Novel Fluorescent Natural Products for Biotechnology

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Abstract

Fluorescent natural products are becoming of interest in the fields of medicine, biotechnology and environmental science. However, while nature is a source of a vast number of bioactive compounds very few fluorescent probes have been discovered from natural products. This research is addressing the discovery of new fluorescent pigments and to expand its application into new domain. A fungus, *Epicoccum nigrum*, was chosen for this project. *E. nigrum* is the sole member of the genus *Epicoccum* (Family Pleosporaceae) and gets its name from its dark colour. The colour is produced by red, blue and purple pigments, one of them (epicocconone) has been commercialised as a total protein stain for proteomics (Deep Purple; GE Healthcare), a fluorescent stain for live cell imaging (LavaCell; Fluorotechnics) and a protein quantification solution (Fluoroprofile; Sigma-Aldrich). This project aims to isolate new pigments from this fungus through the optimisation of growth conditions on agar plates and moving to medium scale fermentation, extraction, purification and structure elucidation of other pigments.

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Declaration

I thereby declare that this research work entitled ‘Novel Fluorescent Natural Products for Biotechnology’ is my own except for quotations and summaries have been dually acknowledged. This thesis has not been accepted for any degree to any other institution rather than Macquarie University and is not concurrently submitted for award for other degree.

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Tahnim Sultana Dated

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1 Introduction

Natural products are a central theme of research at the interface between chemistry and biology. Since the discovery of penicillin, microbes have been a source of novel small molecules useful to mankind, primarily in the area of medicinal chemistry. A large number of natural compounds such as alkaloids, steroids, flavonoids, quinones, xanthones have been isolated from fungal natural products.[1] Fungal endophytes have potential role to promote plant growth through different mechanisms. But, many of fungal endophytes still totally unexplored for biological and ecological sectors.[2] Many of these are also bioactive compounds,[3] such as antibiotics,[4] antiviral,[5] anticancer,[6] immunosuppressive,[7] insecticidal,[8] antidiabetic[9] and also some antioxidants. For example, the flavanols kaempferol and quercetin exhibit significant potency in anticancer and anti-AIDS screens and have low toxicity.[10]

In contrast, relatively few fluorescent natural products have been isolated, primarily because this physical characteristic has not been of high priority. However, fluorescence is becoming increasingly important in biotechnology, medicine and environmental sciences where sensing, detection and quantification of analytes in complex mixtures is required. Fluorescence has almost completely taken over assays previously accomplished with radioactivity and is now expanding its applications into new domains. Take for example the 2014 Nobel Prize in Chemistry for super resolution microscopy. However, the vast majority of current fluorescent probes are based on just four classes of molecules; xanthenes, cyanines, BODIPY and coumarins, the later are based on natural products. To expand the chemical scaffolds available for fluorescent research, one strategy is to turn back to nature.

The recent discovery of the natural product epicocconone from *Epicoccum nigrum*,[11] the first reversible-covalent latent fluorophore, has found wide biotechnological applications in proteomic gel and blot staining, protein quantification, live-cell imaging and monitoring of enzymatic activity.[12-15] This discovery has highlighted the need for new fluorescent scaffolds with unique properties that can extend the use of fluorescence beyond its current boundaries. *Epicoccum. nigrum* has also been found to contain many other natural products including several carotenoids such as β-carotene, γ-carotene, rhodoxanthin, antibiotics such as flavipin, epicorazine, epirodins A and B (Fig. 1.4).[16-17] *E. nigrum* is also characterized by the production of red, yellow or orange pigments of which very little is known. So, the aim of this research work is to isolate new fluorescent pigments from *E. nigrum*.

1.1 Fluorescence: An Overview

Fluorescence is one of the categories of luminescence in which the electronic excited states are divided into different singlet states. With the influence of external sources, the electrons get excited and lifted from ground energy state (S_0) to higher energy states (S_1, S_2…). Electrons in the excited singlet orbital are paired with the electron in the ground state orbital. When the electron returns to the

ground state, rapid emission of a photon takes place. This is called fluorescence. Typically, the rate of emission of fluorescence is 10^{-7}-10^{-9} s. Indicating a lifetime of about (10^{-9} s). This is a fundamental physical property of some molecules with rigid conjugated system that allow the re-emission of light at a longer wavelength. For phosphorescence, transition of electrons from ground state to excited state take place. Alongside the singlet state S_1 some electrons transferred to another energetically favourable state called tripled state (T_1) by intersystem crossing. After releasing energy slowly from this state, electrons returned to the ground state (S_0). This is known as phosphorescence. The whole process is illustrated by Jablonski diagram (Fig. 1.1). But Common fluorescent compounds absorbed ultraviolet light (200-400 nm) and emitted light in the blue region (400-500 nm), which gives the fluorescent substance a distinct colour that can only be seen when exposed to UV light (Fig. 1.1).[18] Quinine was the first natural fluorescent compound discovered. In 1845 Sir John Fredrick William Herschel first observed fluorescence from quinine solution in sunlight.[18] After this many fluorescent compounds have been found such as xanthene derivatives for example, fluorescein, rhodamine, cyanine derivatives such as cyanine, indocarbocyanine and coumarin derivatives. All of them provide scaffolds for applications in biochemistry and biomedical sciences.[19]

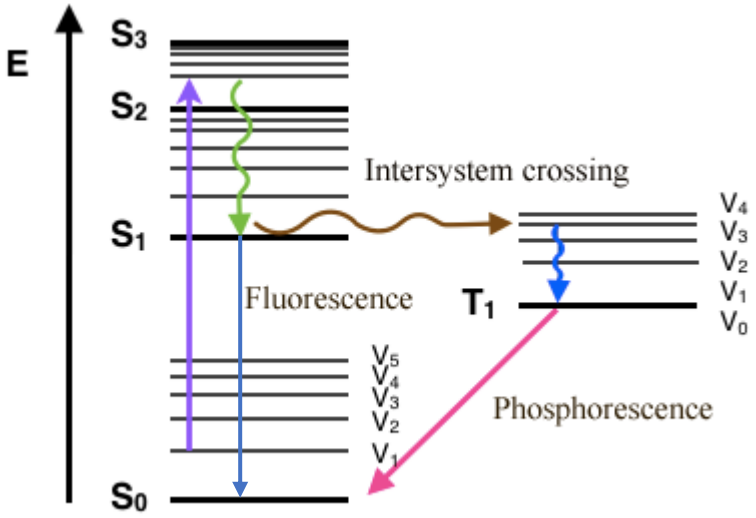

Figure 1.1. Jablonski energy diagram.[20]

The fluorescence process involves the emission of light from a higher energy state. According to the Boltzmann distribution equation (Eq. 1.1) most of the molecules occupy the zeroth vibrational state of the electronic ground state at room temperature.

$$\frac{n}{n_0} = e^{-\frac{\Delta E}{kT}} \qquad 1.1$$

The electronic state and the overall molecular geometry and the distribution of negative charge of a molecule expresses by its electronic states. Every molecule consists of several energy levels which are also subdivided in rotational and vibrational energy levels. The vibrational electronic excitation can access several vibrational levels of the excited electronic states (S_1, S_2, S_3……S_n) leading to vibrational structure in the electronic absorption spectrum which mainly depend on the energy of total electrons and spinning states of electrons.

The singlet state, which is considered as ground electronic state, most of the electrons of organic molecules are found to be spin-paired in this state. The lowest vibrational energy is occupied by electrons at room temperature but whenever they are excited, they shift to a higher energy state. This absorption or relaxation features depend upon the nature and the environment of fluorophores. Whenever the emission of energy take place, several incidents have a possibility to occur such as fluorescence, intersystem crossing, vibrational relaxations etc. (Fig. 1.1). The ratio of photons emitted by a fluorophore is measured as the fluorescence quantum yield (Φ_f). The quantum yield provides the probability of deactivating the excited state either by fluorescence or by a non-radiative mechanism (Eq. 1.2).[21]

$$\Phi f = No.of\ photons\ emitted\ /\ No.of\ photons\ absorbed \qquad 1.2$$

Having high extinction coefficient and high quantum yield[22] allows fluorophores to be used for the biomolecular labelling with great sensitivity.

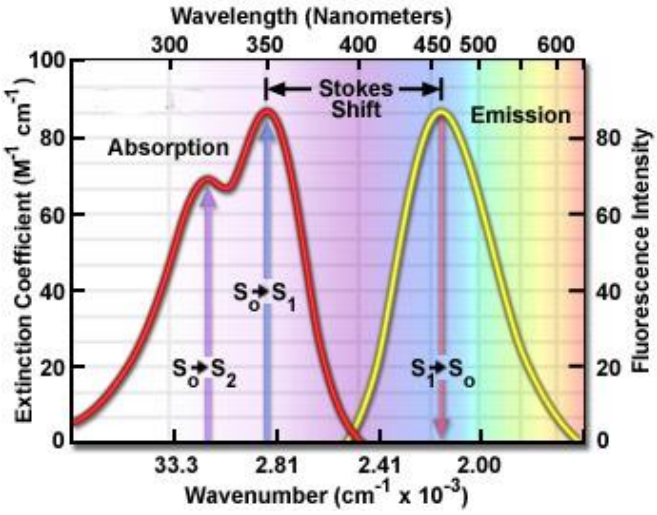

Figure 1.2. Absorption and Emission Spectra of Quinine.[23]

Quinine sulfate in 0.1M H_2SO_4 is used as the standard for determining the efficiency of fluorescence (Φ_f = 0.54) by various comparative methods.[24] The absorption and emission spectra of quinine is presented in Fig. 1.2. As mentioned previously quinine is naturally occurring fluorochrome and is used as an antimalarial agent. According to this Fig. 1.2, it is observed that the mirror image rule for the emission is not followed by quinine at 460 nm, as is evident by inspecting the single peak in the emission spectrum[24] but two peaks in the excitation spectrum, which exhibits S_0-S_1 and S_0-S_2 bands. An internal conversion from S_2-S_1 leads to this asymmetry. The energy of absorption (350 nm = 3.54 eV) is higher than the energy associated with the fluorescence emission (450 nm = 2.76 eV). Thus, the energy spectrum is shifted towards the longer wave length as a result of internal conversion. This phenomenon was explained by Sir George Stokes and known as the Stokes shift.

1.2 Application of Fluorescence

Fluorescent compounds have many applications particularly in biological applications. Cellular functions, cellular components or detection of the whole cells can be carried out using fluorescence compounds. Applications include DNA sequencing, immunofluorescence, cell tracking, confocal microscopy, super resolution microscopy etc.[25-26] Fluorescence *in situ* hybridisation, which is a method to determine the type of gene present in the genome of any organism is a process where fluorescent molecules are used as tags for genes. Moreover, fluorescent molecules have expanded into both *in vivo* and *in vitro* applications. For example, the green fluorescent protein (GFP) can be genetically encoded to label any protein providing versatility in spectroscopic measurements and biological applications.[27] Fluorescent compounds are also used in chemosensing and consider as very important task in molecular biology and biochemistry.[28-29] Chemosensing is a process of detecting the change of analytes through optical or electrochemical properties. Different chemosensors interact with specific analyte and produce a detectable change. In this process, fluorescent indicator and a receptor is bounded through noncovalent interactions and then indicator was displaced by the addition of analyte which make the solution fluorescent. Thus, the analyte signal was recognised by the sharp fluorescence.[30] Similarly the application of fluorescence resonance energy transfer (FRET)[31] is widely distributed in biological research and drug discovery. FRET is mainly based on the distance of the energy transfer between a donor and acceptor fluorophore, by a long-range dipole-dipole interaction in a non-radiative fashion. This technique is used for not only for qualitative measurements but also quantitative measurements on the distance, sensitivity and increased spiral resolution between two macromolecules. Furthermore, the fluorescent properties of molecular beacons are used for the detection of the hybridized target probes in the presence of unhybridized probes.[32]

In the biomedical sector fluorescent biosensor technology is widely used for monitoring health and diseases, for observing the progression of diseases, diagnostic approaches and also for therapeutics responses.[33] New fluorescent stains need to be developed for versatility and new detection capabilities in research areas from pharmacology and immunology to proteomics and genomics.[34-35] For DNA and protein detection in electrophoretic separation fluorescence has taken out from chromogenic detection. Also, large number of molecular antigens and drug from low molecular weight to high molecular weight can be easily detected immunochemically by the fluorescence assays.

1.3 Advantages over Radioactivity

The growing demand for fluorophores is correlated with a diminishing usage of radioactivity because of the hazards associated with working with radioactivity; expensive instruments, limited shelf-life and disposal costs. On the other hand, fluorophores are easy to handle, have a long shelf life, and are easily disposed of.[36-37] Because of the use of fluorescence in clinical and analytical chemistry, interest is growing in the fields of physics and chemistry in an attempt to keep up with demand.

1.4 Development of New Fluorescent Molecule

An ideal fluorescent stain should have a specific reactivity as well as exhibiting bright fluorescence against a low background, be cell permeable and sufficiently small to avoid perturbing the cells, free from cytotoxicity and suitable by common laser sources.

Also some properties like, a large Stokes' shift, high quantum yield and long emission wavelength (to be separable from autofluorescence and to minimize Rayleigh scattering)[38] are considered advantages. But large Stokes' shift dyes generally have large molecular weight or low quantum number yields (or both), which limit their application in many cases, cause large Stokes' fluorescent dyes show relatively low brightness due to the product of the molar extinction coefficient and fluorescence quantum yield and poor photostability.[39] A good fluorophore has high quantum yields and small molecule fluorophore are most suitable as ion indicator, macromolecule labels, cellular stain than heavy molecules.[40] Few fluorescent compounds meet these criteria, so the discovery of new fluorescent scaffolds has the potential of broadening the range and utility of application of small molecules in biology.[34]

1.5 Sources of Fluorescent Molecules

Fluorophores can be classified as extrinsic and intrinsic fluorescent components. Protein tyrosine, tryptophan, other aromatic amino acids, porphyrins and naturally occurring fluorophores such as green fluorescent protein are mainly considered as intrinsic fluorescent molecules. On the other hand,

synthetic or modified pigments such as fluorescein, coumarins xanthones, pyrin, cyanines, attached dansyl etc. are known as extrinsic fluorescent components.[18, 41-42] Fluorescent molecules are commonly used as reporters or tracers in biology and material sciences.[43]

Fluorochromes can be classified both chemically or naturally and used widely in biological detection systems. The available biological sources of fluorescent pigments are plants, animals, microbial and marine organism. The choice of fluorophores is governed by the questions that are to be addressed. For example, to measure the rotational diffusion, the lifetime of the fluorophore depolarization should be associated exclusively with rotational motion, without interference from an excited state process. Fluorophores that can participate on protonation deprotonation equilibria, in the ground and/or excited states, are suitable for the measurement of pH. Only probes with long excitation and emission wave lengths can be used in tissues.[44]

1.6 Synthetic Fluorescent Compounds

To accelerate the discovery of new fluorescent pigments combinatorial chemistry has been applied to generate both receptor and reporter fluorescent molecules and applications have increased in the past few years.[45]

1.6.1 Fluorescein

The most widely used fluorochrome named and showed in Fig. 1.3. In 1800s, it was isolated from coal tar.[46] Oregon Green, Texas Red and Rhodamine are the synthetic analogues of fluorescein (Fig. 1.3). It is used to test for the cell viability.[47] and its pH dependent nature increases its potential in the field of biotechnology.[48]

1.6.2 Xanthene

Xanthene is a yellow organic heterocyclic compound (Fig. 1.3).[49] Because of the special spectroscopic features of the dye, it has attracted considerable interest as fluorescent pigment. It is used as a tracing agent, biological stain, chemical sensitizer and thermochromic and photochromic agents and has broad application as a laser dye.[50]

1.6.3 Coumarin

Coumarin is a natural compound available in some plant extracts and fungi [51] and belongs to the chemical class of benzopyrones. It is a crystalline colourless substance (Fig. 1.3). Cumarine as fluorescent substances are widely spread in living nature and can be found in different genera of plants and fungi. Due to their intensive fluorescent property, they can be easily observed. This gives the opportunity to make exceptions, with low costs, low complexity and without using toxic materials.[52]

1.6.4 BODIPY

Dipyrrometheneboron difluoride, abbreviated as BODIPY (Fig. 1.3), is a class of synthetic fluorescent dyes which are used widely in biotechnology.[53] They are highly notable for absorbing light over a narrow band and show highly intense fluorescence peaks. However, their small Stokes' shifts and solidification in solution limit their use. Though modifications to the BODIPY framework will lead to use it more effectively for labelling reagents, fluorescent dyes also imaging in living cells and whole organisms.[53]

1.7 Natural Fluorescent Compounds

1.7.1 Green Fluorescent Protein

Green Fluorescent Protein (GFP) which is the first genetically encoded dye[54] has found applications in live cell imaging.[55-57] GFP was first extracted from the jellyfish *Aequorea victoria.*[58] As a companion protein to aequorin, which is a chemiluminescent protein that emits blue light (470 nm). This discovery was subjected to the Noble Prize in Chemistry in 2008, to Shimomura for both the discovery and development of GFP. He elucidated the structure of the chromophore *p*-hydroxybenzylideneimidazolidinone (p-HBDI). To generate the chromophore Ser-Tyr-Gly sequence undergoes a cyclization and oxidation to form an imidazolone.[59-60] The outstanding feature of GFP that is that fluorescence generated *in vivo* through gene expression, increase the possibility of conducting cell developmental studies on tagged proteins.[61]

1.7.2 Modified Green Fluorescent Proteins

GFP can be altered to produce different FPs such as yellow fluorescent proteins (YFP), red fluorescent proteins (RFP), cyan fluorescent proteins (CFP) etc. YFP's are one of the variants of green fluorescent protein, showed red shifted emission spectra. The YFPs are acid sensitive and quenched by Cl^- ion.[62] They are liable to produce both fluorescent and non-fluorescent purple blue pigments.[63] This environmental sensitivity makes them useful for biological applications,[64] mainly the physiological activities of the living cells[65] or cell biology.

1.7.3 Epicocconone

Epicocconone (Fig. 1.4) is a natural product and is neutral, non-toxic and appears to diffuse readily into live or fixed cells without the need for permeabilization.[34] It is considered an ideal small molecule fluorophore for biotechnology because of a long Stokes' shift and showing minimal fluorescence in the native state.[66] It was extracted from a fungus, *Epicoccum nigrum* and is the first reversible-covalent latent fluorophore.[11] Latent fluorophore display a unique selectivity and reduce interferences with probe concentration, sensitivity of emission and excitation. The irreversible fluorophores either

react with their target covalently or work by removing a protecting group or quenching group which make it incompatible in protiomics.[66] Thus epicocconone found to have wide applications in proteomic gel staining,[13] blot staining, protein quantification,[12] live-cell imaging,[67] cell tracking[68] and monitoring of enzymatic activity.[15] The fluorescent property of epicocconone are turned on by enamine formation with proteins specifically by reacting (reversibly) with lysine residue of proteins.[69]

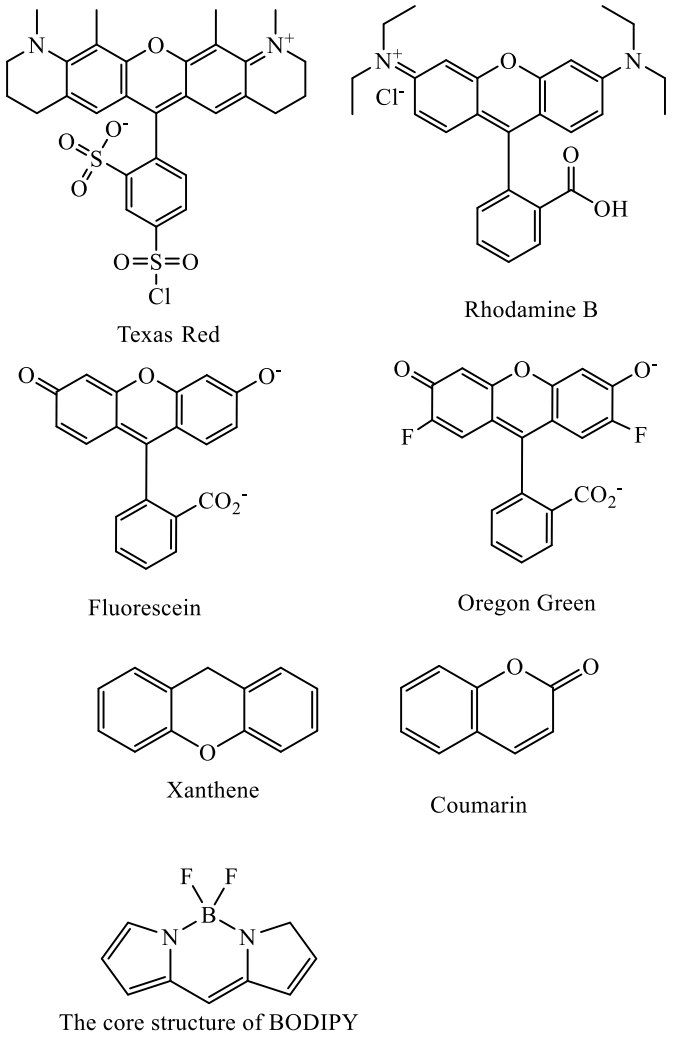

Figure 1.3. Structures of some fluorochrome.

1.8 Selectivity of *Epicoccum nigrum* for Fluorescence

The fungus *Epicoccum nigrum* is known to be a source of fluorescent compounds.[35] Varieties of pigments found including several carotenoids (Fig. 1.4) such as β-carotene, γ-carotene, rhodoxanthin,[70] antibiotics such as flavipin[71] and epicorazine A[72] and B[73], epirodins A and B[74] and phenylalanine derived dimers such as 3,6-dibenzyl-2,5-dioxopiperazine[75] and the polyketide

orevactaene[76-77] (Fig 1.4). *E. nigrum* also secretes unidentified siderophores, a class of high affinity, microbial ferric ion transfer molecules for the purpose of sequestering iron from environment.[78] The isolation of epicocconone from *E. nigrum* made this fungus famous, because of the wide applications of epicocconone in biotechnological sector (sec. 1.7.3). As this fungus is already known to produce fluorescent pigments it is thus the most likely source of other fluorescent pigments.

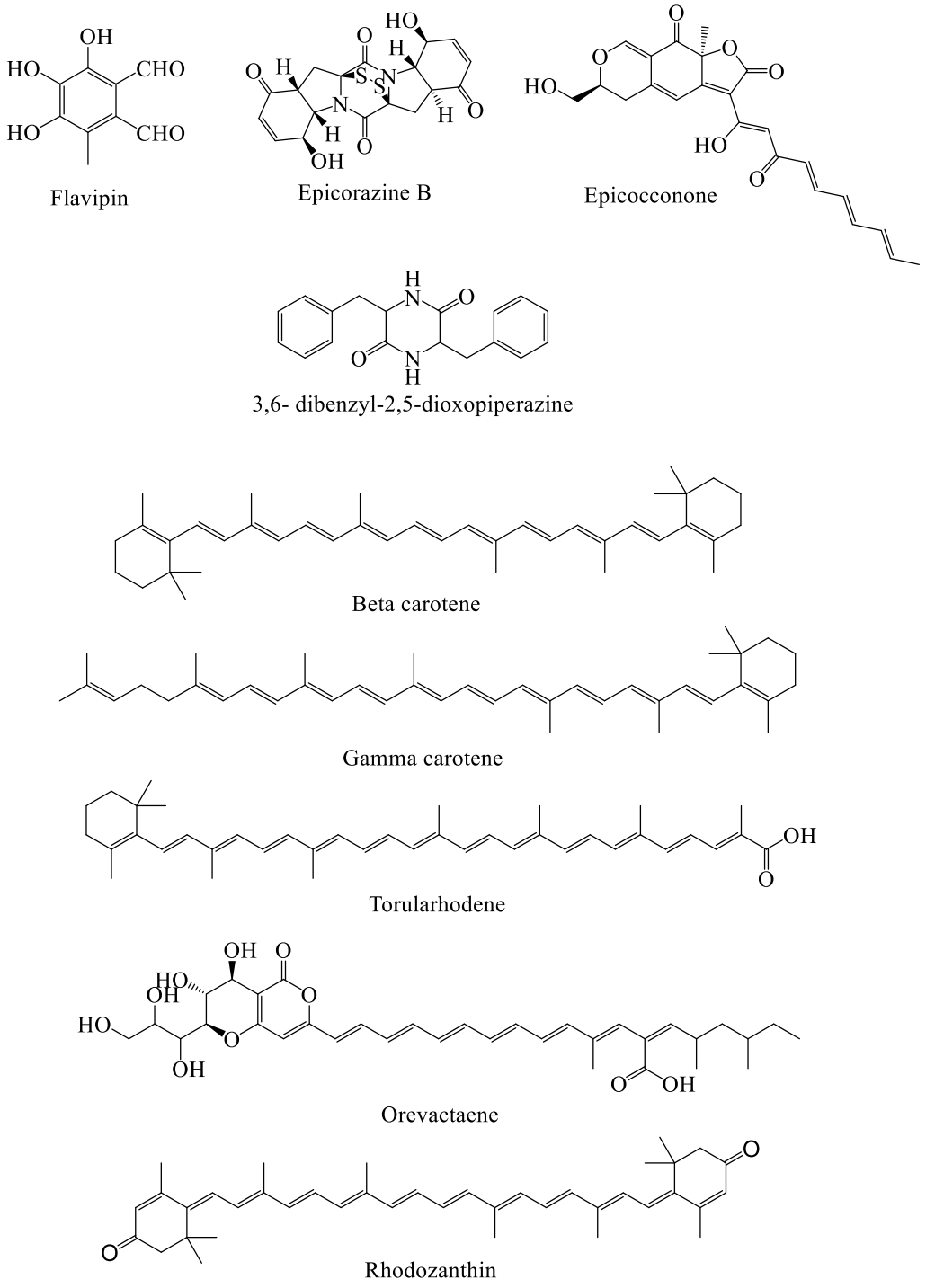

Figure 1.4. Natural products isolated from *E. nigrum.*

1.9 Fungus Culture Condition

Secondary metabolites produced from different sources play a very important role in medical, pharmaceuticals and other biochemical sectors. Thus, the discovery of new drug scaffolds keep researchers looking for new microbial sources of bioactive natural products.[79] Fungi are widely known to produce secondary metabolites, but there are some more factors that play an important role in the production of metabolites. Different types of media as potato dextrose media, yeast extract media, malt dextrose media etc. are used for the growing of metabolites from fungi. Due to the difference in composition of different media, the production rate of metabolites varies in different media. To produce some targeted metabolites specific media must be used. For example, Czapek's agar media was chosen for the production of a blue fluorescent component from *Aspergillus flavus.*[80] Another fungus *Lyophyllum shimeji* cultured on rye extract media to produce more fruiting bodies.[81]

1.10 Other Fluorophores from Fungi

In 1925 two scientists Matsumoto and Tomoyasu isolated a deep red pigment Cercosporin from a fungus of *Cercosporina* species named *Cercosporina Kikuchii Matsumoto et Tomoyasu*. This pigment turned in to green on treatment with alkali and showed bright yellow on reduction with green fluorescence.[82] In 1977, Wong and Bau reported pigment formation property in *Monascus purpureus.*[83] Chinese people used this fungus in food and textiles for millennia. Later Wang and Philip detected monoascidin A, a yellow pigment,[84] which showed antibiotic activity against *Bacillus, Streptococcus,* and *Pseudomona*s. Several fungi are known to produce fluorescent pigments such as *Aspergillus flavus* produces aflatoxin.[80] Hetherington and Raistrick, isolated ciritinin from *Penicillium citrinum* in 1931 which showed fluorescence properties.[85,86] Recently several fungi such as *Scytalidium cuboideum, Scytalidium ganodermophthorum, Chlorociboria aeruginosa, and Chlorociboria aeruginascens* are using as good source of pigment production and also for analysing art pieces.[87]

Fluorescent stains have diverse application over traditional colour and radioactive labels. A variety of instrumentations are available for the detection and quantification of fluorescence such as fluorescence microscopes, fluorometers, fluorescent labelling, biological detector, indicator as intracellular pH, and flow cytometers. Despite the widespread use of fluorescence techniques, very limited number of fluorochrome probes are in use. So there is a need for new fluorophores that can be used to enhance the field of application in multiplex assays [35]. Fungi like *Epicoccum nigrum* is a mitosporic mould, its use is versatile in the production of secondary metabolites like epicoccins, difenylalazines or epicorazines, compounds contain (thio)diketopiperazine skeleton . Its availability and versatility in both phylogeny and physiology make it more suitable for analysis. Also *E. nigrum*

used as a source of novel fluorescent stain as Epicocconone. Also, the study only on *E. nigrum* is seems like accumulating knowledge over the whole *Epicoccum* species.

And of course, searching of new compounds from unusual or unexplored place is an innovative way that leads the researchers to realize the actual meaning of research. And the nature contains infinite stuffs that need to be explored. Finally, it can be predicted that exploring natural products from microorganisms, such as fungi *E. nigrum*, can be consider as relatively less explored resource for the discovery of new fluorophores.

1.11 Aims

Fluorescent stains are much more sensitive than traditional colourimetric methods and safer than radioactive methods. A variety of instrumentations are available for the detection and quantification of fluorescence such as fluorescence microscopes, fluorometers and flow cytometers. Despite the widespread use of fluorescence techniques, very limited number of fluorochrome scaffolds are in use (Fig. 1.3). So there is a need for new fluorophores that can be used to enhance the field of application in multiplex assays.[35] The aim of this 9-month project was to assess the pigment production of *E. nigrum* on a variety of solid media and to translate the best agar method to liquid culture. If time permits, new pigments will be isolated and their structure(s) determined by spectroscopic studies.

2 Experimental

In this project optimization of culture condition will be performed using different solid microbial culture media and migrating these to liquid media. Different types of solid media like, Raw sugar Yeast extract Peptone Agar (RYPA), Czapek-Dox Agar (CzDA), Potato Dextrose Agar (PDA), Sabourad dextrose agar (SDA), Malt Dextrose Agar (MDA) media and Yeast Dextrose Agar (YDA) will be initially screened and used to analyse the production of pigments.[88]

We will start with the RYPA media for pigment analysis as this is known to produce good quantities of epicocconone but will also repeat the work on the above-mentioned media. The general plan is to grow the vegetative form of the fungus on an agar plate for 2-4 days at 25 C or until the plate is 80% covered. The plates will then be move to 4 C for 7 days and extracts (methanol) taken on a daily basis. LC-MS will be used to separate and analyse the pigments. UV spectra will be used to identify the pigments and MS the likely structure.

2.1 General experimental procedure

2.1.1 Chemicals/Materials

Bacteriological agar; Czapek-Dox Agar (CzDA), Potato Dextrose Agar (PDA), Sabouraud dextrose agar (SDA), Malt Dextrose Agar (MDA) media and Yeast Extract Peptone Dextrose Agar (YPD) were provided by Oxoid chemicals. Raw sugar yeast extract peptone agar (RYPA) media was prepared by using raw caster sugar, Bacto yeast extract (BD chemicals) and Bacto peptone (Difco) and finally bacteriological agar (Difco). Methanol (MeOH) and acetonitrile were purchased from Sigma-Aldrich, ethanol (EtOH) and MiliQ water was used to make up all media.

2.1.2 Instruments

Liquid Chromatography Mass Spectra (LCMS) was performed on an Agilent 1260 system, equipped with a Phenomenex Gemini C18 column (150 × 2 mm, 3 μm) in combination with an Agilent 6130 single quadrapole mass detector. A standard LCMS gradient (10-100% acetonitrile with an elution rate 0.5 mL/min over 25 mins) was applied for all LCMS work.

High performance liquid chromatography was conducted on Agilent 1260 infinity quaternary HPLC system equipped with a G1311B quaternary pump and G4212B diode array detector. For analytical HPLC performance a Phenomenex Synergy Hydro-RP 80 Å (250 × 4.6 mm, 4 μm) column was used with a solvent system of 10-100% acetonitrile, 1 mL/min rate for 25 min and semi preparative analysis was conducted on the column Phenomenex Gemini C18, 110 Å (250 × 10 mm, 10 μm) column eluted at 4 mL/min under the same gradient (10-100% acetonitrile over 25 min). Preparative HPLC was conducted on a Gilson 215 Liquid Handler using a Phenomenex Synergy Hydro-RP (250 × 21.20

mm, 10 μm) isocratically with the solvent system of 5% acetonitrile (0.01% TFA) and 95% water (0.01% TFA), rate 4.0 mL/min for 35 min with UV detection (254 nm).

Microscopic analysis (Sec. 6.2) was conducted on a Motic BA300 microscope with colour corrected infinity system.

For the sterilization of media and glassware autoclaving (Getinge PACS 2000) was used at 121 °C for 20 min.

^{1}H-NMR and ^{13}C-NMR were obtained by Bruker Avance AV III-500 MHz spectrometer at 25 °C. The spectra were recorded in 5 mm pyrex tubes (Shigemi, Japan) in DMSO-d_6 and processed on Bruker Topspin 3.5 pl7 (Bruker, Germany).

2.2 Fungal material collection

The fungus *Epicoccum. nigrum* was originally isolated from wild by Jian and Bell and this fungus was revived form frozen stock.

2.3 Growth and preservation of *E. nigrum*

2.3.1 Growth on solid media

Growth of *E. nigrum* on agar media took place mainly on petri dishes (9 cm diameter). Aseptically ½ cm^2 block of mycelial cell was cut from the edge of an *E. nigrum* agar culture plate with the help of disposable sterile blade and placed with the fungus side down onto the middle of the new agar media plate, enclosed with parafilm and incubated for 4 day at 25 °C and stored at 4 °C.

2.3.2 Preparation of glycerol stock of *E. nigrum*

For preparing glycerol stock of *E. nigrum*, 250 mL of glycerol (Biotechnology Grade, Biochemicals) mixed with 250 mL MiliQ water to obtain 50% of glycerol solution and sterilised in an autoclave at 121 °C for 20 mins and stored at room temperature. *E. nigrum* was inoculated from 5 × RYPA plate (4 days old) by cutting into small pieces with a scalpel and placed aseptically in 500 mL Pyrex baffled shaker Erlenmeyer flask containing 100 mL of sterilized RYPB broth (42.5 g/L raw sugar, 12.5 g/L yeast extract and 25 g/L peptone). The fungus was allowed to grow in incubator shaker at 25 °C, 130 rpm for 2 days. This grown culture was sub-cultured by transferring 20 mL of the culturel solution into 2 × 500 mL baffled flask containing 190 mL of RYPB broth and incubated under the same condition for 2 days. After that 120 mL of fungal culture was aseptically mixed with 80 mL of 50% glycerol solution and mixed well. Aliquots of 1.5 mL of the glycerol stock was transferred to 80 × 2 mL sterile cryogenic vials aseptically by using 1 mL Scilogex autoclavable single channel variable pipette and the stocks stored at -80 °C.

2.4 Optimization of pigment producing conditions

The fungus *E. nigrum* from the glycerol stock was grown on six different agar media (Sec. 2.1.1) in an incubator at 25 °C for 4 days and then the plates (7 × 6) were shifted to 4 °C. One plate from each type of medium was removed from the cold temperature every day for seven days. Each removed plate was extracted (6 × 5 mL methanol) over 12 h. Each extract was allowed to soak for 2 h and then combined extracts (30 mL) stored at -80 °C.

The methanol extracts were filtered (0.22 mg) and analysed by LCMS (Sec. 2.1.2) to compare the pigment production over 7 day on 6 different media (Sec. 3.2).

2.4.1 Cultivation and Extraction of *E. nigrum* in RYPA media

RYPA broth (RYPB) was prepared according to the supplementary material (Sec. 6) and sterilized in autoclave at 121 °C for 20 mins. After that 100 mL of RYPB poured in 40 × 500 mL baffled flask in a safety cabinet and again sterilized in autoclave as before. The cultured RYPA plates of *E. nigrum* that had been growing for 4 days at 25 °C and an additional 5 days at 4 °C were used to inoculate the liquid cultures.

The inoculation was created by mixing RYPB (3 mL) with the fungus scraped from each plate (total 40 plates) in a biosafety cabinet. The mixture was Dounce blended and 40 × 500 mL baffled flasks inoculated with 120 mL of inoculum. Then the flasks were incubated on Thermoline orbital shaker (135 rpm) at 25 °C for 2 days, then at 4 °C for another 5 days (200 rpm).

After seven days, the flasks were collected and filtered with Bucher funnel using Advantec 185 mm filter paper in a fume hood. The solid biomass was washed with water (3 × 60 mL) and frozen (-80 °C) and lyophilized (Cheris, Alpha 1-4 LD Plus)

The mass of the dry biomass was recorded and ground finely in a mortar and pestle (fume hood) and then packed into a glass chromatography column (15 × 40 cm). MeOH was percolated as a solvent slowly through the column for 2 days with the extract kept at -80 °C (acetone/dry ice) and under nitrogen. The combined extracts were partitioned against hexane, to remove any fatty materials and the methanol fraction reduced to dryness (rotary) and weighed.

The waxy precipitate formed on concentration was separated with a C-18 Sep-Pak (Grace) with 50% methanol and eluted with 80% methanol.

The residue was dissolved in MeOH (5 mL) and applied to a Sephadex LH-20 column (88 × 2 cm) and eluted with MeOH (14 hours) to yield 100 fractions. Each fraction was analysed by LCMS and similar fractions combined to yield three main fractions. Crude fractions were stored at -80 °C.

2.5 Isolation of metabolites

In total 5 batches of extractions were conducted. Thus, the cultivation and extraction process (mentioned above) was done for 5 times. The first 3 batches and next 2 batches were mixed separately and run in the Sephadex column separately. The collected fractions were divided in 3 fractions, as red, purple, and light red and analysed their LCMS data. The red fractions were found to contain largest amount of material (Table 4). The red and purple fractions were sub-fractionated on a Gilson HPLC by semi-preparative column Gemini C18 (Sec. 2.1.2) and analysed by ^{1}H and ^{13}C NMR.

2.6 Waste disposal

All the waste media plates, biological pipette, cryogenic vials, filter paper and other biological wastes were collected in a bin and autoclaved at 121 °C for 20 min. After completing the autoclaving, the waste was collected and transferred in another autoclave bin for disposal. All the sharps (surgical blades, needles) were collected in a small yellow bin and transferred to medical waste disposal bin for disposal.

3 Results and Discussion

Microorganisms can generate a large verities of secondary metabolites but the number and concentration of these metabolites depends on the growth conditions, including medium,[89-90] temperature,[91] atmosphere,[92] light[93] and many other factors.[94] Accordingly, the emphasis of this research work was to focus on the best medium for pigment production in the fungus *Epicoccum nigrum*.[11] The effects of various media, temperature and time were assessed relative to the production of pigments with the aim of isolating new pigments from this fungus.

3.1 Culture of *E. nigrum* on 6 different media

Media in agar plates were prepared according to supplementary material (Sec. 6.1). Seven plates for each medium were prepared in a safety cabinet and then stored at 4 °C. The plates were inoculated with *E. nigrum* from glycerol stocks (−80 °C). After incubation at 25 °C for the 4 days the plates were moved to 4 °C and one plate was extracted with methanol each day for 7 days (Sec. 3.2). LCMS analysis showed the production of secondary metabolites during the 7-day incubation period (Sec. 3.2).

Analysis on the LCMS results indicated the presence of five major metabolites (**A**, **B'**, **B**, **C** and **D**; Sec. 3.2).

3.2 Results for various media

3.2.1 MDA media

3.2.1.1 Culture in agar media

E. nigrum cultured Malt Dextrose Agar (MDA) (pH 5.4 ± 0.2) medium was evaluated over 7 days (Fig. 3.1). The change in colour indicated the production of pigments after day 4.

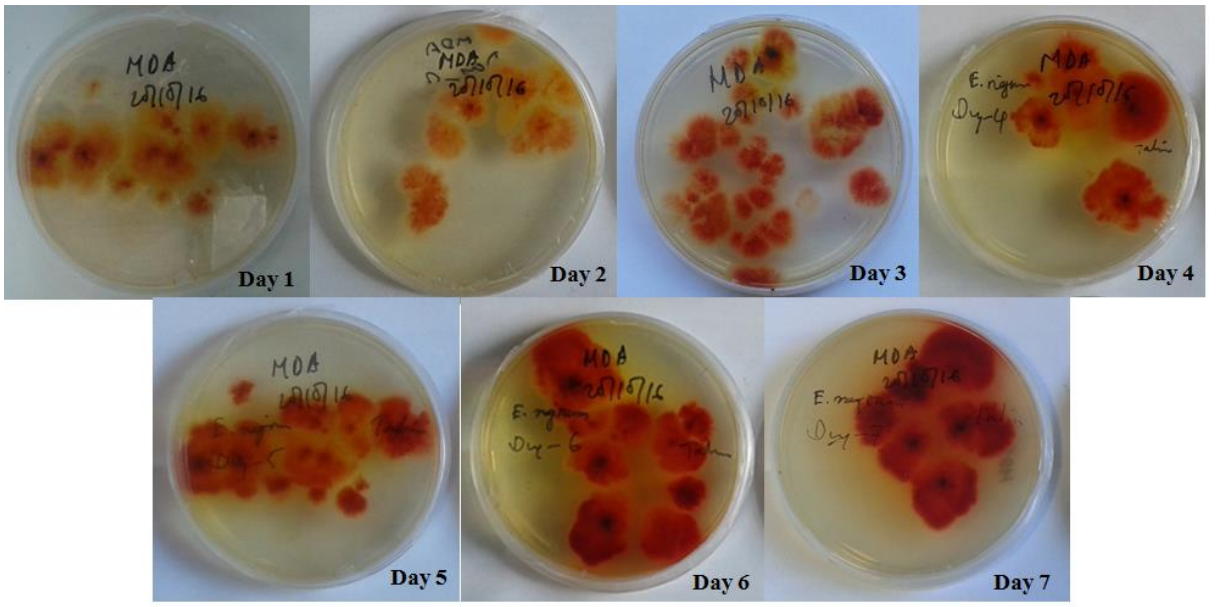

Figure 3.1. 7-day culture of *E. nigrum* in MDA after 4 days at 25 °C.

3.2.1.2 UV analysis of seven-day culture

Plates were extracted with methanol (MeOH) at day 1-7 by adding 5 mL of solvent to the plate and incubating at room temperature for every 2 h. Total 30 mL solvent applied for incubating 12 h for each plate. Observing the MeOH extract under long wave (365 nm) ultraviolet light (Fig. 3.2) indicated the production of green fluorescent pigment on day 1 and an orange fluorescent pigment on day 7.

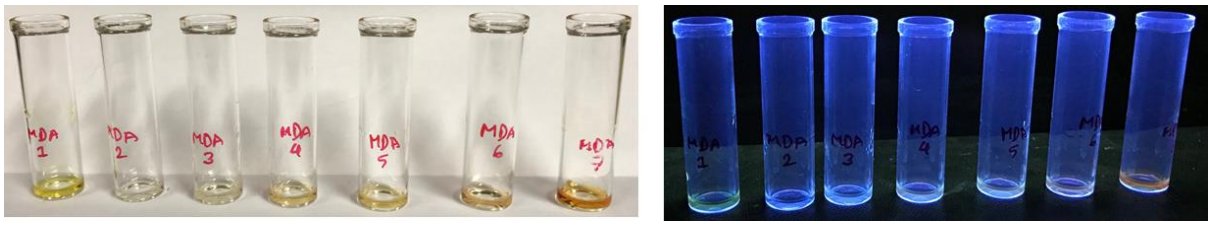

Figure 3.2. Analysis of extractions under UV-light.

3.2.1.3 LCMS analysis of seven-day culture

By LCMS on extracts, on MDA medium. *Epicoccum. nigrum* produced 4 major compounds (Fig. 3.3). Compound **A** was constant whereas **B** and **D** were produced on day 1 but decreased over time. **B'** was only observed at low level from day 4 onwards. Compound **C** was the most concentrated on the last day and production began only after day 3.

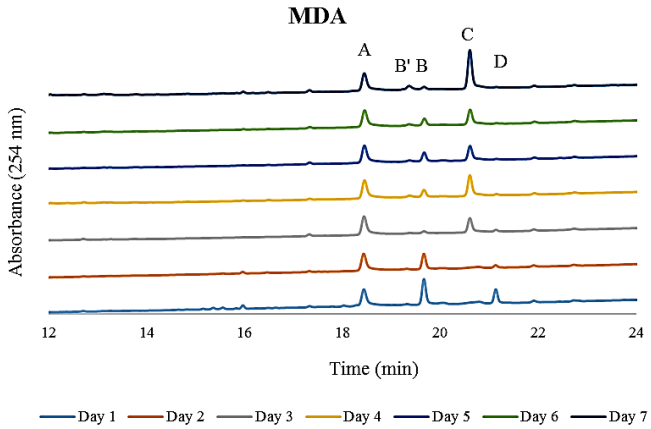

Figure 3.3. LCMS (Phenomenex Gemini, 0.2 mL/min) of seven-day *E. nigrum* on MDA medium (λ_{max} = 254 nm).

3.2.2 PDA media

3.2.2.1 Culture in agar media

Potato Dextrose Agar (PDA) media (pH 5.6 ± 0.2) of *E. nigrum* showed very bright and deep colour from the day 1. The change in intensity of colour during this period was easily visible from the agar plate (Fig. 3.4).

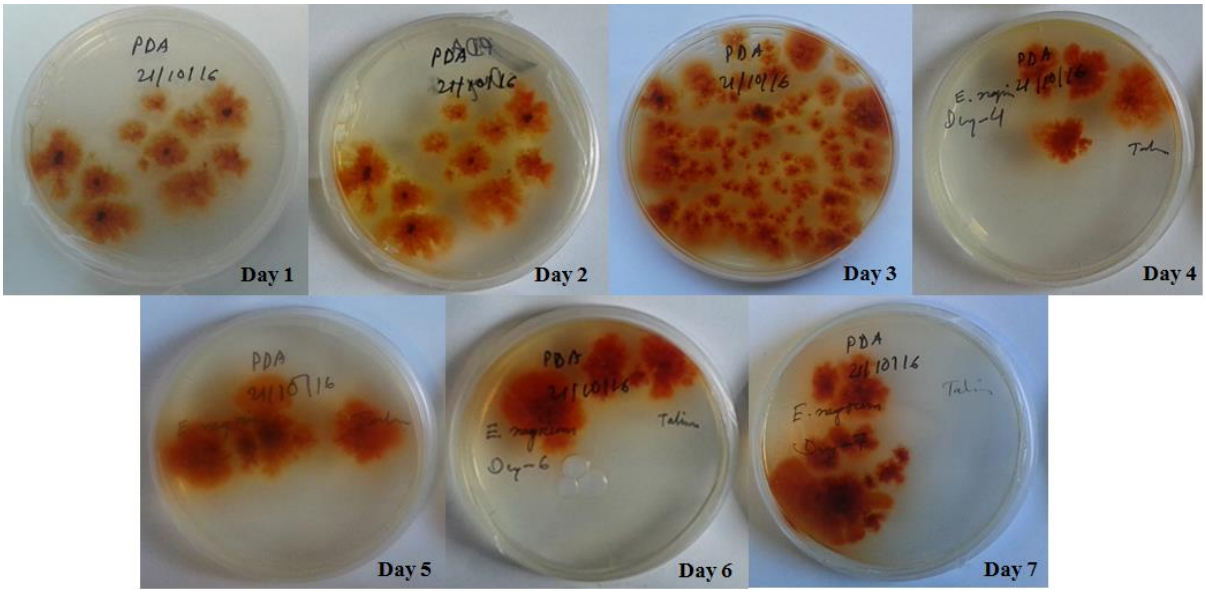

Figure 3.4. 7-day culture of *E. nigrum* in PDA after 4 days at 25 °C.

3.2.2.2 UV analysis of seven-day culture

On the 1st day the colour was light orange and in the 2nd day was colourless but under UV there was no noticeable change (Fig. 3.5). On the 3rd day green fluorescence was obtained under UV light from a yellow coloured extract. Day 4, 5 and 6 showed greenish orange, orange, and bright pink fluorescent respectively.

Figure 3.5. Analysis of extractions under UV-light.

3.2.2.3 LCMS analysis of seven-day culture

The LCMS results of *E. nigrum* in PDA media showed quite similar result to MDA media. The presence of 4 major compounds **A**, **B**, **C**, and **D** were obtained but their abundance varied from day to day (Fig. 3.6). A was constant from day 1 to 7 but **B** was observed from day 3 and gradually

decreased to day 7. Compound C was also constant but seemed to vary from day-to-day, whereas **D** was only noticeable on day 1 and 6.

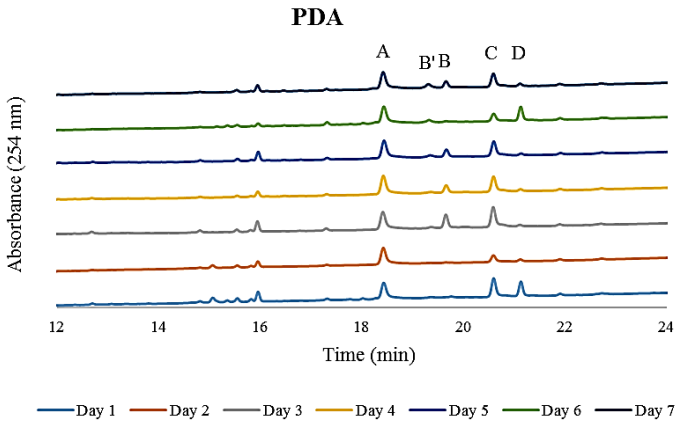

Figure 3.6. LCMS (Phenomenex Gemini, 0.2 mL/min) of seven-day *E. nigrum* on PDA medium (λ_{max} = 254 nm).

3.2.3 SDA media

3.2.3.1 Culture in agar media

Sabouraud Dextrose Agar (SDA) media (pH 5.6 ± 0.2) media showed the change of colour intensity from day 1 to day 7. It clearly indicated the production of pigment producing spores increased with time (Fig. 3.7).

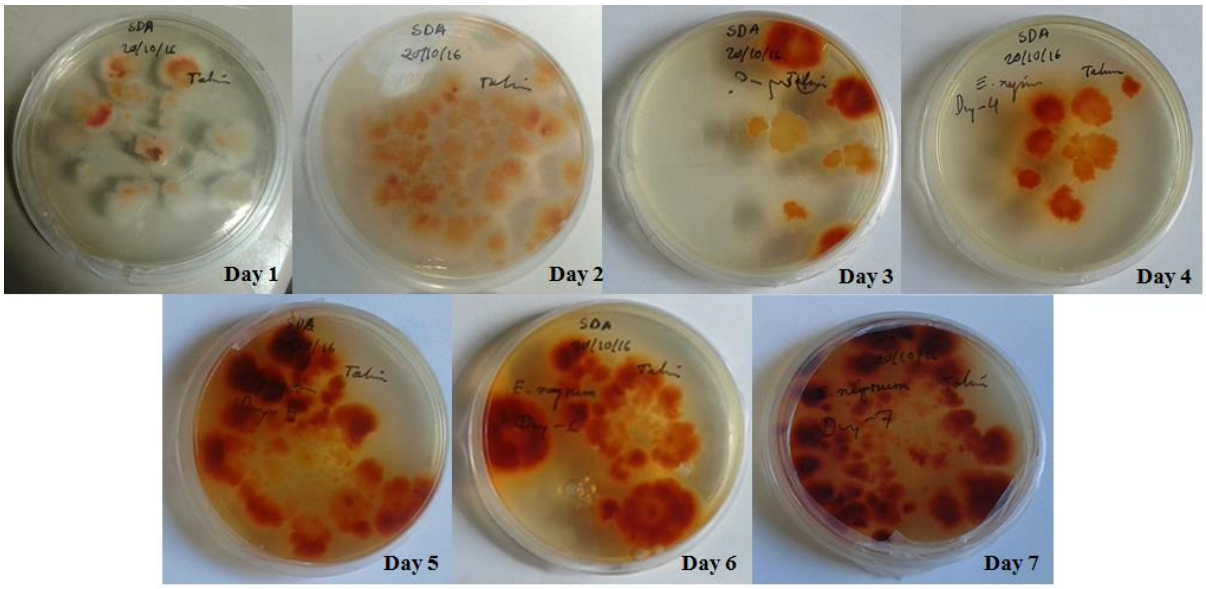

Figure 3.7. 7-day culture of *E. nigrum* in SDA after 4 days at 25 °C.

3.2.3.2 UV analysis of seven-day culture:

Analysis of the extraction from SDA showed the increasing intensity of pink/red colour. That was obtained in the UV-light also. Day 1, 2 and 3 showed almost colourless solution but in UV light day

2 and 3 were found to produce light green fluorescent. Day 4 and 5 showed very light orange and orange coloured solution but in UV, light orange and pink fluorescent was obtained respectively. Day 6 displayed light red colour that was orange fluorescent in UV and Day 7 produced the deep pink/red fluorescent (Fig. 3.8).

Figure 3.8. Analysis of extractions under UV-light.

3.2.3.3 LCMS result of seven-day culture:

LCMS results from SDA medium of *E. nigrum* produced A in each 7-day culture but with the slight decrease in abundance. The existence of **B'** was noticeable from day 5 onwards. Compound **C** and **D** were produced in day 1 and 6 but **C** was observed in large extent on last 3 days (Fig. 3.9).

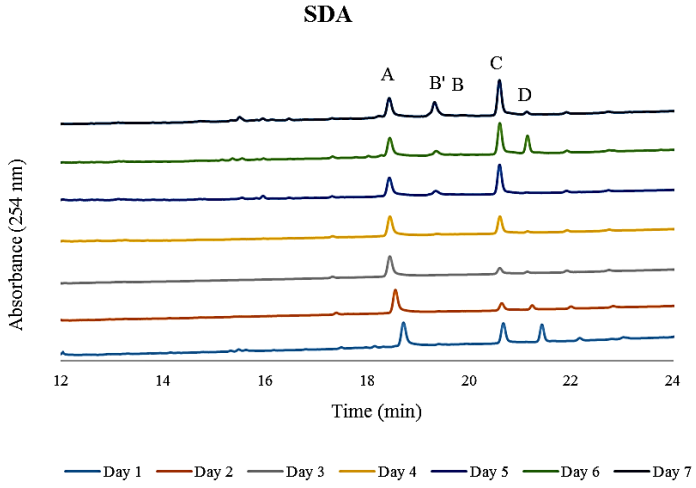

Figure 3.9. LCMS (Gemini, 0.2 mL/min) of seven-day *E. nigrum* on SDA medium (λ=254 nm).

3.2.4 YPD media

3.2.4.1 Culture in agar media

Yeast extract Peptone Dextrose (YPD) agar media (pH 6.5 ± 0.2) of *E. nigrum* showed intensive colour form the day 1(Fig. 3.10). The increased colour intensity was obtained in next 7 days.

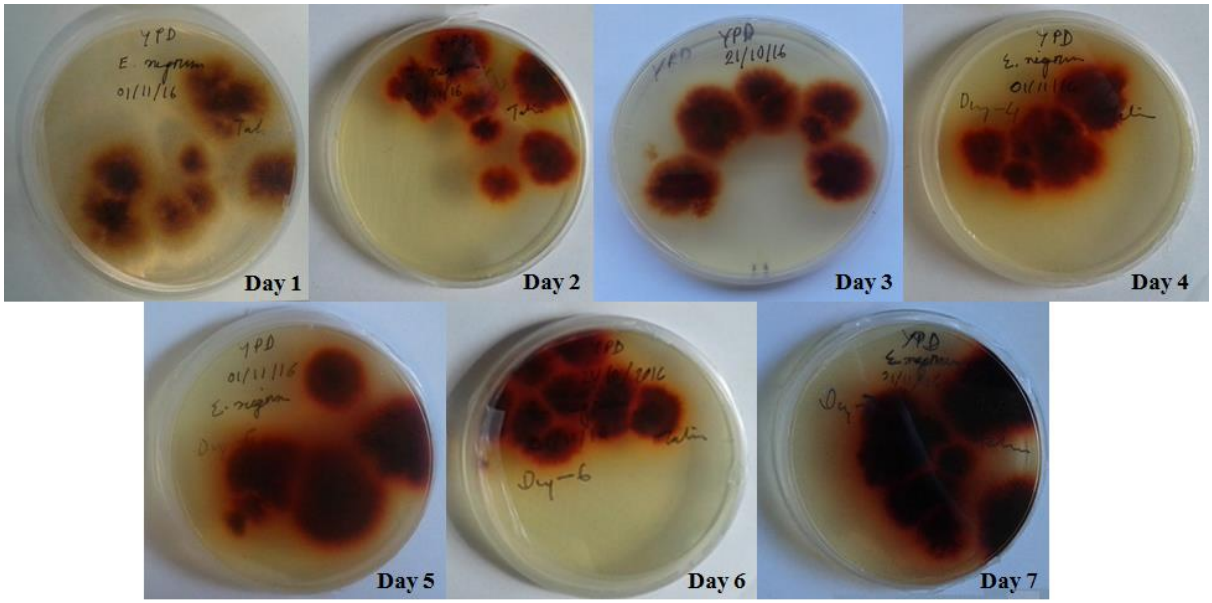

Figure 3.10. 7-day culture of *E. nigrum* in YPD after 4 days at 25 °C.

3.2.4.2 UV analysis of seven-day culture:

The extraction from YPD media showed a beautiful trend of changeable coloured pigment from day 1 to 7 (Fig. 3.11). Day 1 and 2 showed green fluorescent in UV, whereas light orange and intense orange solution of day 3 and showed yellowish green and orange fluorescent. The increasing intensity of red pigment production was obtained from day 5 to day 7 that produced red fluorescence with increasing intensity.

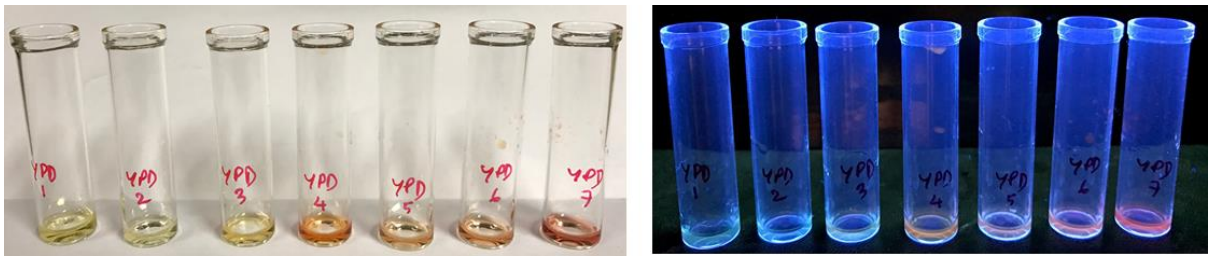

Figure 3.11. Analysis of extractions under UV-light.

3.2.4.3 LCMS result of seven-day culture

LCMS result of *E. nigrum* in YPD media showed the constant existence of metabolites **A** and **B** constantly for 7 days (Fig. 3.12). Compound **D** was obtained in day 1 and 4 but **B'** was found from day 5 to 7. Metabolite **C** showed very low intensity during these 7 days.

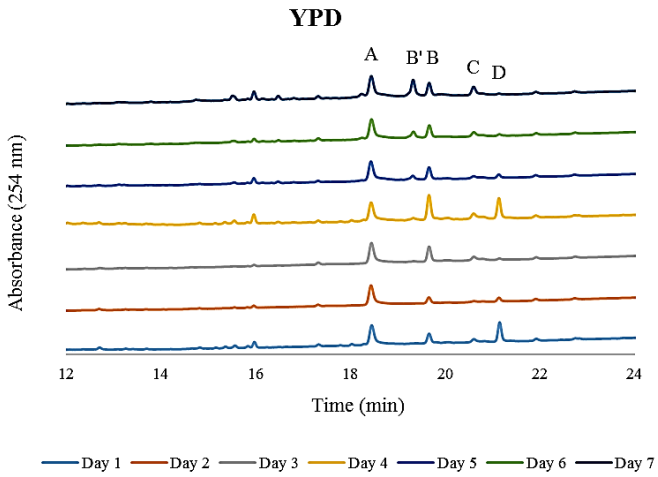

Figure 3.12. LCMS (Gemini, 0.2 ml/min) of seven-day *E. nigrum* on YPD medium (λ=254 nm).

3.2.5 CzDA media

3.2.5.1 Culture in agar media

Epicoccum nigrum culture in Czpak Dextrose Agar (CzDA) media (pH 6.8 ± 0.2) showed to produce yellow colour from day 1 to day 3 (Fig. 3.13). Then From day 4 red pigment started producing and the growth increased till day 7.

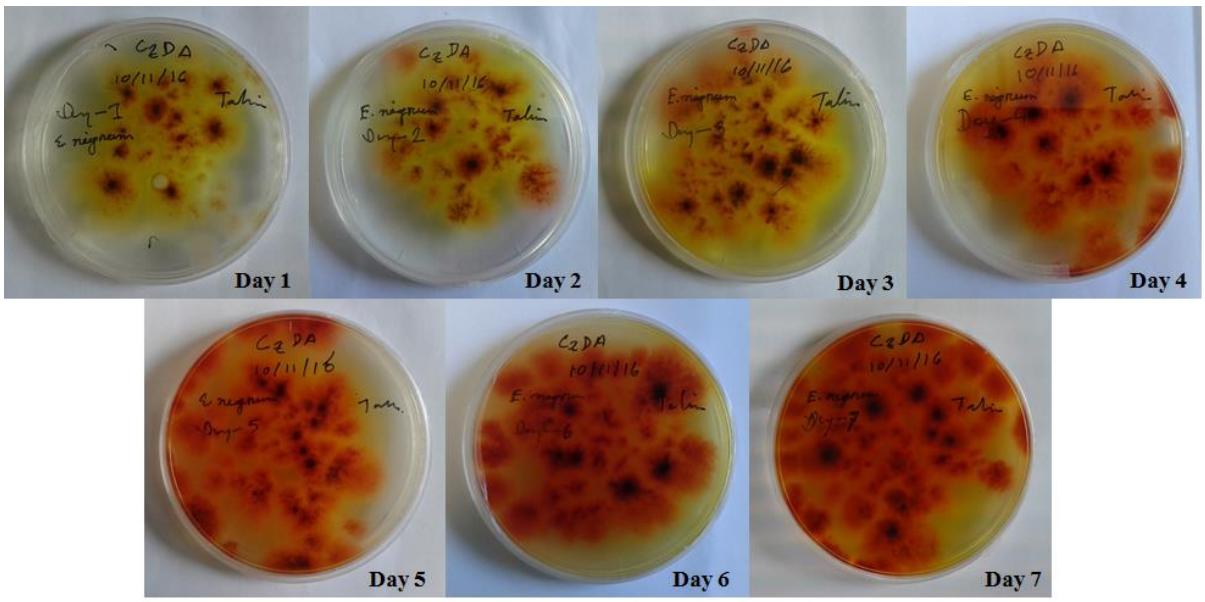

Figure 3.13. 7-day culture of *E. nigrum* in CzDA after 4 days at 25 °C.

3.2.5.2 UV analysis of seven-day culture

Extractions from CzDA media plate showed almost same colour (Fig. 3.14). From day 1 to day 4 the green colour intensity increased and reduced in day 5 and 6. But day 7 exhibited orangish green.

Under UV light, day 1 was almost colourless and the green fluorescent intensity increased to day 5. Day 4 to 7 showed almost similar type of fluorescent in UV.

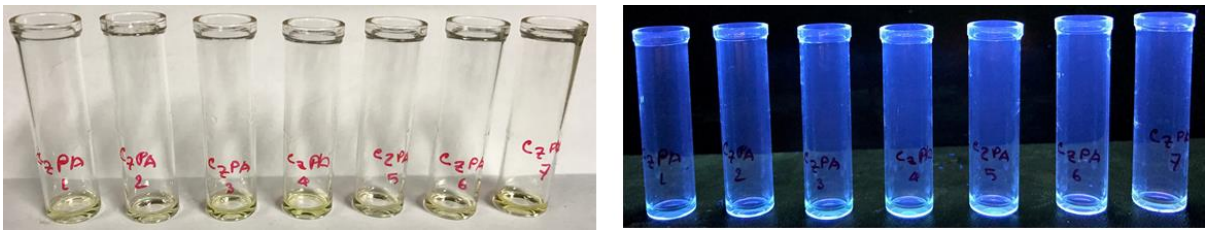

Figure 3.14. Analysis of extractions under UV-light

3.2.5.3 LCMS result of seven-day culture

CzDA media of *E. nigrum* showed very low existence of mentioned metabolites (Fig. 3.15). The presence of **A** and **B** was found to consistent from day 1 to 7 and metabolite **D** obtained with low intensity in day 1 and **C** was absent.

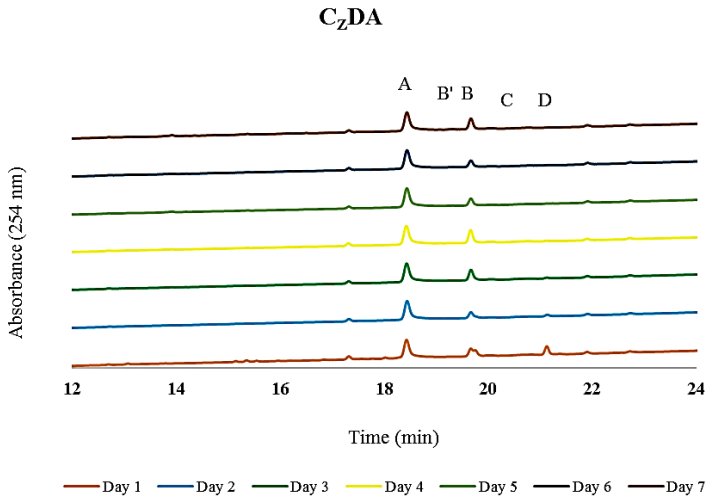

Figure 3.15. LCMS (Gemini, 0.2 ml/min) of seven-day *E. nigrum* on CzDA medium (λ=254 nm).

3.2.6 RYPA media

3.2.6.1 Culture in agar media

Raw sugar Yeast extract Peptone Agar (RYPA) media showed excellent pigment production from day 1 to day 7 (Fig. 3.16). The colour intensity found to increase untill day 5.

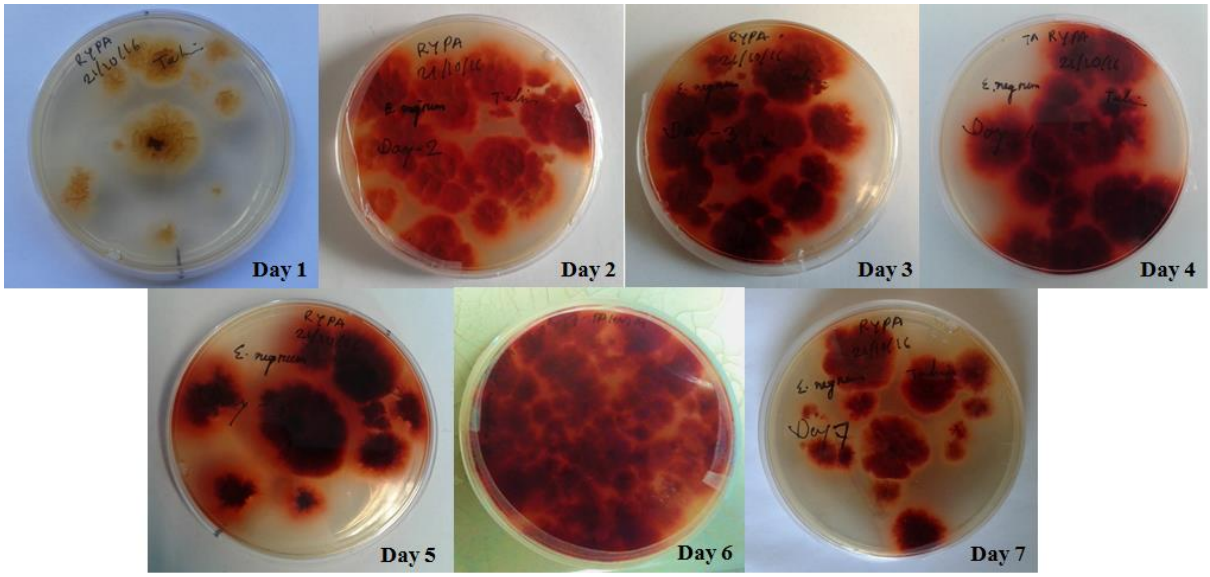

Figure 3.16. 7-day culture of *E. nigrum* in RYPA after 4 days at 25 °C.

3.2.6.2 UV analysis of seven-day culture

All 7-day RYPA media exhibited different types of pigment production (Fig. 3.17). From day 1 extraction yellowish green colour fluorescent. Day 2, 6 and 7 showed very light pink colour solution and similar fluorescent in UV. The most noticeable red colour fluorescent was obtained from day 3 to day 5 with the high intensity in day 5.

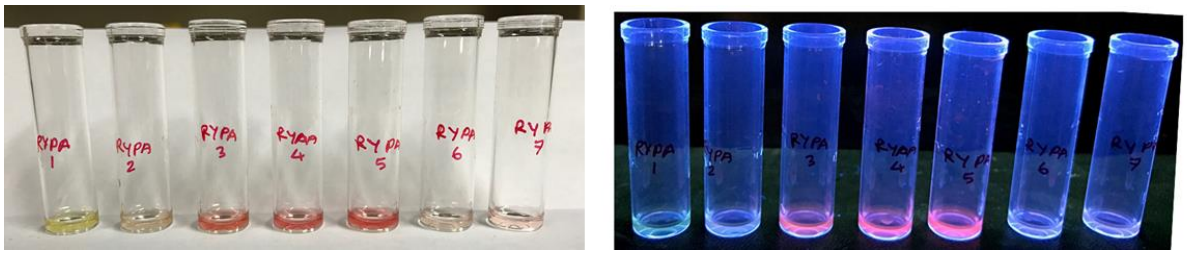

Figure 3.17. Analysis of extractions under UV-light

3.2.6.3 LCMS result of seven-day culture

LCMS analysis of 7-days *E. nigrum* extraction in RYPA media showed the presence of **A** and **B'** and **C** in all seven-day extraction (Fig. 3.18). Component **D** showed its existence only in Day 1. The intensity of **C** was observed higher than any other media.

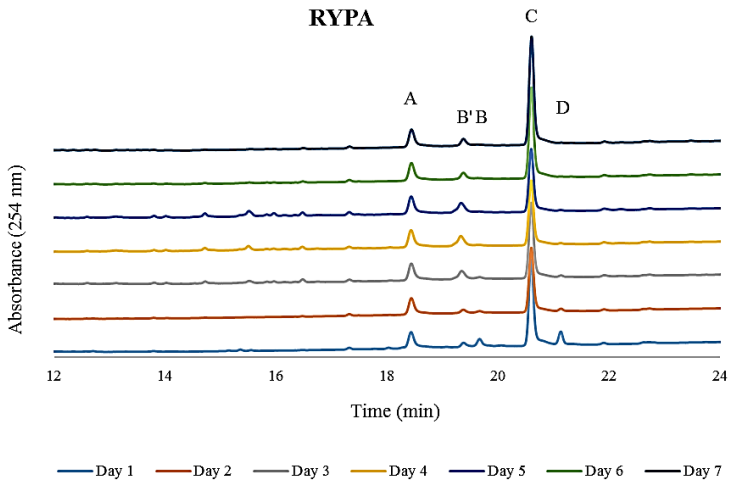

Figure 3.18. LCMS (Gemini, 0.2 ml/min) of seven-day *E. nigrum* on RYPA medium (λ=254 nm).

The most noticeable part is that different media showed the production of same types of metabolites with different intensities and different types of absorbance from day one to day seven. So, it reveals the effect of different type of media on the growth of *E. nigrum*.

3.3 Measuring of solid content from each media

Extraction of all media plates was continued for 12 h with 30 mL of methanol each day. So, depending on the pigment production ability, the extracted solid content of individual media plates varied from 0.03 to 1.15 mg. The combined MeOH extracts were evaporated and weighed (Fig. 3.19). No specific trend was observed in the dried weight of extract.

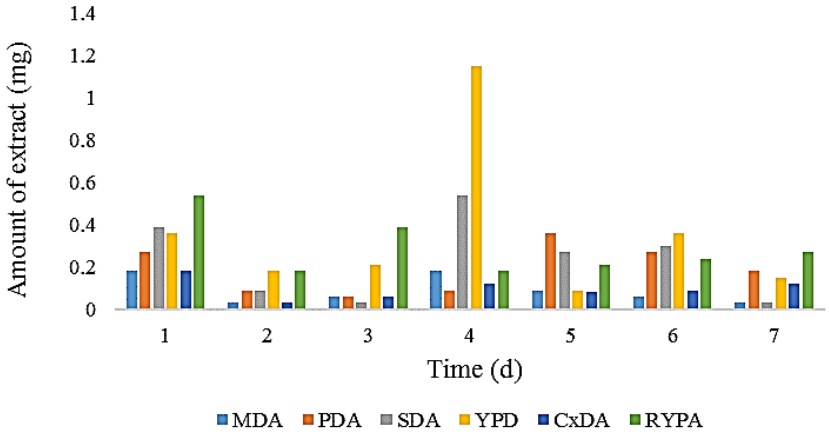

Figure 3.19. Solid content of each media.

3.4 UV-Vis spectra of the metabolites

From LCMS data, the wave length (λ_{max}) of metabolites **A**, **B**, **B'**, **C** and **D** were found to quite different, ranging from 240-440 nm with the molecular weights (*m/z*) of 124, 411, 558 and 248 amu respectively (Table 1). Among all the wave length only component **B** showing absorbance in the visible region and *m/z* value indicated the presence of epicocconone as component **B**.

Table 1: Analysis of spectral behaviour of 4 major metabolites.

Components	**λ_{max}**	**Predictable *m/z***
A	255	124
B	440	411
B'	440	520
C	285	558
D	240	248

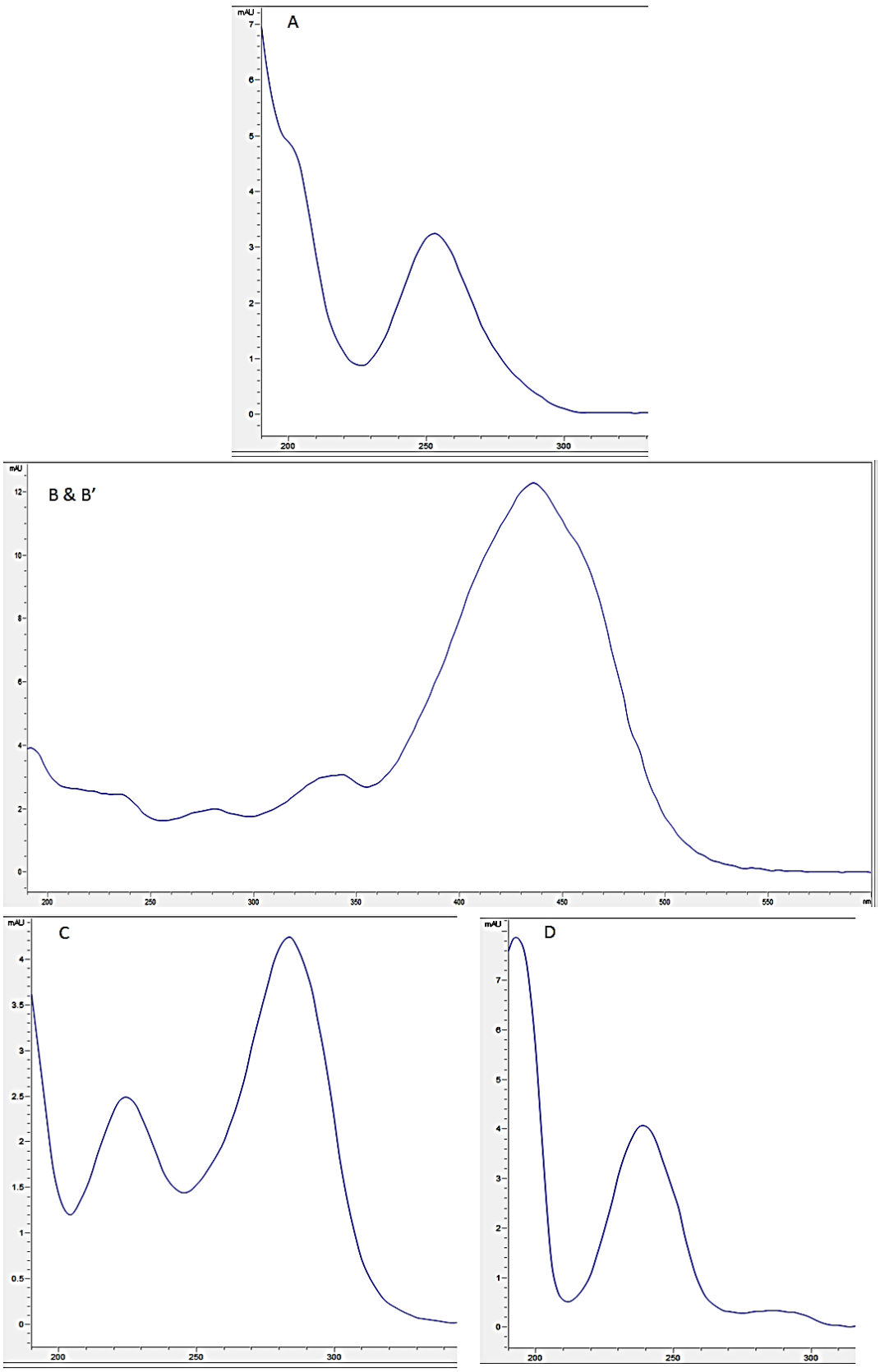

Figure 3.20. UV spectra of metabolites **A**, **B**, **B'**, **C** and **D**.

3.5 Extraction from RYPA media

RYPA media was selected for the liquid culture of *E. nigrum* because of the attractive red fluorescence and intensity of the compound C (Fig. 18). Also, an interesting peak obtained at λ_{max} = 530 nm. In day 3,4 very low intensity red fluorescence was obtained in UV but day 5 showed deep red fluorescence. So, from all the cultures, day 5 considered to be the optimal growth time on agar plates and LCMS was conducted at 400 nm for the purpose of finding the red fluorescent component.

The plate conditions were transferred to liquid culture and 5 batches were grown and assessed for pigment production (Table 2).

Table 2: Inoculation and Extraction details

Batch No	First 2 days		Second 5 days			Biomass (g)	Extracted amount (g)
	Temperature (°C)	RPM	Temperature (°C)	RPM	Time (h)		
1	27	150	4	200	117	3.0	0.8
2	25.5	130	4	200	119	5.0	3.51
3	29	135	4	200	117.5	0.83	0.48
4	23.5	135	4	190	122	6.2	1.67
5	24	135	4	190	120.2	8.2	2.30

According to table 1, the 2nd batch was found to produce the largest amount of extract. Batch 4 and 5 also produced relatively good yields and batch 3 was particularly poor. This was due to poor temperature control on this occasion. These results suggest that initial growth temperature should be between 23-25 °C. Extracts were purified by size exclusion chromatography (Table 3) using Sephadex LH-20 with methanol. The pigments eluted last (Fig. 3.22) as a dark purple band.

Table 3: Solid content obtained from Sephadex fraction.

Batch no	Retention time per tube (mL/min)	Red fraction		Purple fraction		Diluted red fraction	
		Solid content (g)	Tube no.	Solid content (g)	Tube no	Solid content (g)	Tube no.
1+2+3	4	0.5184	25	0.0456	5	0.0296	3
4+5	2	2.2433	46	0.1254	18	0.0135	12

*Tube vol. 28 mL

3.6 LCMS analysis of each batch

LCMS analysis of each batch showed the presence of epicocconone, B (Fig. 3.21) as a peak with *m/z* = 411. The intensity of epicocconone was the highest in batch 5. The reason could be, that, for batch 5, a 21-day old *E. nigrum* agar plate was used to initiate growth on fresh agar plate rather than directly from glycerol stock. As conidiation of *E. nigrum* production improved at reduced water availability,[95] the old plates may have increased conidiation that was maintained in the liquid cultures.

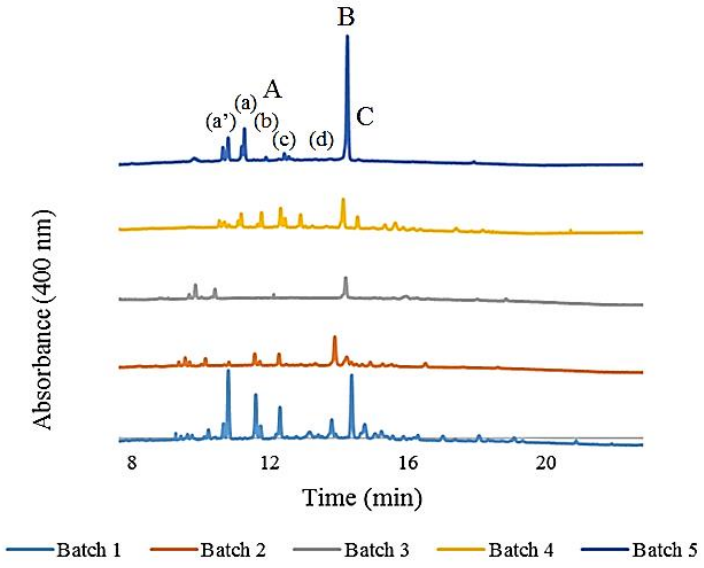

Figure 3.21. LCMS analysis of all extractions showing new metabolites a', a, b, c, and d.

The LCMS result of these extractions showed some interesting features. The presence of A, B and C metabolites were found but the intensity was variable. Also, the presence of some more components were visible in here (Fig. 3.21). These components were indicated as a', a, b, c, and d whereas 'a' obtained at λ_{max} 350 nm but the rest showed highest absorption at 530 nm. In every extraction, some additional peaks were also obtained but their absorption range and mass intensity was very low. So those were not taken in account for analysing the existence of fluorescent metabolites.

Additionally, metabolite A was present in batches 3 and 5 only. The highest peak was the peak of metabolite B, which showed λ_{max} at 440 nm and *m/z* = 411 and considered to be epicocconone (Table 4).

Table 4: Analysis the peaks with λ_{max} from extractions:

Components	**λ_{max} (nm)**	**Predicted MW from MS**
A	255	123
B	440	410
B’	440	519
C	285	557
D	240	246
a’	350	465
a	530	448
b	530	294
c	530	449
d	440	329

3.7 Separation of new component

Initial separation was carried out with size-exclusion column chromatography (Sephadex LH-20) using MeOH as eluent. Concentrated crude extract was eluted with methanol. A slight red colour eluted first. Containing highly fluorescent but high molecular weight polymers. Next, red and purple fractions eluted, which contained low molecular weight compounds. LCMS of each fraction was conducted and mixed on the basis of profile similarity. All fractions were divided into three groups; red, purple and light red. Semi-prep HPLC was used for purification of extracts based on absorbance at 254 nm.

Figure 3.22. Sephadex LH-20 column separation of *E. nigrum* extractions.

From HPLC, 13 fractions were collected with fraction 8 containing the most material (Table 5). This fraction was also lightly fluorescent (Fig. 3.24).

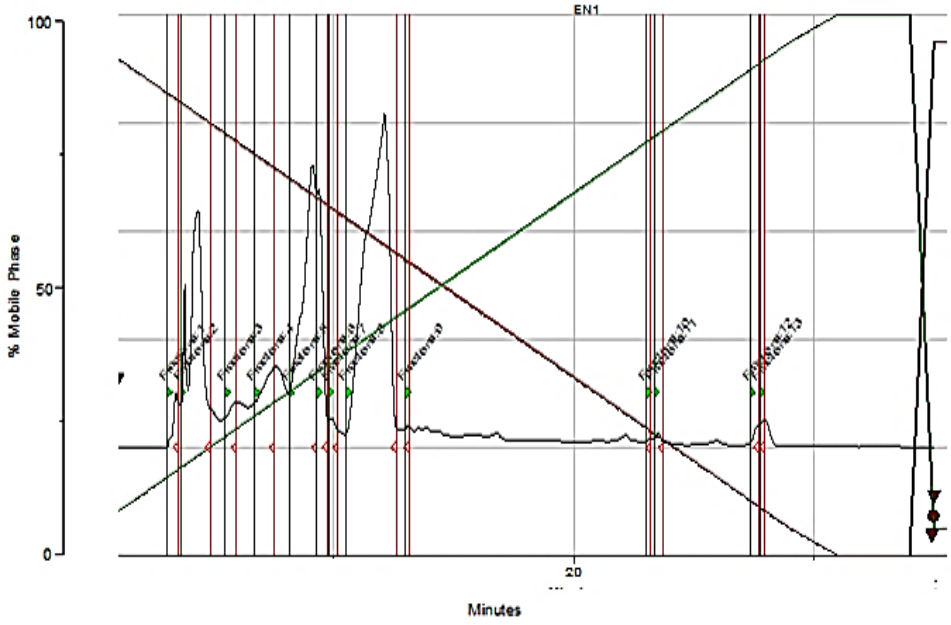

Figure 3.23. HPLC of the red fraction.

Table 5. Amount of solid content in each fraction form HPLC of the red fractions.

Fraction No.	Solid Content (mg)
4	1.3
5	1.5
6	1.5
7	1.4
8	7.8
9	0.9
10	0.2
11	0.4

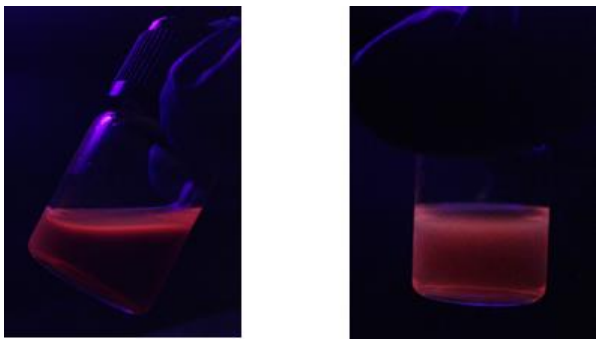

Figure 3.24. UV image of red fraction from Sephadex (left) and fraction 5 from HPLC (right).

From LCMS analysis, fraction 8 indicating the presence of a metabolite with *m/z* 298, indicative of a new compound containing an odd number of nitrogens. NMR analysis of ^{1}H and ^{13}C were carried out on this fraction (Sec. 6.4), which unfortunately proved to be a mixture of two compounds (Fig. 3.25). According to Figure 3.25, the presence of two compounds were clearly obtained at λ_{max} = 254 and 400 nm at retention time (t_R) 2.602 and 2.711 min.

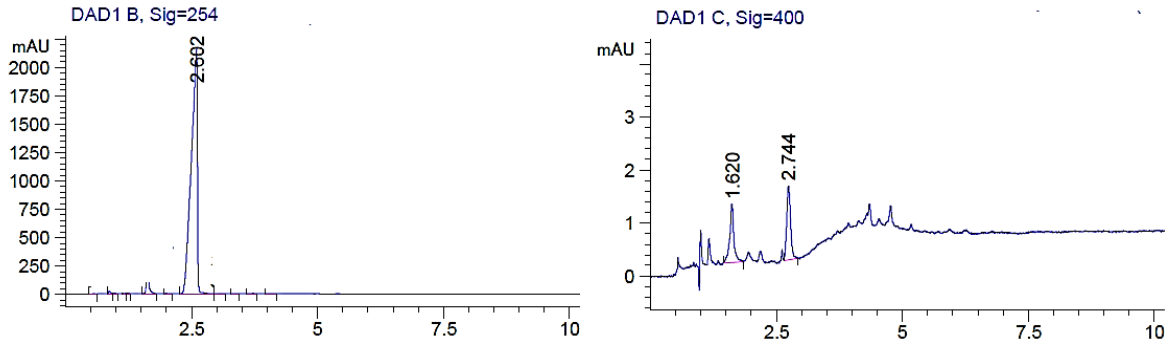

Figure 3.25. LCMS analysis of the compound (fraction 8) at 254 and 400 nm.

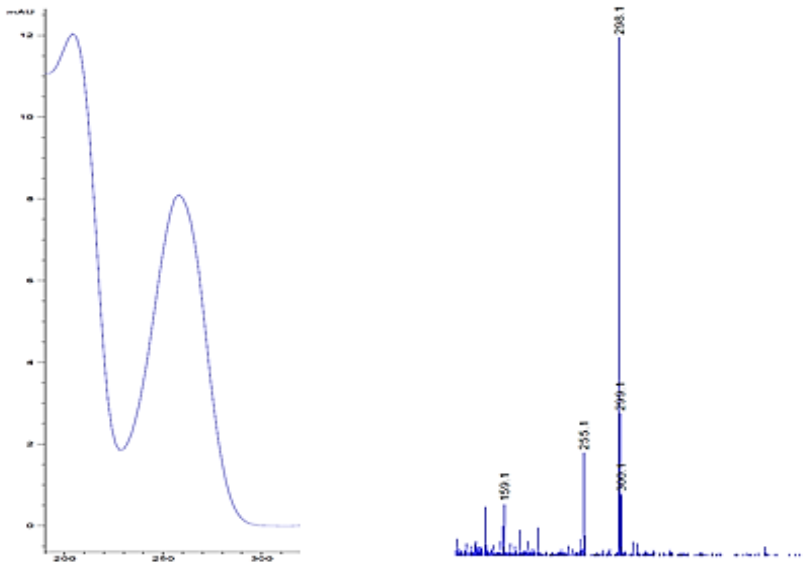

Figure 3.26. UV and MS of the isolated compound (t_R = 2.602 min).

3.8 Summary

The target of this research was to develop the best medium for growing *E. nigrum* to produce new metabolites and also to try to isolate and analyse new fluorescent compounds. Growing fungal cultures is a generally time consuming task and maintenance of aseptic condition for 1-2 weeks is a requirement[96]. During extraction and separation temperature needs to be kept low and oxygen excluded. Our results show that a small change in cultivation temperature can cause large changes in metabolite yields. It took quite a while to perfect the aseptic techniques, without adding any antibacterial agents. The RYPA medium was selected because it had the best pigment production. It is noteworthy that in the view of producing the known compound, epicocconone, RYPA was also the best medium.

Comparing the solid content of each batch, in the first three batches the agar plates were inoculated from the glycerol stock of *E. nigrum* whereas in the last two batches agar plates were inoculated from another agar plate that was 21 days old. This could have affected the production of metabolites. But all the growth conditions tested showed the presence of various pigments having λ_{max} at 350 nm, 440 nm and 530 nm which may extend the possibility of finding some of new pigments.

One of the limitation of this research was the poor results due to instability of the fluorescent compounds. Consequently, analysing the compounds after storage led to decomposition (data not shown). The isolation of a new metabolite was achieved albeit with an impurity. This proved to be inseparable by the HPLC method used. Other solvents or a different column should be able to separate the two components but this was not possible in the short time available for this project.

This project was mainly focusing on the searching of new fluorescent material and leading lots of scope for discovering a new way of extraction process. But it took a long time to understand and maintain the process on *E. nigrum* Its behaviour changes of fluorescent compounds depending on various factor as light, time and also in different medias was not also analysed properly because of short duration of time.

But in future, if I get a chance to work in this project of PhD, I will be more aware about my experiments. As now I am quite used with the working process and handling process of fungus extraction and analysing methods, it would be more enthusiastic for me to look forward for discovering new fluorescent components.

4 Conclusion

Natural products display the greatest diversity of structure and biological activity of any group of compounds and have deservedly attracted the attention of chemists and biologist. However, the discovery of fluorescence is usually associated with synthetic chemistry, not natural products, even though the first fluorescent compounds discovered (e.g. quinine) were natural products. The link between synthetic chemistry and fluorophores that was forged during the late 19th century through the German dye industry and propagated to the end of the 20th century through companies like Molecular Probes (Eugene, Or). However, synthetic fluorophores are based on a relatively small number of scaffolds (e.g. xanthene, BODIPY, coumarin) that are beginning to limit new applications of fluorescence.

The recent discovery of epicocconone from the fungus *Epicoccum nigrum* and the development of 11 commercial products from this structure, marketed by several companies (e.g. GE Healthcare, Sigma-Aldrich, SERVA, Acquistain and Fluorotechnics), highlighted natural products as a viable source on new fluorescent scaffolds with unique properties. For example, epicocconone was the first reversible covalent, turn-on fluorophore discovered. To expand the palette of natural fluorophores we undertook a screen of culture conditions of *E. nigrum* in an attempt to produce other related structures that may also find utility in biotechnology. Thus, in this research, the optimisation of growth conditions and pigment production of *E. nigrum* and purification of new fluorescent pigments has proven to be a time consuming and laborious process that cannot be conducted in a short-time frame. In this work, we have determined the best conditions for pigment production and used LCMS to identify a range of new compounds, some with odd molecular weights, indicating alkaloids. One such fluorescent alkaloid was isolated and studied by NMR spectroscopy but was shown to be a mixture. The NMR spectra revealed that the minor compound of the mixture was related to epicocconone.

5 References

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6 Supplementary Materials

6.1 Protocol for microbiological media

Raw Sugar Yeast Extract Peptone Agar Media	
Ingredients	**Quantity**
Raw Sugar (CSR)	42.5 g
Yeast extract	12.5 g
Peptone	25 g
Agar	16 g
Mili Q water	1000 mL
Autoclave	121 °C
Time	20 min

Malt Dextrose Agar Media	
Ingredients	**Quantity**
Malt Dextrose Agar (Oxoid)	10 g
Mili Q water	200 mL
Autoclave	121 °C
Time	20 min

Sabouraud Dextrose Agar Media	
Ingredients	**Quantity**
Sabouraud Dextrose Agar (Oxoid)	13.2 g
Mili Q water	200 mL
Autoclave	121 °C
Time	20 min

Potato Dextrose Agar Media	
Ingredients	**Quantity**
Potato Dextrose Agar (Oxoid)	7.8 g
Mili Q water	200 mL
Autoclave	121 °C
Time	20 min

Yeast Extract Peptone Dextrose Agar Media	
Ingredients	**Quantity**
Yeast Extract Peptone Dextrose Agar (Oxoid)	13 g
Mili Q water	200 mL
Autoclave	121 °C
Time	20 min

Czapek Dox Agar Media	
Ingredients	**Quantity**

Czapek Dox Agar (Oxoid)	9.1 g
Mili Q water	200 mL
Autoclave	121 °C
Time	20 min

6.2 Microbial image of *E. nigrum*

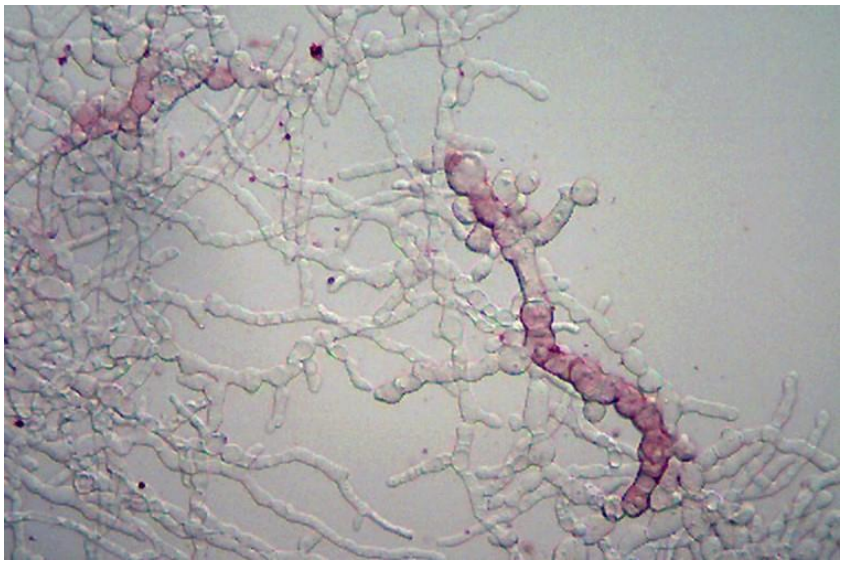

Figure 6.1. *E. nigrum* of Day 4 at 25 °C (During incubation).

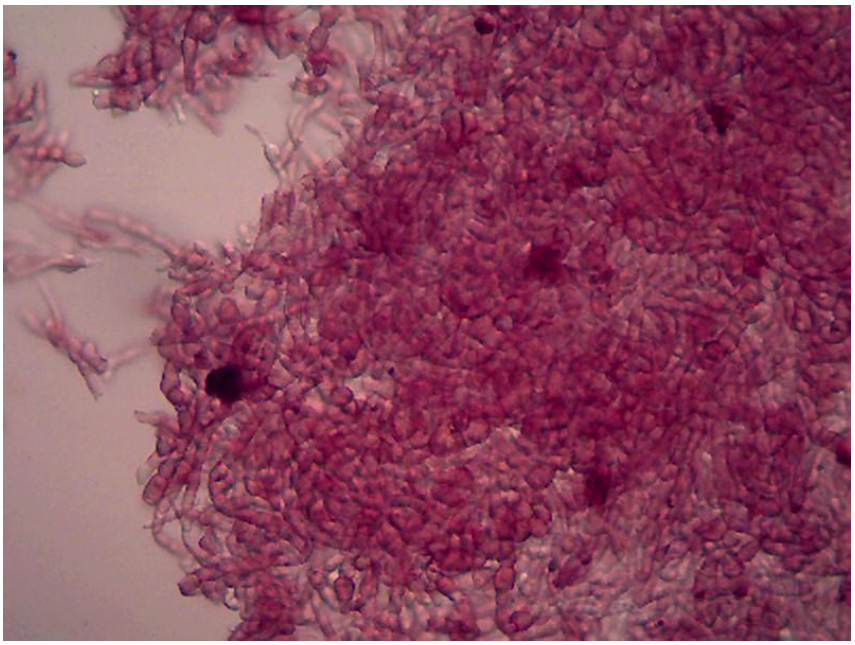

Figure 6.2. *E. nigrum* of Day 5 at 4 °C.

6.3 UV-Vis data of metabolites a, a', b, c and d

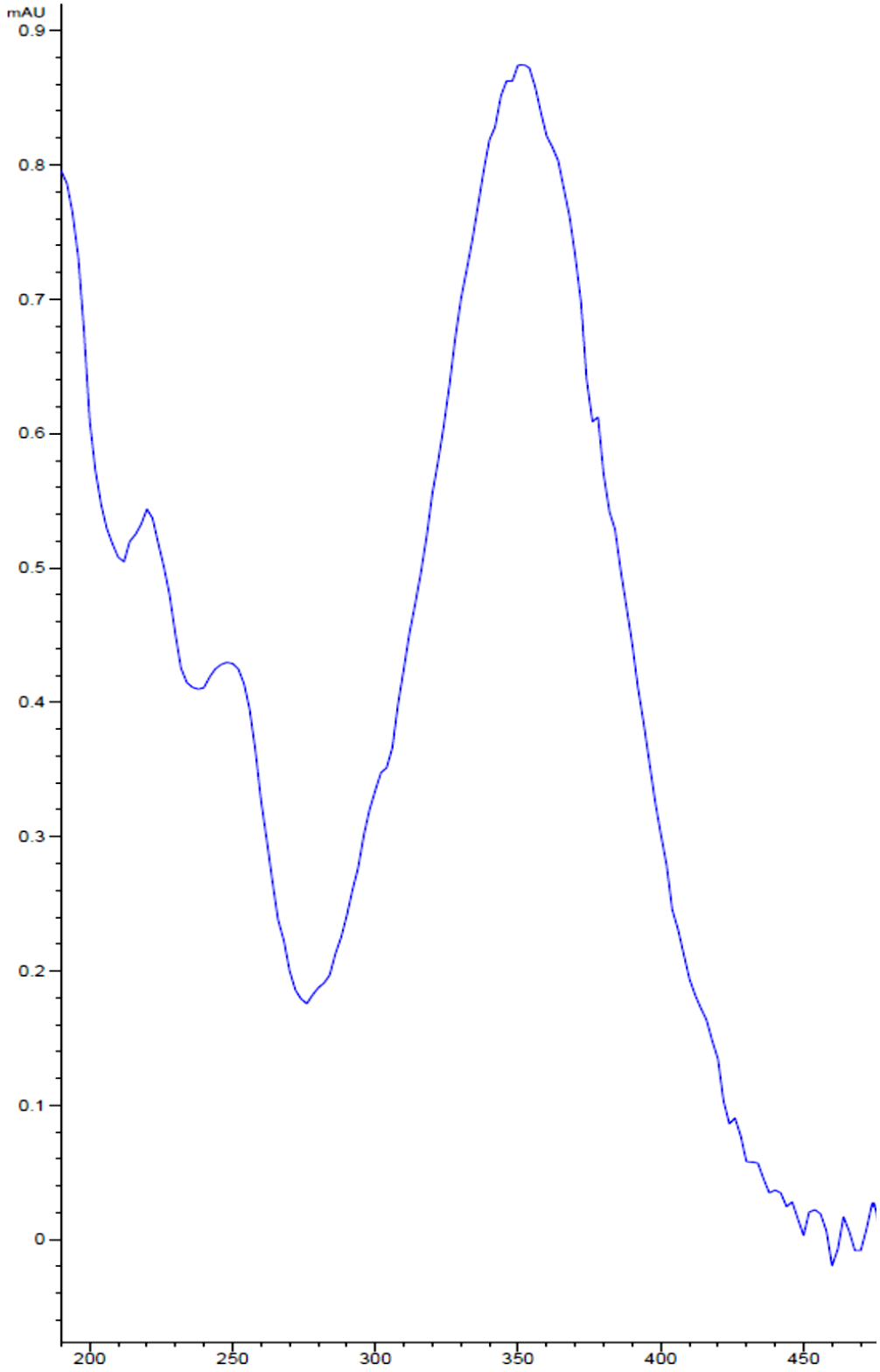

Figure 6.3. UV of metabolite **a**.

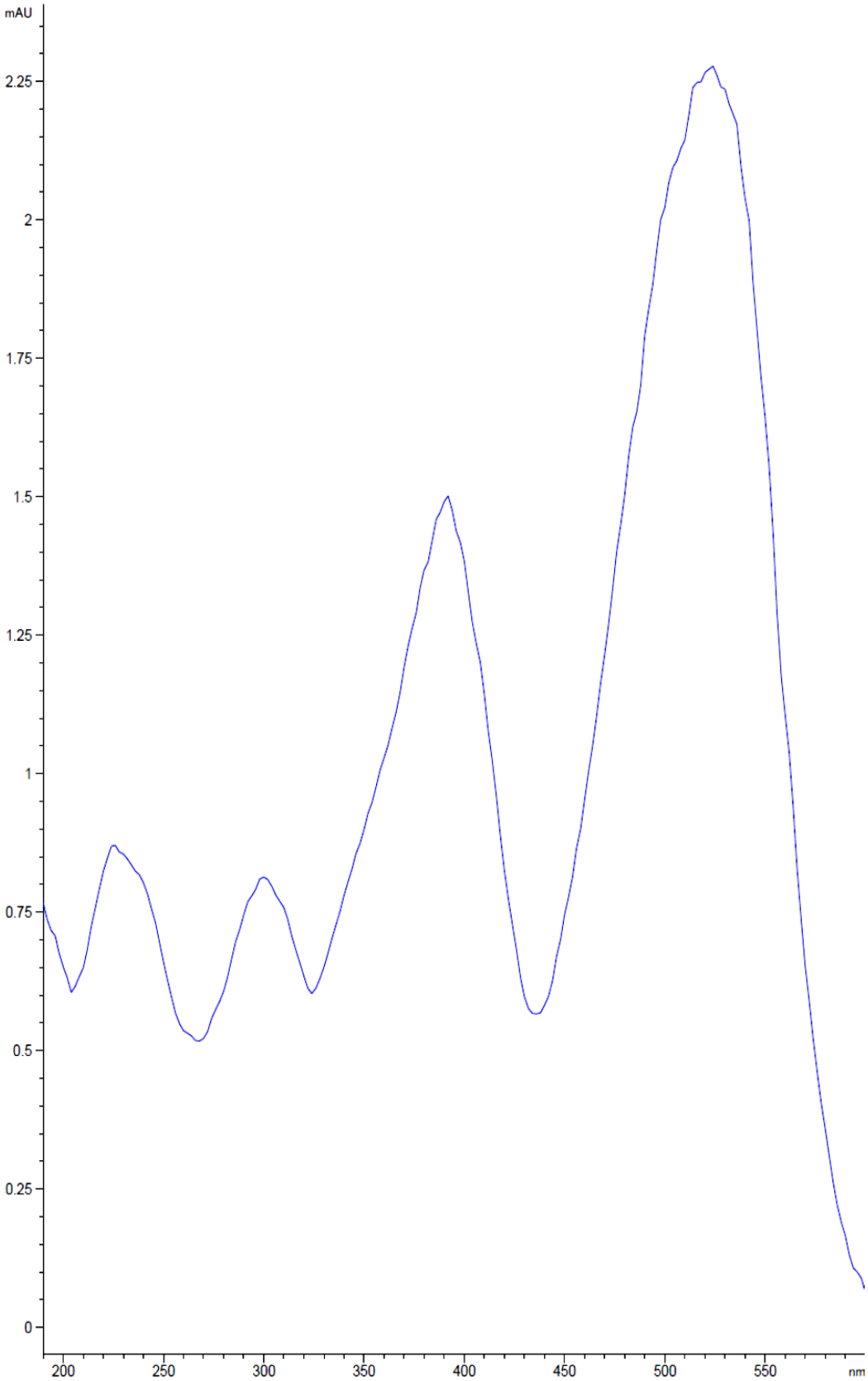

Figure 6.4. UV of metabolite **a'**.

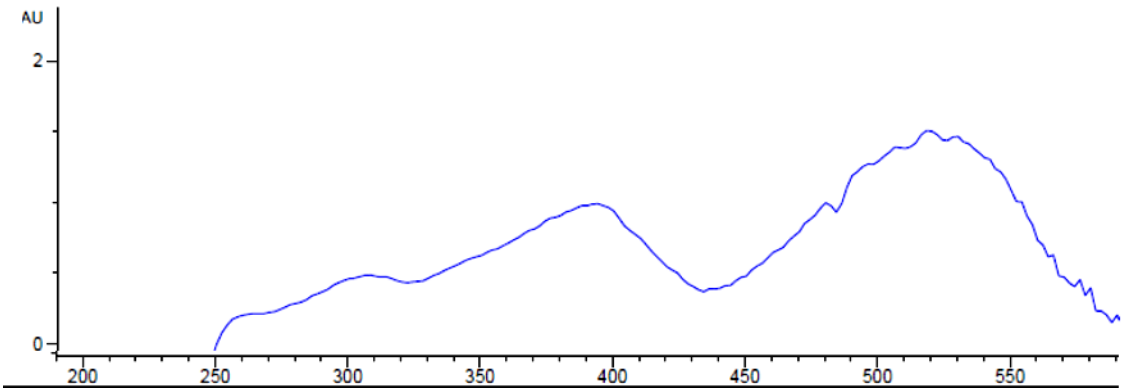

Figure 6.5. UV of metabolite **b**.

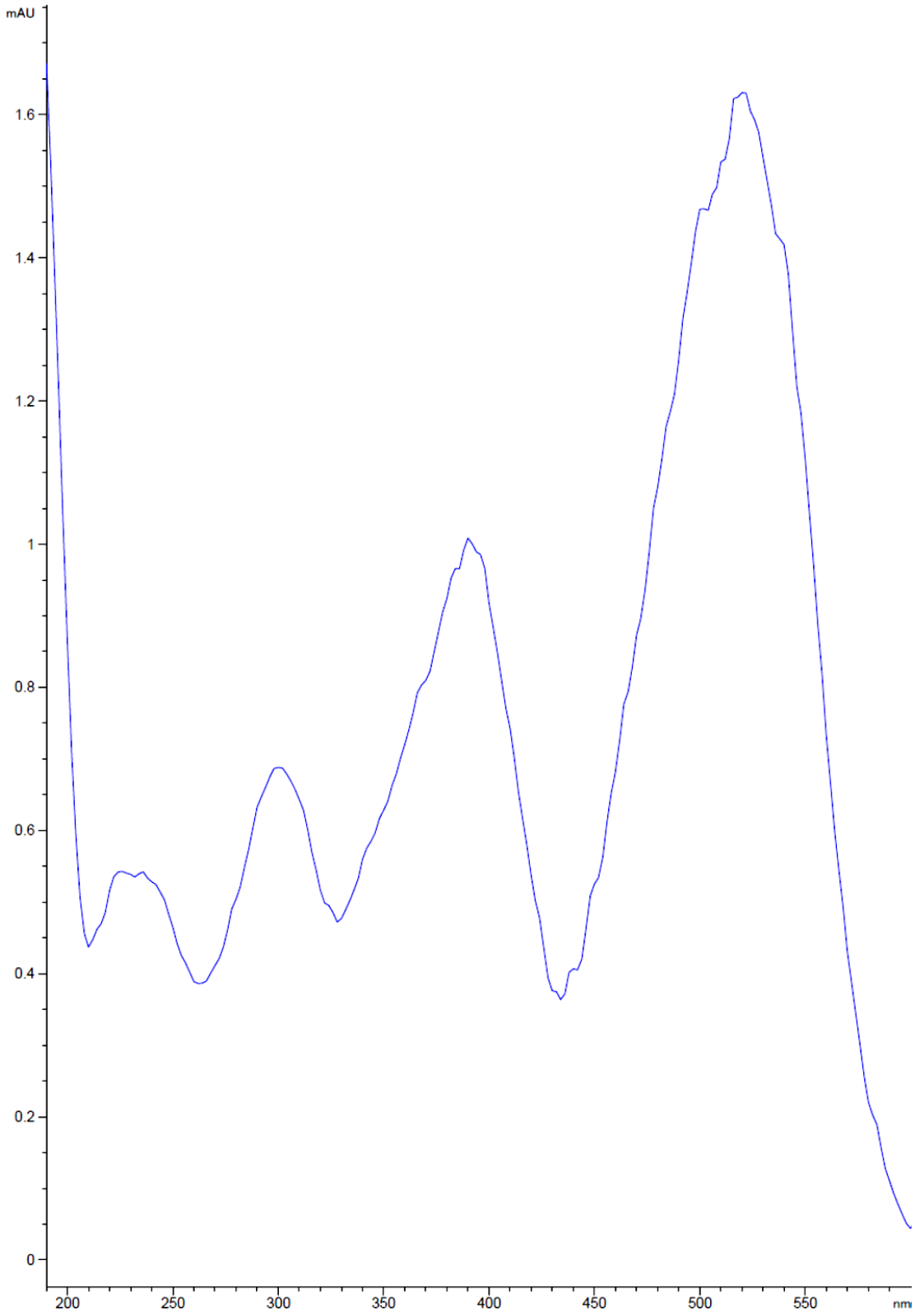

Figure 6.6. UV of metabolite **c**.

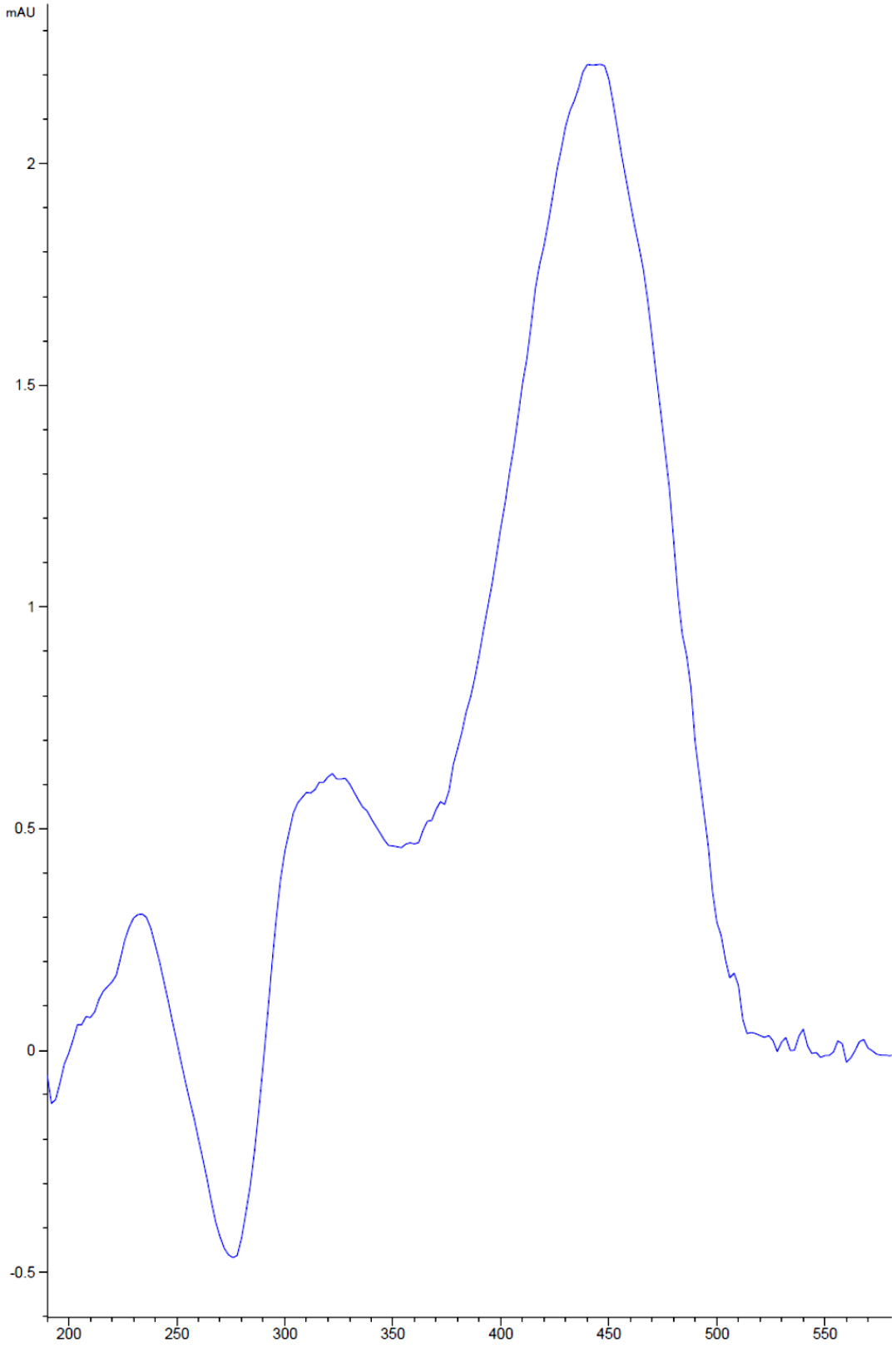

Figure 6.7. UV of metabolite **d**.

6.4 NMR spectra of fraction 8

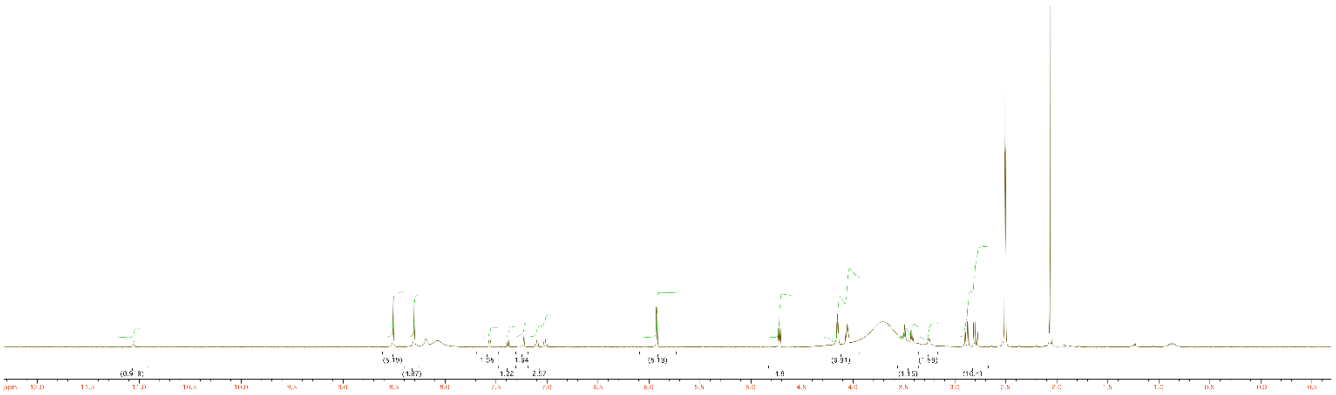

Figure 6.8. ^{1}H NMR spectrum of fraction 8.

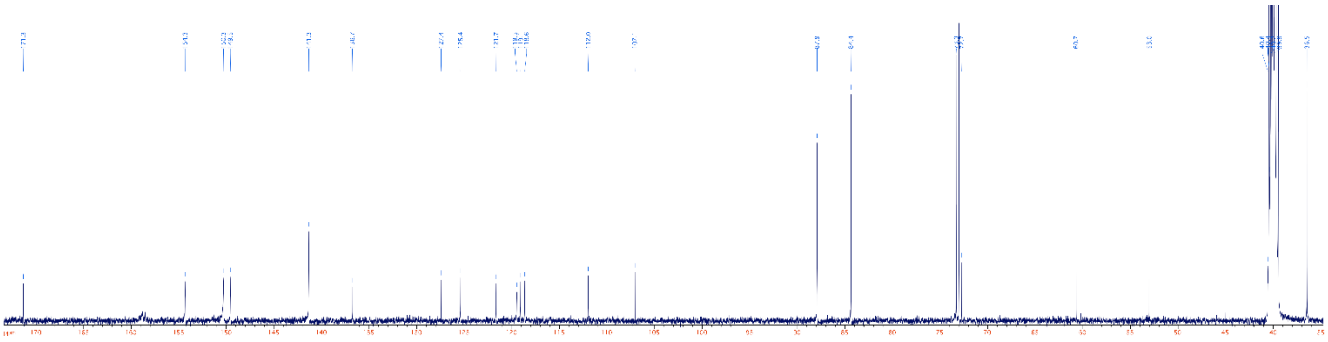

Figure 6.9. ^{13}C NMR spectrum of fraction 8.

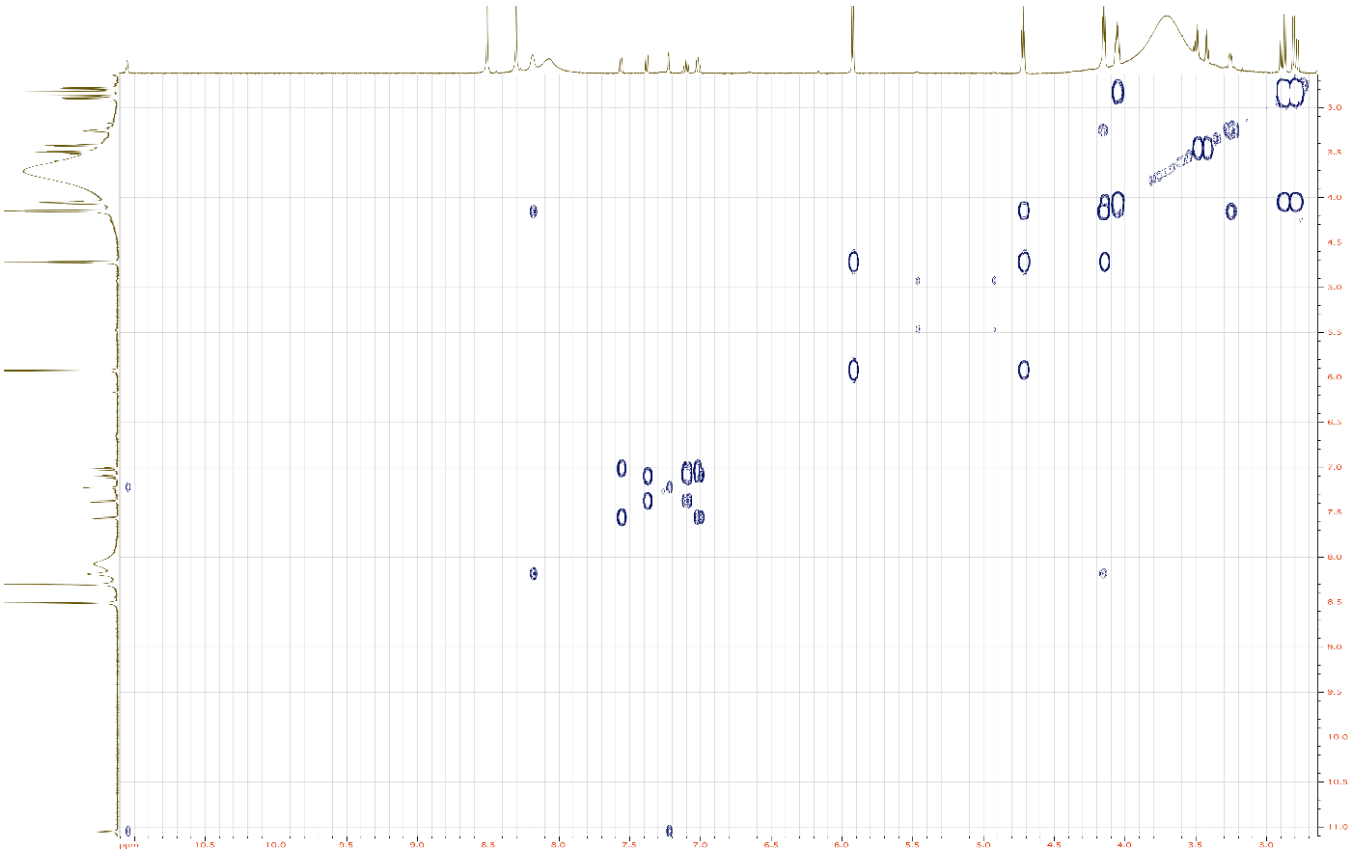

Figure 6.10. COSY spectrum of fraction 8.

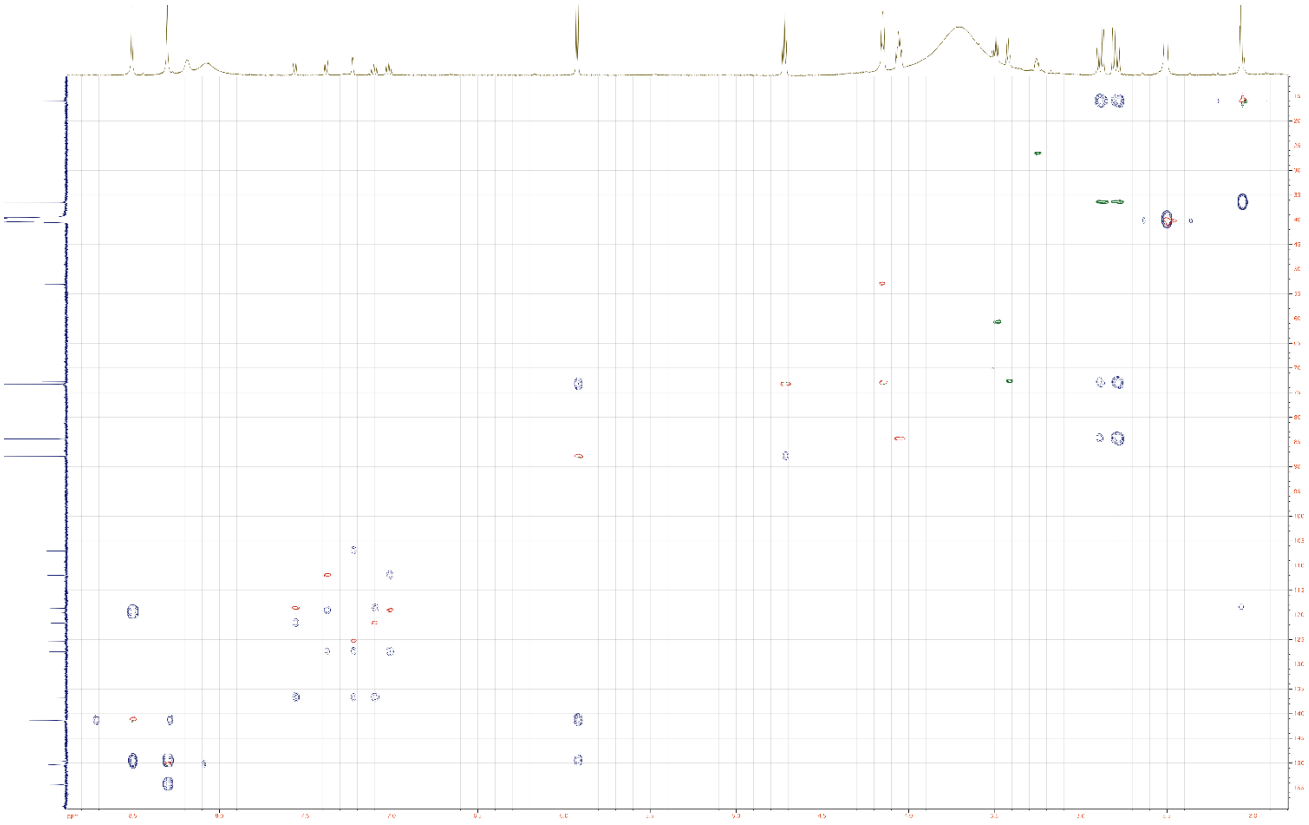

Figure 6.11. HSQC (red and green) and HMBC (blue) overlayed spectra of fraction 8. Green contours indicate methylenes and red contours are methines or methyls.

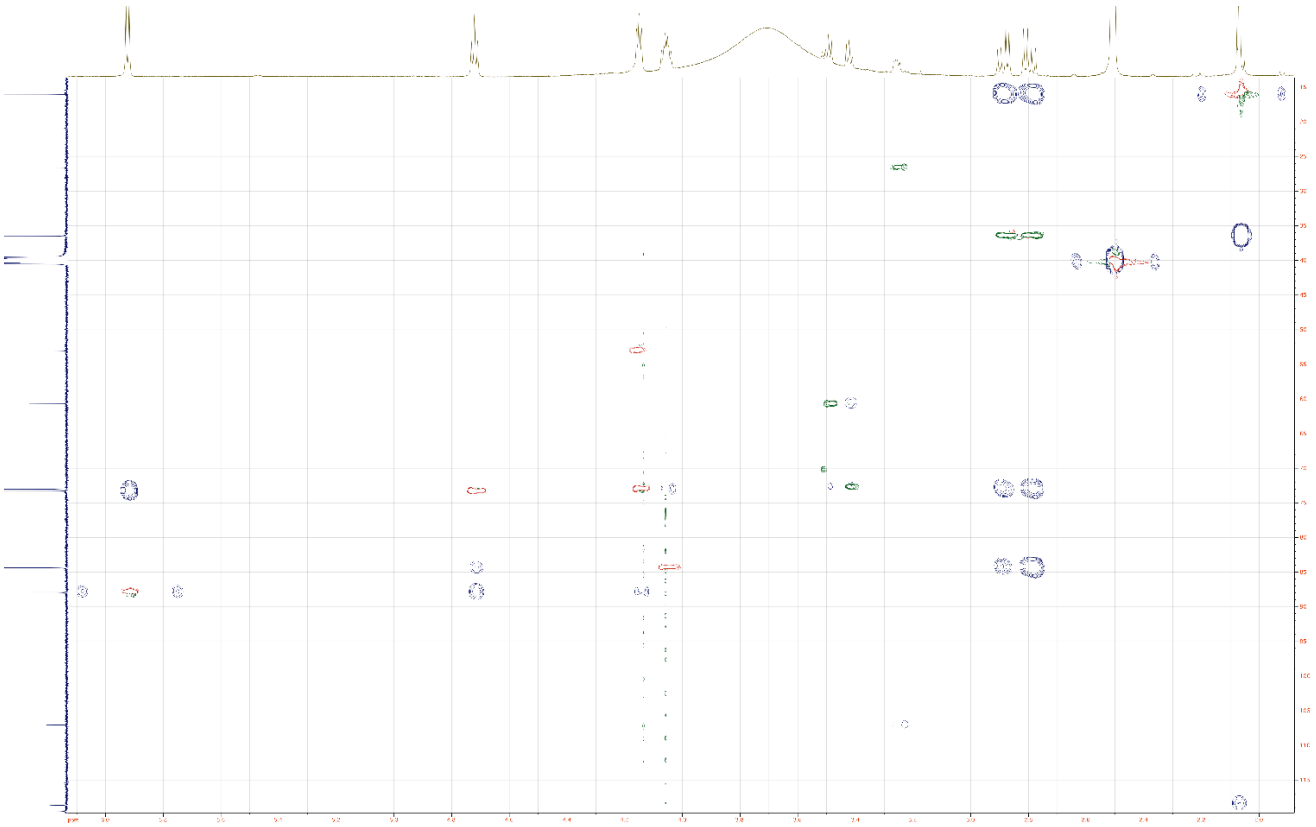

Figure 6.12. HSQC (red and green) and HMBC (blue) overlayed spectra of fraction 8. Green contours indicate methylenes and red contours are methines or methyls. Detail of the aliphatic region.

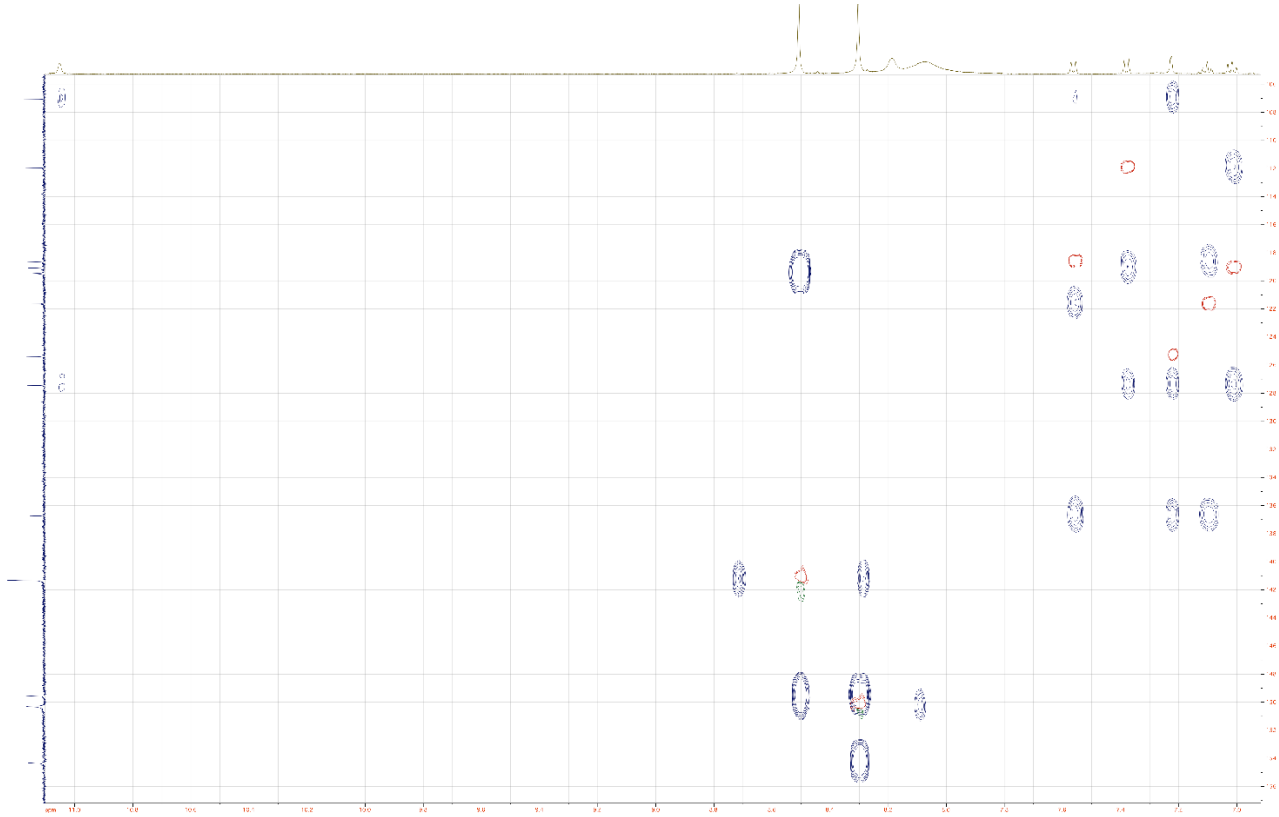

Figure 6.13. HSQC (red and green) and HMBC (blue) overlayed spectra of fraction 8. Green contours indicate methylenes and red contours are methines or methyls. Detail of the aromatic region.

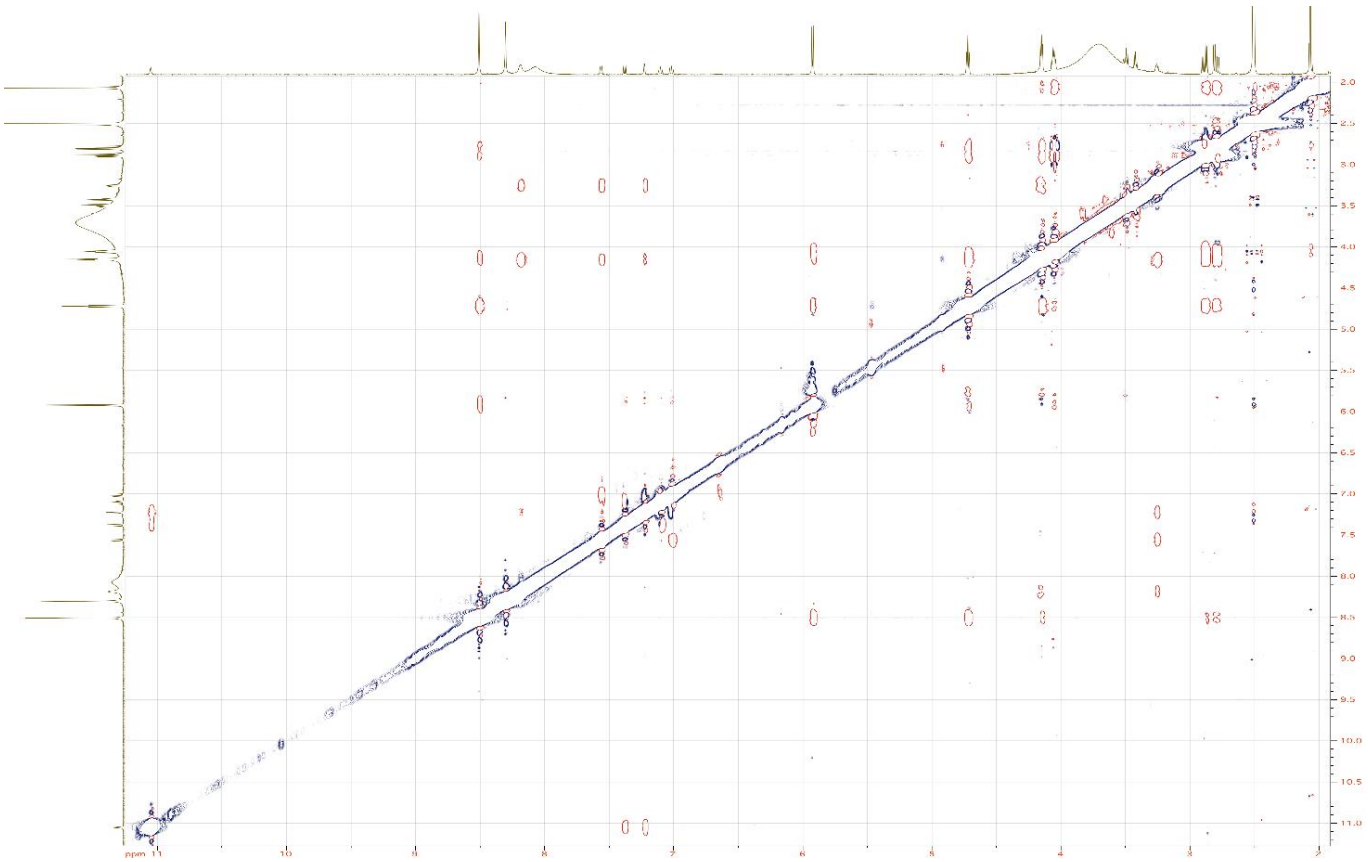

Figure 6.14. ROESY spectrum of fraction 8.

Section 6.5 - Ethical clearance - of this thesis has been removed as it may contain sensitive/confidential content