- HPLC chromatogram
2- HPLC spectra
3- LC-MS

# NMR result of Tensidol B and its chemical structure

AN103-semi-Peak8



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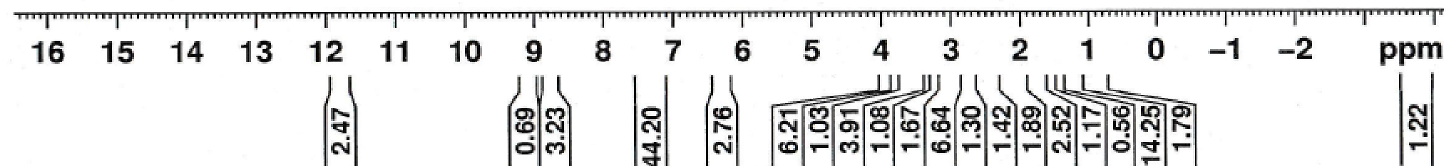
| PULPROG | zg30 |
| TD | 65536 |
| SOLVENT | CDCl3 |
| NS | 16 |
| DS | 2 |
| SWH | 6188.119 Hz |
| FIDRES | 0.094423 Hz |
| AQ | 5.2953587 sec |
| RG | 362 |
| DW | 80.800 usec |
| DE | 6.50 usec |
| TE | 298.0 K |
| D1 | 1.00000000 sec |
| TD0 | 1 |

======== CHANNEL f1 ========

| | |
|---|---|
| NUC1 | 1H |
| P1 | 9.00 usec |
| PL1 | -2.00 dB |
| PL1W | 17.95463371 W |
| SFO1 | 300.1318534 MHz |
| SI | 32768 |
| SF | 300.1300000 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 0.30 Hz |
| GB | 0 |
| PC | 1.00 |



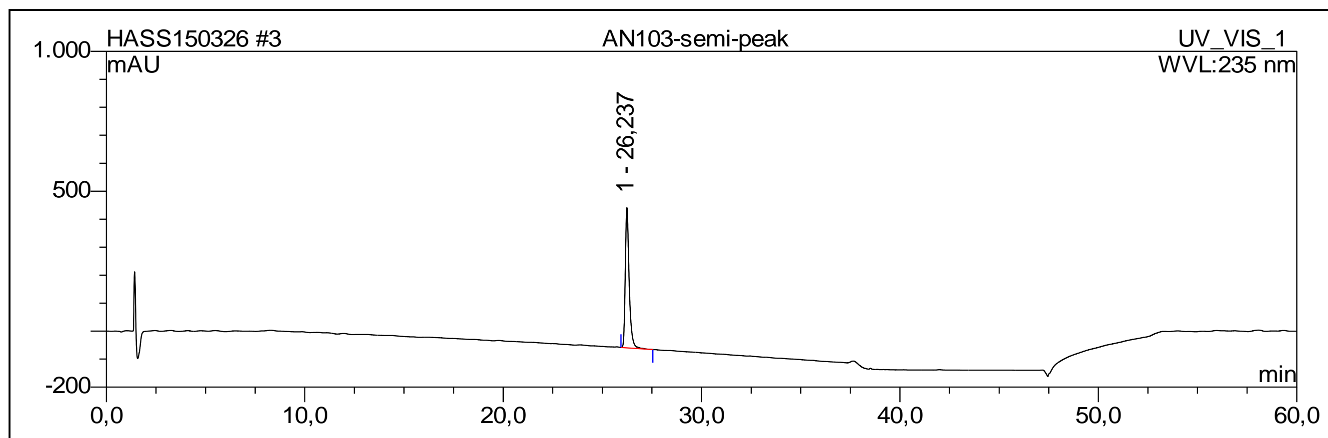
# Compound 6

## Asperazine

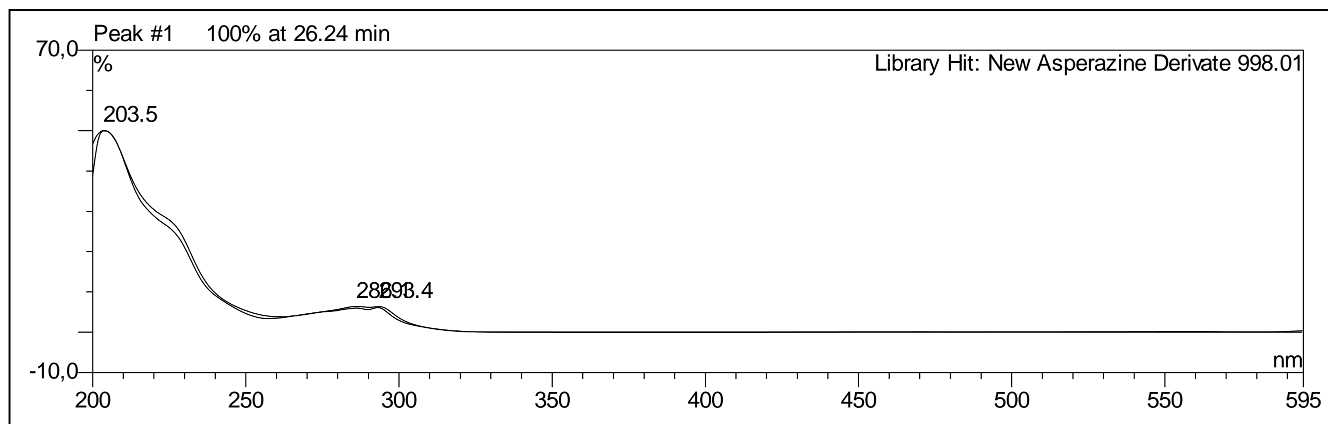
## $C_{40}H_{36}N_6O_4$

1- HPLC chromatogram
2- HPLC spectra
3- LC-MS

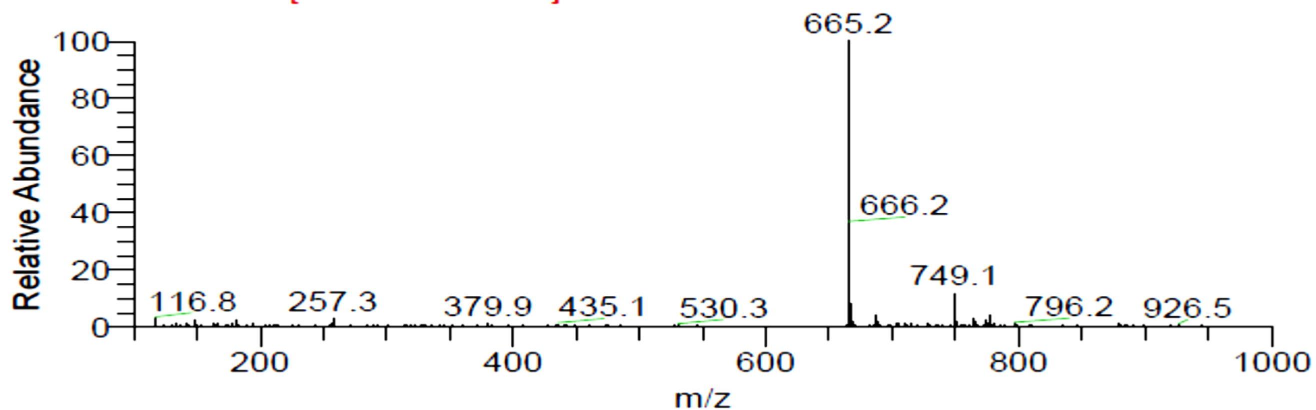
1




2

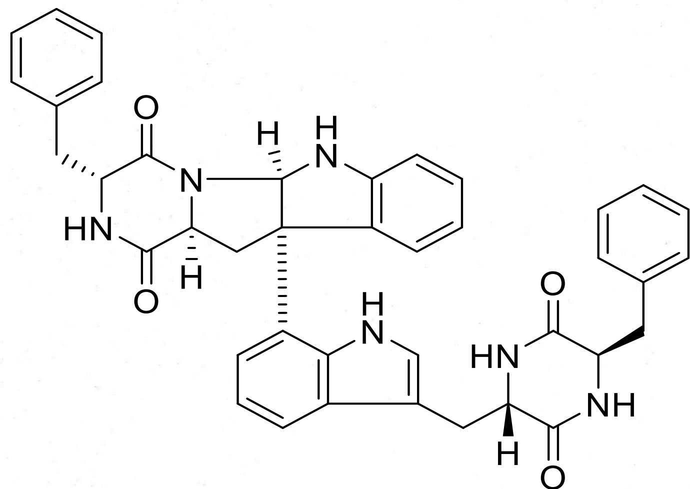


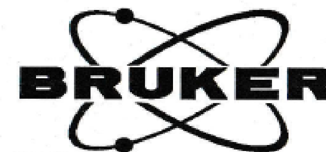

3



# NMR result of Asperazine and its chemical structure

AN103-Semi-Peak10




| | |
|---|---|
| NAME | HassanAwad |
| EXPNO | 130 |
| PROCNO | 1 |
| Date_ | 20150420 |
| Time | 12.15 |
| INSTRUM | spect |
| PROBHD | 5 mm PABBO BB- |
| PULPROG | zg30 |
| TD | 65536 |
| SOLVENT | DMSO |
| NS | 16 |
| DS | 2 |
| SWH | 6188.119 Hz |
| FIDRES | 0.094423 Hz |
| AQ | 5.2953587 sec |
| RG | 256 |
| DW | 80.800 usec |
| DE | 6.50 usec |
| TE | 298.0 K |
| D1 | 1.00000000 sec |
| TD0 | 1 |

======== CHANNEL f1 ========

| | |
|---|---|
| NUC1 | 1H |
| P1 | 9.00 usec |
| PL1 | -2.00 dB |
| PL1W | 17.95463371 W |
| SFO1 | 300.1318534 MHz |
| SI | 32768 |
| SF | 300.1300000 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 0.30 Hz |
| GB | 0 |
| PC | 1.00 |

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 −1 −2 ppm

0.81 0.84 1.69 0.88 15.14 0.88 1.61 1.23 73.34 1.29 1.60 0.68

## Biological activity of isolated compounds:

Cytotoxicity and antimicrobial tests were carried out at Institut für Physiologische Chemie und Pathobiochemie, University of Mainz, Mainz The cytotoxicity was tested against L5178Y mouse lymphoma cells using the microculture tetrazolium (MTT) assay. And antimicrobial against *Staphylococcus aureus* and *Mycobacterium tuberculosis* TB

| Compound tested | L5178Y growth in % (Conc. 10 µg/mL) | Antimicrobial activity | |
|---|---|---|---|
| | | *Mycobacterium tuberculosis* (µg/ml) | *Staphylococcus aureus* (µg/ml) |
| Fonsecin | 79.6 | >100 | >100 |
| TMC 256 A1 | 98.6 | >100 | >100 |
| Pyranonigrin A | 95.6 | >100 | >100 |
| Tensidol A | 79.3 | >100 | >100 |
| Tensidol B | 102.7 | >100 | >100 |
| Asperazine | 129.7 | >100 | >100 |

**Appreciation:**

Associate Prof. Peterson A Mikhaylovna
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Prof. Dr. Peter Proksch
Head of Institute of Pharmaceutical Biology
and Biotechnology, Heinrich Heine University
Düsseldorf, Germany.

# Thank you for your kind attention!