Natural Products with anticancer activity from Moroccan plant *Thymelaea lythroides* and its endophyte *Chaetomium aureum*

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Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel **„Natural Products with anticancer activity from Moroccan plant *Thymelaea lythroides* and its endophyte *Chaetomium aureum*"** selbst angefertigt habe. Außer den angegebenen Quellen und Hilfsmitteln wurden keine weiteren verwendet. Diese Dissertation wurde weder in gleicher noch in abgewandelter Form in einem anderen Prüfungsverfahren vorgelegt. Weiterhin erkläre ich, dass ich früher weder akademische Grade erworben habe, noch dies versucht habe.

Wuppertal, den 21.10.2013
Fatima Zahra Kabbaj

I dedicated this work to my husband and my parents

Acknowledgement

First and foremost thanks to the Almighty God "ALLAH" who has granted me all these graces to fulfill this work and blessed me by His power, mercy and patience during my life. To Him I extent my heartfelt thanks.

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Zusammenfassung

Heilpflanzen und endophytische Pilze produzieren Naturstoffe mit einer großen Vielfalt von chemischen Strukturen, die für bestimmte medizinische Anwendungen geeignet sein könnten. Die meisten dieser Sekundärmetaboliten zeigen biologische Aktivitäten in pharmazeutisch relevanten Bioassay-Systemen und stellen daher potentielle Leitstrukturen dar, die optimiert werden könnten, um wirksame therapeutische und bioaktive Wirkstoffe zu ergeben.

Das Hitze-Schock-Protein 90 spielt eine entscheidende Rolle bei der Aufrechterhaltung der Homöostase onkogener Proteine. Hsp90 Inhibitoren bietet großes Versprechen in der Behandlung einer Vielzahl von soliden und hämatologischen Malignomen. Zahlreiche natürliche Hsp90-Inhibitoren wurden in den letzten Jahren entwickelt, von denen einige ausgezeichnete Antitumor-Aktivitäten zeigen und in klinische Studien eingetreten sind.

Ziel dieser Arbeit war die Isolierung von Sekundärstoffen aus der ausgewählten Pflanze *Thymelaea lythroides* und deren endophytischen Pilz *Chaetomium aureum*, gefolgt von Strukturaufklärung und Untersuchung der Antitumor-Aktivitäten isolierter Substanzen durch das Screening ihrer inhibierenden Aktivität gegen der Hsp90 Maschinerie.

Die medizinische Pflanze *Thymelaea lythroides* wurde aus 55 Heilpflanzen, die als traditionelle Medizin von den Patienten des „National Institute of Oncology" in Marokko verwendet werden, ausgewählt. Fünf endophytische Pilzstämme (*Alternaria sp., Chaetomium sp., Cladosporium sp.* und *Pleospora sp.*) wurden aus *Thymelaea lythroides* isoliert. Der Pilz *Chaetomium aureum* wurde zur weiteren Untersuchung ausgewählt und in Wickerham-Flüssigmedium sowie auf 11 festen Medien für einen Zeitraum von drei bis vier Wochen angezogen. Die Extrakte wurden anschließend verschiedenen chromatographischen Trennverfahren unterzogen, um die Sekundärmetaboliten zu isolieren. Zur Strukturaufklärung der isolierten Substanzen wurden moderne Verfahren, einschließlich der Massenspektrometrie (MS) und der Kernresonanzspektroskopie (NMR) eingesetzt. Zusätzlich wurden chirale Derivatisierungsreaktionen bei optisch aktiven Verbindungen angewendet, um deren absolute Konfiguration zu ermitteln.

Schließlich wurden die erhaltenen Verbindungen verschiedenen Biotests unterzogen, um ihre Antitumor-Aktivität zu untersuchen, wobei ihre Fähigkeit die Chaperon Hsp90 Maschinerie

zu hemmen, geprüft wurde. Außerdem wurden die isolierten Verbindungen auf ihre antimikrobiellen, antifungiziden und zytotoxischen Eigenschaften, sowie die Wirkung als Inhibitoren verschiedener Proteinkinasen, getestet.

1 . Isolierte Verbindungen aus der Pflanze *Thymelaea lythroides*

Acht Sekundärmetabolite wurden aus der Heilpflanze *Thymelaea lythroides* isoliert, darunter ein Depsipeptid (**1**; Bassiatin), zwei Coumarine (**2**; Daphneon und **3**; Daphneolon), ein Dicoumarin (**4**; Daphnoretin), zwei Lignane (**5**; Wikstromol und **6**; δ-Sesamin), ein Flavonoidglukosid (**8**; *trans*-Tilirosid) und ein Dicoumaringlukosid (**7**; Rutarensin).

Bassiatin (**1**) ist ein ungewöhnlicher pflanzlicher Sekundärmetabolit, der zuvor ausschließlich aus endophytischen Pilzen isoliert wurde. Folglich ist dies der erste Bericht dieser Verbindung aus *Thymelaea lythroides.*

Daphneon (**2**) zeigte eine starke zytotoxische Aktivität gegen der L5178Y Maus-Lymphom-Zelllinie. Eine Hemmung mehrerer der getesteten Proteinkinasen wurde nur bei *trans*-Tilirosid (**8**) und Rutarensin (**7**) beobachtet. Alle aus *Thymelaea lythroides* isolierten Verbindungen waren inaktiv gegen die Hsp90 Chaperon-Maschinerie.

2 . Isolierte Verbindungen aus dem endophytischen Pilz *Chaetomium aureum*

Sechs neue Verbindungen wurden aus diesem Pilzstamm erhalten, einschließlich des neuen Alkylresorcinolderivats (**18**; Chaetoresorcinol), vier neue Xanthoradonderivate (**14**; Xanthoradon D_1, **15**; Xanthoradon D_2, **16**; Xanthoradon E_1 und **17**; Xanthoradon E_2), und ein Pyrochromonderivat (**13**; SB 236050), zusammen mit vier bekannten Azaphilonderivaten (**9**; Sclerotiorin, **10**; Isochromophilon VII, **11**; Sclerotioramin und **12**; Isochromophilon VI).

(+)-Sclerotiorin (**9**), (+)-Isochromophilon VII (**10**), (+)-Sclerotioramin (**11**) und Isochromophilon VI (**12**) waren nicht aktiv im Zytotoxizitätstest gegen der L5178Y Maus-Lymphom-Zelllinie. Allerdings war (+)-Sclerotiorin (**9**) gegen alle getesteten Proteinkinasen außer MEK1wt aktiv, während seine Derivate, Isochromophilone VI (**12**) und Sclerotioramin (**11**), nur wenige der getesteten Enzyme hemmen konnten. Darüber hinaus wurden alle Verbindungen im Hsp 90 Chaperon-Maschinerie Hemmungs-Assay getestet. Nur (+)-Sclerotiorin (**9**) zeigte herausragende Aktivität mit einer dem bekanntenHsp-90-Inhibitor 17-AAG (Geldanamycinderivat) ähnlichen Wirksamkeit.

Chaetoresorcinol (**18**) zeigte eine mittelmäßige fungizide Wirkung gegen *Aspergillus fumigatus*, während die anderen isolierten Verbindungen in den antibakteriellen und fungiziden Tests nicht aktiv waren.

Table of Contents

1. General introduction

Drug discovery and development has a long history and dates back to the early days of human civilization. In those ancient times, drugs were not just used for physical remedies but were also associated with religious and spiritual healing.

Even today, after more than 100 years of research in pharmaceutical industries, there is still a great need for innovative drugs. Only one third of all diseases can be treated efficiently (Müller *et al.*, 2000). The role of natural products in drug discovery is demonstrated by an analysis of the number and sources of anticancer and antiinfective agents, reported mainly in the Annual Reports of Medicinal Chemistry from 1984 to 1995 (Cragg, 1997). It was observed that over 60% of the approved drugs and pre-NDA (New Drug Applications) candidates (for the period 1989-1995), excluding biologics (vaccines, monocloneals, etc. derived from mammalian sources), developed in these disease areas are of natural origin.

The nature itself, has constantly supplied mankind with a broad and structurally diverse array of pharmacologically active compounds that continue to be utilised as highly effective drugs to combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature (Proksch *et al.*, 2002).

The ancient medical literature reports that surgery was performed but that physicians also recommended the use of some natural, and especially plant products, which represent an interesting point of comparison with current knowledge. Natural products play a relevant role in cancer therapy today with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products from various sources such as plants, animals and microorganisms. Large-scale anticancer drug discovery and screening programs such as those promoted by the National Cancer Institute (NCI) have played an important role in the development of anticancer natural compounds (Stefania *et al.*, 2009).

The molecular chaperone Hsp90 has been a promising target for cancer therapy. Cancer is a disease marked by genetic instability. Thus specific inhibition of individual proteins or signaling pathways holds a great potential for subversion of this genetic plasticity of cancers.

1.1. Aim and scopes of the study

The aim of this study is to look for natural lead molecules with anticancer activity specialy the inhibitors of Hsp90 machine. For this, the drug discovery process was divided into 4 steps namely (Figure 1.1):
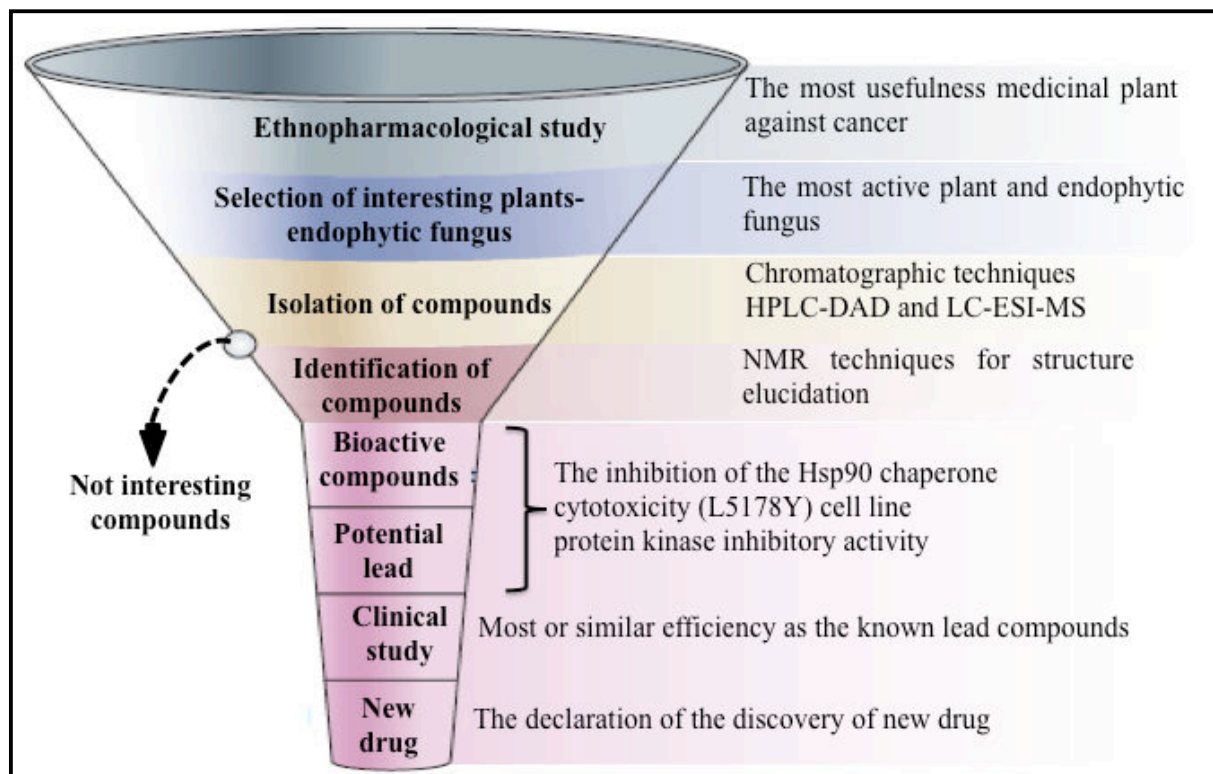
Figure 1.1. Steps of drug discovery process

1- *The selection of the interesting natural sources* from an ethnopharmacological study about the most usefulness medicinal plant against cancer, then from the selected plant, a screening of the most active endophytic fungus; this step is basically of two types of natural sources. Firstly, the macro-organism source which are plants, and secondly, the micro-organism source which are endophytic fungi. The plant *Thymelaea lythroides* and the endophytic fungi *Chaetomium aureum* were suspected for this study.

2- *The isolation, characterization and structure elucidation of the secondary metabolites*. The raw extracts of the selected natural sources have to be fractionated and separated using various chromatographic techniques and their fractions have to be analyzed by HPLC-DAD for their purity and ESI-LC/MS for their molecular weight and fragmentation patterns. The pure compounds should then submitted to state-of-the-art one- and two-dimensional NMR techniques for structure elucidation.

3- *The selection of the potential lead compounds*. The pure compounds were subjected to selected bioassays to determine their pharmaceutical potential. Thus, the application of secondary metabolites for the inhibition of the Hsp90 chaperone pathway will be carried out by Prof. A. Chadli at the Cancer Research Center, Molecular Chaperones Biology, Georgia Regents University in USA. The cytotoxicity will be studied *in vitro* using mouse lymphoma (L5178Y) cell line. Moreover, the pure compounds will also be tested for their protein kinase inhibitory activity, whereas, antimicrobial activity will be studied using the agar diffusion assay. The latter three assays will be conducted in cooperation with Prof. W. E. G. Müller, Mainz, ProQinase, Freiburg and Prof. U. Hentschel, Würzburg, respectively.

4- *The clinical study*. Sclerotiorin showed similar efficiency to that of 17-AAG, the classical inhibitor of Hsp90, which make it the first suspect for the clinical essays.

2. Literature review

2.1. Secondary metabolites as natural products

The compounds known as secondary metabolites were defined in 1891 as materials that were relatively unimportant in the overall physiology of plants and not absolutely essential to the life and growth of the producing organism (Bentley, 1999). From 1900 to 1950 the term secondary metabolites received little use and was restricted to plants. It was not until 1961 that secondary metabolite was applied to microbial products; soon thereafter a new terminology "natural product" was used by organic chemists to refer to materials derived from terrestrial plants, bacteria, filamentous fungi, marine creatures, etc. In the strict sense of the term, natural products encompass also the primary metabolites. As stated by Davies (1992), "a secondary metabolite is a natural product, but a natural product isn't necessarily a secondary metabolite" (Bentley R., 1999). Secondary metabolites are classified depending on their structural scaffold. These classes include among others polyketides (PKs), non ribosomal peptides (NRPs), terpenoids, alkaloids and hybrid metabolites of the different classes. The function of secondary metabolites in their hosts has not yet been exploited to its fullest; however, they seem to have several functions including defense, communication, and signaling (Calvo *et al*., 2002; Fox *et al*., 2008; Kempken and Rohlfs, 2010; Rohlfs and Churchill, 2010).

Although there are notable exceptions (e.g., palytoxin, maitotoxin), most secondary metabolites have relative molecular masses less than 1500 Da. The range of chemical structures is breathtaking from simple aliphatic acids (e.g., itaconic acid, $C_5H_6O_4$) to complex structures such as alkaloids and toxins (e.g., palytoxin, $C_{129}H_{223}N_3O_{54}$). This wide range of chemical types has made secondary metabolites a major topic for study by organic chemists (Bentley, 1999).

2.2. Medicinal application of natural products

Medicinal application of natural products has a long history and dates back to the early days of human civilization. In traditional medicine, natural products, mainly botanical drugs, were and are used on the basis of empirical experiences rather than pharmacological knowledge. The World Health Organization has estimated that almost 80% of the world's inhabitants depend mainly on traditional medicines for their primary health care (Arvigo and Balich, 1993; Farnsworth et al., 1985; Newman, 2008), which Morocco is one of them. Moroccan traditional pharmacopoeia is one of the richest and varied in the world (Bellakhdar *et al*.,

1978; Bellakhdar *et al.*, 1982; Bellakhdar *et al.*, 1991; Benjelloun, 1997; Bounejmate, 1995). Clinical, pharmacological, and chemical studies of these traditional medicines, which were derived predominantly from plants, were the basis of most early medicines such as the antipyretic and antimalarial active alkaloid quinine (2) (from *Cinchona pubescens*) or the cardiac glycoside digitoxin (3) (from *Digitalis purpurea*). Even more drugs are derived from natural products, like the world-famous aspirin (4) aspirin (acetylsalicylic acid), which is derived from the glycoside salicin (5) and is found in many species of the plant genera *Salix* and *Populus*. Aspirin is only one, albeit the best known example for the prosperous interaction of synthetic and natural products chemistry.
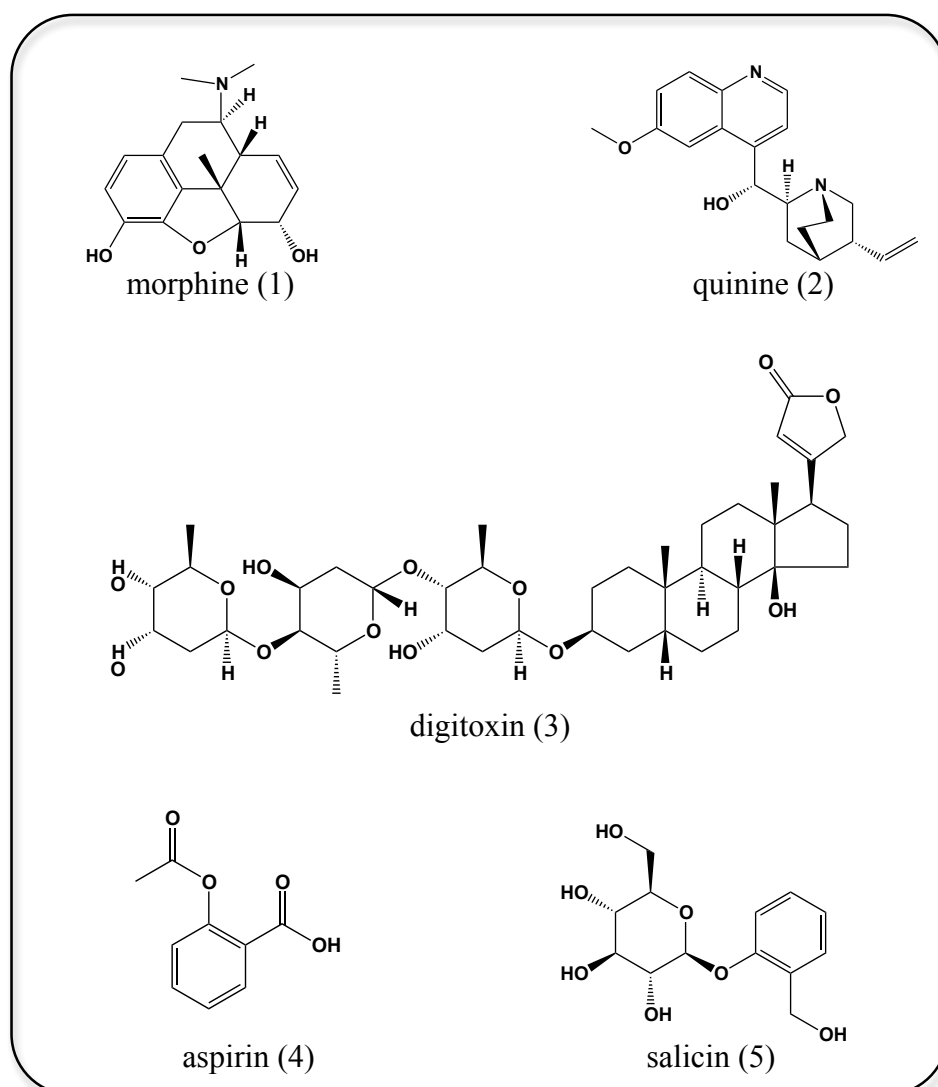
Figure 2.1. Natural products of medicinal importance

With the increase of this knowledge and the better understanding of the mode of action of different remedies, more and more distinct drugs were used and chemically modified. Additionally, scores of novel active compounds were introduced to medicines that are produced by means of traditional or combinatorial synthetic chemistry. In fact, around 60% of the new drugs registered during the period 1981-2002 by the FDA as anticancer and anti-hypertensive agents were either natural products or based on them (Newman *et al.*, 2003). Likewise, more recent studies demonstrate that natural products constantly play an important role in drug development (Butler, 2008; Newman and Cragg, 2007; Sneader, 1996).

Hence, a large number of examples from medicine reveal the innovative potential of natural compounds and their impact on progress in drug discovery and development (Tejesvi *et al.*, 2007).

2.3. The use of traditional medicine or phytotherapy

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank *et al.*, 1982; Vulto and Smet, 1988; Mentz and Schenkel, 1989). This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that "natural" products are harmless. However, the use of these substances is not always authorised by legal authorities dealing with efficacy and safety procedures, and many published papers point to the lack of quality in the production, trade and prescription of phytomedicinal products. Nevertheless, the phytomedicines are freely marketed and, in underdeveloped or developing countries, the use of medicinal plants is widely accepted. This can result in toxic accidents resulting from the use of plants as food or for therapy or from accidental ingestion by children or animals. Toxicity can result from highly concentrated doses or from the state of conservation of plants and the form of use.

The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries (Rates, 2001).

2.4. Plants as sources of bioactive products

The analysis of data of prescriptions dispensed from community pharmacies in the United States from 1959 to 1980 revealed that approximately 25% contained plant extracts or active principle from higher plants (Newman *et al.*, 2000). About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). The vast majority of the drugs isolated from plants cannot yet be synthesised economically and are still obtained from wild or cultivated plants.

In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al.*, 1996).

However, the potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250,000–500,000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties; in most cases, only pharmacological screening or preliminary studies have been carried out. It is estimated that 5000 species have been studied for medical use (Payne *et al.*, 1991). Between the years 1957 and 1981, the NCI screened around 20,000 plant species from Latin America and Asia for anti-tumour activity, but even these were not screened for other pharmacological activities (Hamburger and Hostettman, 1991).

2.5. Endophytic fungi as sources of bioactive products

The word endophyte means "in the plant" (from gr. Endo = within, phyton = plant). ''Endophytism'' is, thus, a unique cost-benefit plant-microbe association defined by ''location'' (not ''function'') that is transiently symptomless, unobtrusive, and established entirely inside the living host plant tissues (Kusari *et al.*, 2012). During this association, none of the interacting partners is discernibly harmed, and the individual benefits depend on both the interacting partners.

The existence of fungi inside the organs of asymptomatic plants has been known since the end of the 19th century (Guerin, 1898) and the term ''endophyte'' was first proposed in 1866 (de Bary, 1866).

Since endophytes were first described in the Darnel (*Lolium temulentum*) (Kusari, 2012), they have been isolated from various organs of different plant species.

The significance of fungi as unconventional sources of bioactive compounds was first realized by the discovery of penicillin from *Penicillium notatum*, by Alexander Fleming in 1928. Since then, natural product discovery from fungi has gained considerable attention and momentum, especially after the large-scale production of penicillin during World War II.

For the past 50 years, fungal secondary metabolites have revolutionized medicine yielding blockbuster drugs and drug leads of enormous therapeutic and agricultural potential (Amal *et al*., 2011).

Recent development of screening technologies revealed the great potential of endophytes as a major source of biologically active compounds with promising medicinal or agricultural applications (Zhang *et al*. 2012; Aly *et al*., 2010).

In addition, natural products from endophytic fungi represent a huge and largely untapped resource of unique chemical structures that have been optimized by (co-)evolution and probably play an important role for communication or adaptation as a response to habitat and environmental changes (Gunatilaka, 2006). It has been estimated that approximately 1.5 million fungal species are present on earth of which only about 7% have been described so far (Aly *et al*., 2010). Whereas between 1987 and 2000 approximately 140 new natural products were isolated from endophytic fungi, a similar number was subsequently characterised in half of this time span, i.e. between 2000 and 2006 (Zhang *et al*., 2006). Since then, especially fungi isolated from soil samples have been identified as a rich source of biologically active secondary metabolites. Beside other well known antimicrobial agents like fusidic acid (6) (Aly *et al*., 2010) and griseofulvin (7) (Kjier, 2009), novel semisynthetic antifungal drugs like anidulafungin (8) (Eraxis) and caspafungin (Cancidas) are likewise derived from fungal metabolites (Kjier, 2009). With the discovery of cyclosporine (9) isolated from

Tolypocladium inflatum in 1971, an important step in immunopharmacology was made because this substance prevents rejection after organ or tissue transplantations.

fusidic acid (6)

griseofulvin (7)

anidulafungin (8)

cyclosporine (9)
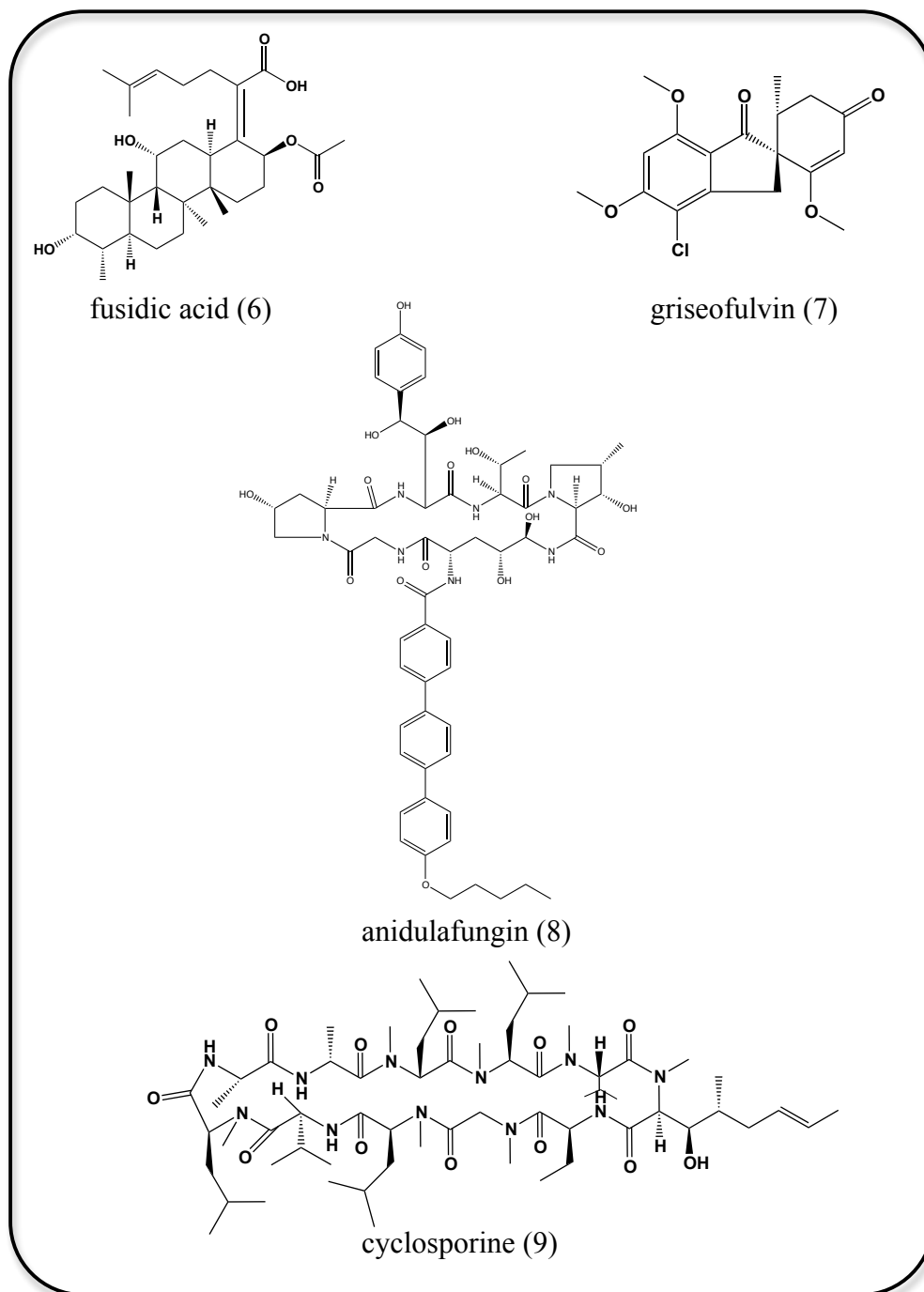
Figure 2.2. Bioactive fungal metabolites used in medicine

Probably the most economical important fungal metabolites represent antilipidemic drugs collectively known as "statins", with their parent compounds mevastatin and lovastatin (10) isolated from *Penicillium citrinum* and *Aspergillus terreus*, respectively. Statins reduce blood cholesterol levels by inhibiting the rate limiting enzyme HMG-CoA reductase in the

mevalonate pathway of cholesterol synthesis, and are used for the treatment of cardiovascular diseases (Dewick, 2006).
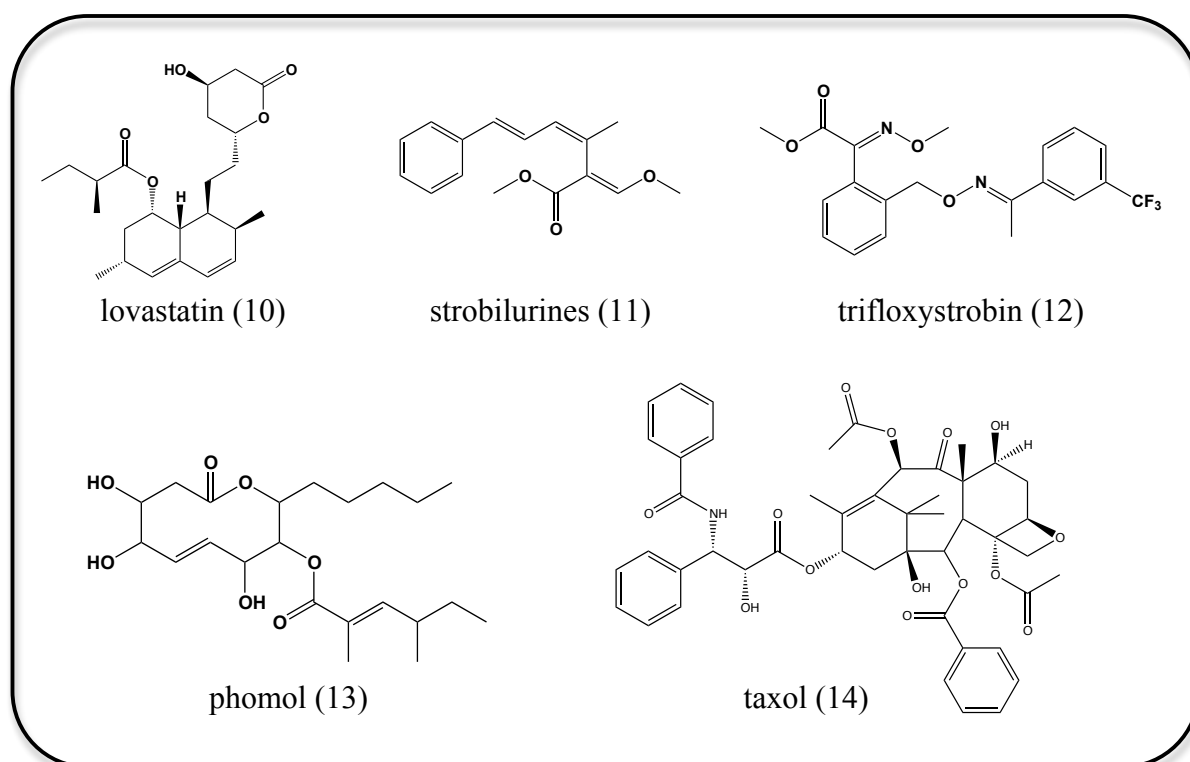
Figure 2.2. Bioactive fungal metabolites used in medicine

Fungal metabolites are, however, not only indispensable for medicine but are also important for plant protection as demonstrated by the discovery of the strobilurines (11), that were first isolated from *Strobilurus sp*. and served as lead compounds for synthetic fungicidals such as trifloxystrobin (12) (Flint[®]) (Kjier, 2009).

From the medicinal plant *Erythrina crista-galli* the endophyte *Phomopsis sp.* was isolated, which produced the anti-inflammatory as well as antifungal and antibacterial active polyketide lactone, phomol (13) (Weber et al. 2004).

But it is not only new compounds being isolated from endophytes that are promising. The well known plant metabolite taxol (14), the "world's first billion-dollar anticancer compound" (Strobel, 2004), was originally isolated from the bark of the endemic Pacific yew tree, *Taxus brevifolia*. It interferes with the normal function of microtubule breakdown. Specifically, taxol binds to the ß-subunit of tubulin and thereby interrupts the dynamic rearrangement of this important component of the cytoskeleton. This adversely affects cell function due to the

shortening and lengthening of microtubules is necessary for their function as a mechanism to transport other cellular components, e.g. during mitosis. Thus taxol affects dividing cells, especially fast dividing ones like cancer cells. For the treatment of one patient suffering from cancer, 2 g taxol are required, which represents an amount equivalent to twelve trees and thereby posing a challenge to the limited natural resources, since the isolation from the inner bark implies the destruction of trees. Thus, the demand for taxol greatly exceeds the supply that can be sustained by isolation from its natural source and alternative sources of the drug have been sought for a long time. Although the highly functionalized, polycyclic diterpene has been prepared by total synthesis, the process is too complex and not economically feasible. Currently, the supply of the compound is achieved by a successfully implied partial synthetic route based on baccatin III or its 10-deacetyl congener, which are isolated from the needles of other *Taxus* species and thus from a renewable resource. However, the extraction process of these precursors is tedious and costly. In the ongoing search for alternative sources of taxol, the group of Gary Strobel discovered taxol production in a hitherto undescribed endophytic fungus associated with Taxus brevifolia, identified as *Taxomyces andreanae* (Stierle et al., 1993). Although initially controversial, these findings prompted further studies, and it is nowadays an emerging picture that the ability to produce taxol upon fermentation seems to be a rather widespread feature among endophytic fungi. So far, more than 10 different fungal strains from at least 6 different host plants, most of them only distantly (if at all) related to *Taxus*, have been identified. However, it is worth mentioning that in all cases the resulting yields are minuscule, so far preventing any commercial exploitation (Strobel *et al*., 2004).

2.6. The relationship between the endophyte and the host plant

Any plant-fungal interaction is preceded by a physical encounter between a plant and a fungus, followed by several physical and chemical barriers that must be overcome to successfully establish an association. The ''balanced antagonism'' hypothesis (Schulz *et al*., 1999; Schulz and Boyle, 2005) was initially proposed to address how an endophyte avoids activating the host defenses, ensures self-resistance before being incapacitated by the toxic metabolites of the host, and manages to grow within its host without causing visible manifestations of infection or disease (Arnold, 2005, 2007, 2008; Schulz and Boyle, 2006) (Figure 2.3.A). This hypothesis led to the proposal that the interactions between host plants and endophytes in natural populations and communities are described as a balanced symbiotic continuum ranging from mutualism through commensalism to parasitism (Tan and Zou, 2001;

Kogel *et al.*, 2006). Endophytes and pathogens both possess many virulence factors that are countered by plant defense mechanisms. If fungal virulence and plant defense are balanced, the association remains apparently asymptomatic and a virulent. Although the genetic basis of symbiotic communication is not yet known, studies examining the relation between host genotype and symbiotic lifestyle expression revealed that individual isolates of some fungal species can express either parasitic or mutualistic lifestyles depending on the host genotype colonized (Redman *et al.*, 2001; Unterseher and Schnittler, 2010). Some endophytes are generally viewed at as mutualists; by receiving nutrition and protection from their host plants, the endophytes enhance resistance of the host plant against insect herbivores or pathogens (Clay, 1990). Studies showed that vertically transmitted (systemic) endophytes, growing within seeds, are more likely to be mutualistic, while horizontally transmitted (non-systemic) endophytes, via spores, tend to be more antagonistic to the host (Schardl *et al.*, 1991; Saikkonen *et al.*, 1998). Furthermore, upon leaf aging or senescence, endophytic fungi can shift to the pathogenic side of the continuum, thus becoming more widespread and causing external infections (Saikkonen *et al.*, 1998).

The environmental factors play a major role to destabilize the delicate balance of antagonisms. If the plant defense mechanisms completely counteract the fungal virulence factors, the fungus will perish. Conversely, if the plant succumbs to the virulence of the fungus, a plant-pathogen relationship would lead to plant disease (Figure 2.3.B). Because many endophytes could possibly be latent pathogens, they might be influenced by certain intrinsic or environmental factors to express factors that lead to pathogenicity (Arnold, 2008) (Figure 2.3.C).

Recently, it was revealed that the plant-endophyte interaction might not be just equilibrium between virulence and defense, but a much more complex and precisely controlled interaction (Figure 2.3.D). According to the plant-endophyte coevolution hypothesis (Ji *et al.*, 2009), it might be possible for endophytes to assist the plant in chemical defense by producing bioactive secondary metabolites. Two parallel intriguing propositions have been made. Conferring to the ''mosaic effect'' theory, endophytes might protect host plants by creating a heterogeneous chemical composition within and among plant organs that are otherwise genetically uniform (Carroll, 1991). Consequently, these organs would vary arbitrarily in lusciousness or worth for herbivores, and in terms of infectivity for pathogens. The other

theory holds that endophytes might assist their corresponding host plants as ''acquired immune systems'' (Arnold *et al.*, 2003).
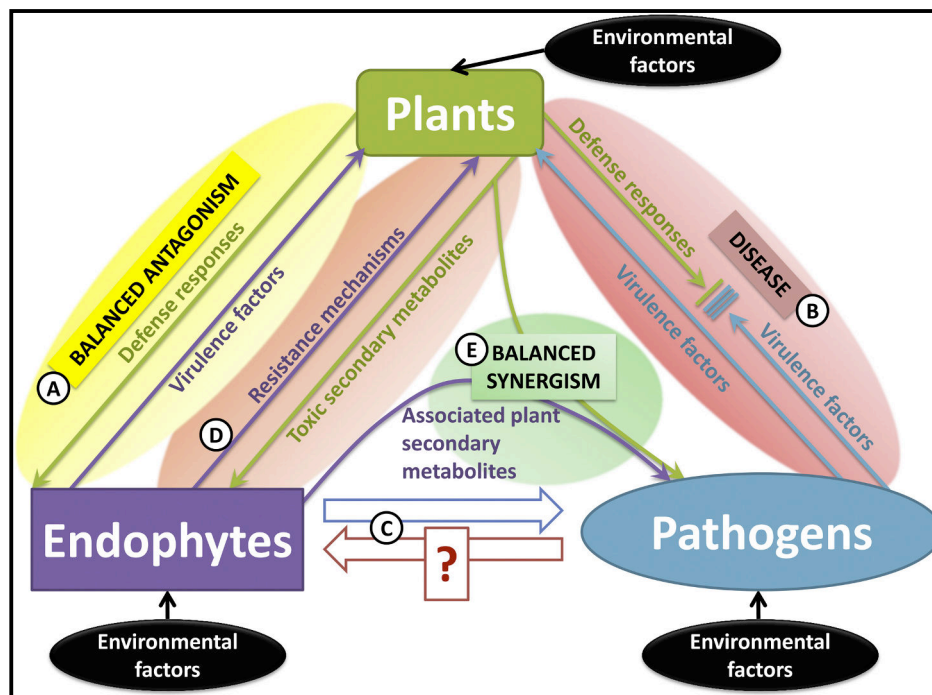
Figure 2.3. Chemical-Ecological Schematic Interpretation of Plant-Fungus Cost-Benefit Interactions with Emphasis on Endophytic Fungi (Kusari *et al*., 2012)

(A) Balanced antagonism hypothesis is shown.
(B) Plant disease caused by pathogenic fungi is presented.
(C) Endophyte-pathogen reciprocity is demonstrated. The question mark (?) indicates that this phenomenon might not be universal, and further research is necessary for verification.
(D) Endophyte survival strategy is illustrated.
(E) Balanced synergism is shown.

2.7. Pathway to new drug discovery

New medical discoveries and advancement of associated technology is further widening the gap between traditional concepts of diseases, their treatment by healers and the present day physicians. A physician interested in ethnopharmacology could fill this gap and offer modern explanation of old concepts of healing. However, all this requires a modest and collaborative attitude of physicians with healers, botanists and anthropologists which is the key to successful acquisition of the information (Lozoya, 1994).

2.7.1. Ethnopharmacological study

Ethnopharmacology and natural product drug discovery remains a significant hope in the current target-rich, lead-poor scenario. Many modern drugs have origin in traditional medicine and ethnopharmacology.

Enormous ethnopharmacological research was carried out by physicians with expertise or interest in chemistry, pharmacology, botany or anthropology during the early period of medicinal plant research 250 years ago. The classic example is of Dr. William Withering, who in 1775 discovered the use of foxglove in the treatment of 'dropsy' (i.e. edema) due to cardiac ailment (now known as congestive heart failure). The plant was used for the cure of 'dropsy' in the form of aqueous tea of 20 or more herbs by an old woman in Shropshire. Withering combined his medical expertise and knowledge of botany and discovered that foxglove was the active ingredient, and that only dropsy related to heart ailment was curable (Raza, 2006).

Table 2.1. Selected physicians and their contributions in ethnopharmacological investigations (Raza, 2006)

Willem William Withering (1741–1799)	Medicinal uses of Pilocarpus jaborandi
Pies (1611–1678)	Use of Foxglove in "Dropsy" (congestive heart failure)
Robert Christison (1797–1882)	Toxicology of Physostigma venenosum
John Hutton Balfour (1808–1884)	Description of Physostigma venenosum
Claude Bernard (1813–1878)	Pharmacological investigation of curare
Paolo Mantegazza (1831–1910)	Medicinal uses of coca
John Kirk (1832–1922)	Effect of African arrow poison (Strophanthus sp.) on CVS
Symphronio Olympio Cezar Coutinho (1832–1887)	Investigation of medicinal uses and introduction of *Pilocarpus jaborandi* in medical practice
Douglas Argyll Robertson (1837–1909)	Introduction of Physostigma venenosum in ophthalmic medicine
Thomas Richard Fraser (1841–1920)	Pharmacology of Physostigma venenosum
Nagai Nagayoshi (1844–1929)	Chemistry and pharmacology of ephedrine
John Raleigh Briggs (1851–1907)	Investigation of peyote
Arthur Heffter (1860–1925)	Chemistry and pharmacology of peyote alkaloids
Thomas Moreno Y Maiz (1868)[a]	Pharmacological investigation of cocaine

[a] Year of completion of thesis.

Several investigators (Table 2.1) who played leading roles in the discovery and/or use of physostigmine, cocaine, ephedrine, emetine, pilocarpine, strychnine, etc. from traditional sources were physicians (Raza, 2006).

Moreover, to determine whether the chemical efforts were stimulated by ethnomedical claims and to correlate current uses for the compounds with such ethnomedical claims, in 2001 Fabricant et al., conducted a study on a total of 122 compounds; 80% of these compounds were used for the same (or related) ethnomedical purposes (Table 2.2). Further, it was discovered that these compounds were derived from only 94 species of plants.

Table 2.2. Drugs derived from plants, with their ethnomedical correlations and sources.
(Fabricant, 2001).

Drug	Action or clinical use	Plant source Acetyldigoxin
Acetyldigoxin	Cardiotonic	Digitalis lanata Ehrh.
Adoniside	Cardiotonic	Adonis vernalis L.
Aescin	Anti-inflammatory	Aesculus hippocastanum L.
Aesculetin	Antidysentery	Fraxinus rhynchophylla Hance
Agrimophol	Anthelmintic	Agrimonia eupatoria L.
Ajmalicine	Circulatory disorders	Rauvolfia serpentina (L.) Benth ex. Kurz
Allyl isothiocyanate	Rubefacient	Brassica nigra (L.) Koch
Andrographolide	Bacillary dysentery	Andrographis paniculata Nees
Anisodamine	Anticholinergic	Anisodus tanguticus (Maxim.) Pascher
Anisodine	Anticholinergic	Anisodus tanguticus (Maxim.) Pascher
Arecoline	Anthelmintic	Areca catechu L.
Asiaticoside	Vulnerary	Centella asiatica (L.) Urban
Atropine	Anticholinergic	Atropa belladonna L.
Berberine	Bacillary dysentery	Berberis vulgaris L.
Bergenin	Antitussive	Ardisia japonica Bl.
Bromelain	Anti-inflammatory; proteolytic agent	Ananas comosus (L.) Merrill
Caffeine	CNS stimulant	Camellia sinensis (L.) Kuntze
(+)-Catechin	Haemostatic	Potentilla fragaroides L.
Chymopapain	Proteolytic; mucolytic	Carica papaya L.
Cocaine	Local anaesthetic	Erythroxylum coca Lamk
Codeine	Analgesic; antitussive	Papaver somniferum L.
Colchicine	Antitumor agent; antigout	Colchicum autumnale L.
Convallotoxin	Cardiotonic	Convallaria majalis L.
Curcumin	Choleretic	Curcuma longa L.
Cynarin	Choleretic	Cynara scolymus L.
Danthron	Laxative	Cassia spp.
Deserpidine	Antihypertensive; tranqulizer	Rauvolfia canescens L.
Deslanoside	Cardiotonic	Digitalis lanata Ehrh.
Digitalin	Cardiotonic	Digitalis purpurea L.
Digitoxin	Cardiotonic	Digitalis purpurea L.
Digoxin	Cardiotonic	Digitalis lanata Ehrh
Emetine	Amoebicide; emetic	Cephaelis ipecacuanha (Brotero) A. Richard
Ephedrine	Sympathomimetic	Ephedra sinica Stapf.
Etoposide	Antitumour agent	Podophyllum peltatum L.
Gitalin	Cardiotonic	Digitalis purpurea L.
Glaucaroubin	Amoebicide	Simarouba glauca DC.
Glycyrrhizin	Sweetener	Glycyrrhiza glabra L.
Gossypol	Male contraceptive	Gossypium spp.
Hemsleyadin	Bacillary dysentery	Helmsleya amabilis Diels
Hydrastine	Hemostatic; astringent	Hydrastis canadensis L.
Hyoscamine	Anticholinergic	Hyoscamus niger L.
Kainic Acid	Ascaricide	Digenea simplex (Wulf.) Agardh
Kawain	Tranquilizer	Piper methysicum Forst. f.
Khellin	Bronchodilator	Ammi visnaga (L.) Lamk.
Lanatosides A, B, C	Cardiotonic	Digitalis lanata Ehrh.
Lobeline	Smoking deterrent; respiratory stimulant	Lobelia inflata L.
Monocrotaline	Antitumor agent	Crotolaria sessiliflora L.
Morphine	Analgesic	Papaver somniferum L.
Neoandrographolide	Bacillary dysentery	Andrographis paniculata Nees
Noscapine	Antitussive	Papaver somniferum L.
Ouabain	Cardiotonic	Strophanthus gratus Baill.
Papain	Proteolytic; mucolytic	Carica papaya L.
Phyllodulcin	Sweetener	Hydrangea macrophylla (Thunb.) DC
Physostigmine	Cholinesterase inhibitor	Physostigma venenosum Balf.

Picrotoxin	Analeptic	Anamirta cocculus (L.) W.&A.
Pilocarpine	Parasympathomimetic	Pilocarpus jaborandi Holmes
Podophyllotoxin	Condylomata acuminata	Podophyllum peltatum L.
Protoveratrines A & B	Antihypertensive	Veratrum album L.
Pseudoephedrine	Sympathomimetic	Ephedra sinica Stapf.
Pseudoephedrine, nor-	Sympathomimetic	Ephedra sinica Stapf.
Quinine	Antimalarial	Cinchona ledgeriana Moens ex. Trimen
Quisqualic	Acid Anthelmintic	Quisqualis indica L.
Rescinnamine	Antihypertensive; tranqulizer	Rauvolfia serpentina (L.) Benth ex. Kurz
Reserpine	Antihypertensive; tranqulizer	Rauvolfia serpentina (L.) Benth ex. Kurz
Rhomitoxin	Antihypertensive	Rhododendron molle G. Don
Rorifone	Antitussive	Rorippa indica (L.) Hochr.
Rotenone	Piscicide	Lonchocarpus nicou (Aubl.) DC.
Rotundine	Analgesic; sedative	Stephania sinica Diels
Salicin	Analgesic	Salix alba L.
Santonin	Ascaricide	Artemisia maritima L.
Scillarin A	Cardiotonic	Urginea maritima (L.) Baker
Scopolamine	Sedative	Datura metel L.
Sennosides A & B	Laxative	Cassia spp.
Silymarin	Antihepatotoxic	Silybum marianum (L.) Gaertn.
Stevioside	Sweetener	Stevia rebaudiana Bertoni
Strychnine	CNS stimulant	Strychnos nux-vomica L.
Teniposide	Antitumor agent	Podophyllum peltatum L.
Tetrahydropalmatine	Analgesic; sedative	Corydalis ambigua (Pallas) Cham. & Schltal
Theobromine	Diuretic; bronchodilator	Theobroma cacao L.
Theophylline	Diuretic; bronchodilator	Camellia sinensis (L.) Kuntze
Trichosanthin	Abortifacient	Thymus vulgaris L
Tubocurarine	Skeletal muscle relaxant	Chondodendron tomentosum R. & P.
Valepotriates	Sedative	Valeriana officinalis L.
Vincamine	Cerebral stimulant	Vinca minor L.
Xanthotoxin	Leukoderma; vitiligo	Ammi majus L.
Yohimbine	Aphrodisiac	Pausinystalia yohimbe (K.Schum.) Pierre
Yuanhuacine	Abortifacient	Daphne genkwa Seib. & Zucc
Yuanhuadine	Abortifacient	Daphne genkwa Seib. & Zucc.

The ethnopharmacology knowledge can always serve as an innovative and powerful discovery engine for newer, safer and affordable medicines.

2.7.2. Screening of the promising natural sources for drug discovery

To the discovery of natural products with biologic activity three areas are involved, the *observation*, *description*, and *experimental investigation* of indigenous drugs and their biologic activities. It is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines (anthropology, archaeology, history, and linguistics). Several approaches to drug discovery for selecting plants as candidates for drug discovery programs have been published (Phillipson, 1989; Kinghorn, 1994; Vlietinck, 1991; Farnsworth, 1966; Farnsworth, 1977; Harvey, 2000; Farnsworth, 1988); some of these approaches are :

- *Random selection followed by chemical screening.* These so-called phytochemical screening approaches [i.e., for the presence of cardenolides/bufadenolides, alkaloids, triterpenes, flavonoids, isothiocyanates, iridoids, etc. (Farnsworth *et al.*, 1966; Fabricant *et al.*, 2001)] have been used in the past and are currently pursued mainly in the developing countries. The tests are simple to perform, but it is usually impossible to relate one class of phytochemicals to specific biologic targets; for example, the alkaloids or flavonoids produce a vast array of biologic effects that are usually not predictable in advance.

- *Random selection followed by one or more biologic assays.* In the past, plant extracts were evaluated mainly in experimental animals, primarily mice and rats. From 1960 to 1981, more than 35,000 species were screened *in vitro* and later *in vivo* at National Cancer Institute (NCI). Taxol and camptothecin were discovered in this program. In 1986 the NCI program continued to collect and screen plants using a battery of 60 human tumor cell lines. Calanolide A, currently in Phase I clinical trials, was developed from this program (Fabricant *et al.*, 2001).

- *Follow-up of biologic activity reports.* These reports showed that the plant extracts had interesting biologic activity, but the extracts were not studied for their active principles. The literature from the 1930s through the 1970s contains these types of reports.

- *Follow-up of ethnomedical (traditional medicine) uses of plants.* Several types of ethnomedical information are available:

- *Plants used in organized traditional medical systems.* The individual arrangements all emphasize education based on an established, frequently revised body of written knowledge and theory. These systems are still in place today because of their organizational strengths, and they focus primarily on multicomponent mixtures (Bannerman *et al.*, 1983; Fabricant *et al.*, 2001).

- *Herbalism, folklore, and shamanism.* An apprenticeship system of information passed to the next generation through a shaman, curandero, traditional healer, or herbalist. The plants that are used are often kept secret by the practitioner, so little information about them is recorded; thus there is less dependence on scientific evidence as in systems of traditional medicine that can be subject to scrutiny. The shaman or

herbalist combines the roles of pharmacist and medical doctor with the cultural/spiritual/religious beliefs of a region or people, which are often regarded as magic or mysticism. This approach is widely practiced in Africa and South America (Rastogi *et al*., 1982; Fabricant *et al*., 2001).

- *Use of databases.* The NAPRALERT database (Fabricant *et al*., 2001) currently contains information on 43,879 species of higher plants covering ethnomedical, chemical, and pharmacologic (including clinical studies) uses. Of these, 13,599 species contain ethnomedical data, distributed among 3,607 genera and 273 plant families. Thus it is possible to correlate ethnomedical use with experimental biochemical or pharmacologic activities (*in vitro, in vivo*, or *in humans*) to identify plants having both types of activity for a given effect-e.g., anticancer, antidiabetic, antimalarial.

- *Ethnomedical approach.* In 1985 an approach based on ethnomedical information to experimentally pursue plants as a source of drugs was established. The approach was designed primarily for implementation by developing countries, where lack of hard currency often prevents sophisticated types of research from being conducted. The possibility of drug development in the form of stable, standardized crude extracts and eventual development of the active principles from these plants was envisioned (Fabricant *et al*., 2001) (Figure 2.4).

Some examples of drugs from plants that served as models for the next generation of drugs are exemplified as follows: Khellin [from *Ammi visnaga (L.) Lamk*.] was used as a bronchodilator in the United States until it was shown to produce nausea and vomiting after prolonged use. In 1955 a group of chemists in England set about to synthesize khellin analogs as potential bronchodilators with fewer side effects. This eventually led to the discovery of chromolyn (used as sodium chromoglycate), which stabilized cell membranes in the lungs to prevent the allergen induced release of the substance ultimately causing bronchoconstriction in allergic asthma patients (Fabricant *et al*., 2001).
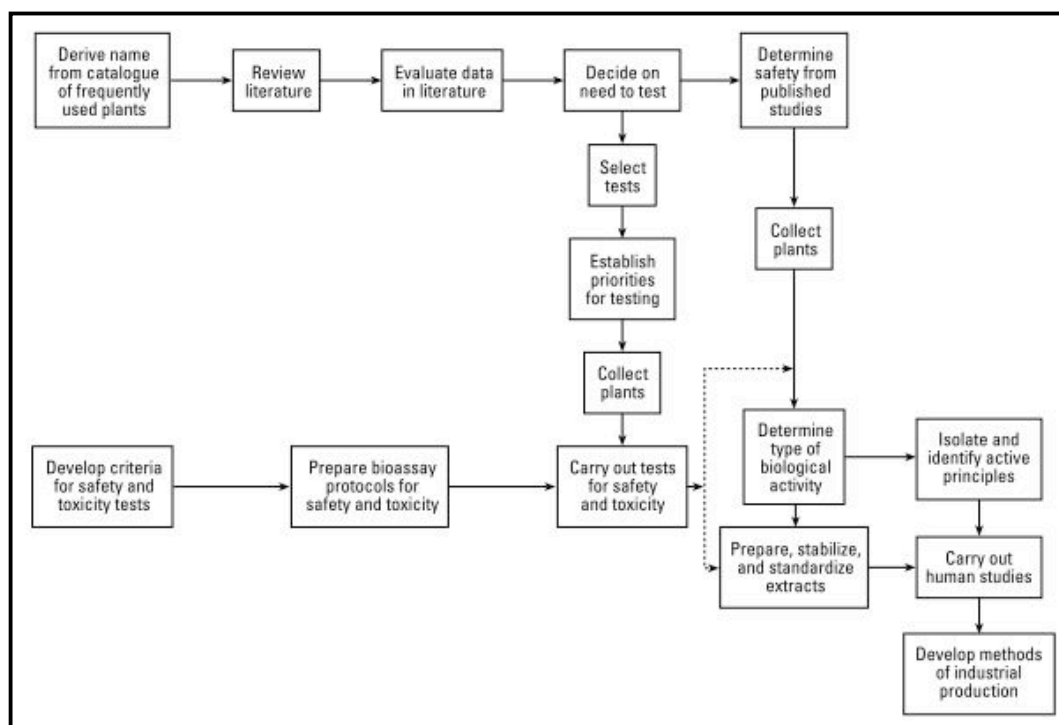
Figure 2.4. Flow chart of sequence for the study of plants used in traditional medicine. Adapted from Farnsworth et al. (Farnsworth, 1985)

2.8. Anticancer drug discovery

2.8.1. Cancer statistics

Worldwide, over ten million new cases of cancer (all sites excluding non-melanoma skin), with over six million deaths, were estimated in the year 2000 (Parkin, 2001; Parkin *et al.*, 2001). Since 1990 there has been a 22% increase in cancer incidence and mortality with the four most frequent cancers being lung, breast, colorectal, and stomach and the four most deadly cancers being lung, stomach, liver, and colorectal (Parkin *et al.*, 2001). Cancer is the second leading cause of death in developed countries, surpassed only by cardio-vascular disease (Jemal *et al.*, 2005).

In Morocco, cancer is a major public health problem and is responsible for 56% of morbidity related to chronic diseases (Tazi *et al.*, 2013). A total of 2,473 new cases were registered among residents in Rabat during the period 2006–2008. The overall world age-standardised rate (ASR) for all sites combined was 136.6/100,000 for men and 114.5/100,000 for women (Tazi *et al.*, 2013). About 176,620 new cancer cases are expected to be diagnosed among African Americans in 2013, including 94,540 cases among men and 82,080 cases among women (Figure 2.5). Prostate cancer is expected to be the most commonly diagnosed cancer in men and breast cancer the most common in women. Cancers of the lung and colorectum

will be the second and third most commonly diagnosed cancers in both African American men and women (Desantis *et al*., 2013).

Estimated New Cases						
			Males	**Females**		
Prostate	35,430	37%		Breast	27,060	33%
Lung & bronchus	13,110	14%		Lung & bronchus	10,980	13%
Colon & rectum	9,280	10%		Colon & rectum	8,830	11%
Kidney	4,400	5%		Uterine corpus	5,690	7%
Liver & intrahepatic bile duct	3,270	3%		Thyroid	3,850	5%
Non-Hodgkin lymphoma	2,960	3%		Kidney	3,030	4%
Pancreas	2,670	3%		Pancreas	2,930	4%
Oral cavity & pharynx	2,460	3%		Non-Hodgkin lymphoma	2,590	3%
Urinary bladder	2,380	2%		Myeloma	2,170	3%
Leukemia	2,220	2%		Uterine cervix	2,060	3%
All Sites	**94,540**			**All Sites**	**82,080**	

Estimated Deaths						
			Males	**Females**		
Lung & bronchus	9,430	29%		Lung & bronchus	6,830	21%
Prostate	4,980	15%		Breast	6,080	19%
Colon & rectum	3,600	11%		Colon & rectum	3,250	10%
Liver & intrahepatic bile duct	2,240	7%		Pancreas	2,390	7%
Pancreas	2,110	6%		Uterine corpus	1,500	5%
Stomach	1,100	3%		Ovary	1,330	4%
Leukemia	1,040	3%		Myeloma	940	3%
Myeloma	950	3%		Leukemia	890	3%
Esophagus	900	3%		Liver & intrahepatic bile duct	830	3%
Kidney	820	2%		Stomach	760	2%
All Sites	**32,970**			**All Sites**	**31,910**	

Figure 2.5. Leading Sites of New Cancer Cases and Deaths Among African Americans, 2013 Estimates* (Desantis *et al*., 2013)

*Estimates are rounded to the nearest 10 and exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder.

Although these figures are disquieting, some progress has been made in cancer diagnosis and treatment as evident through the high incidence of lung, breast and prostate, as compared with their relatively lower mortality (Parkin, 2001; Jatoi and Miller, 2003; Jemal *et al*., 2005). From 2000 to 2009, incidence rates decreased by 1.4% per year among African American men; however, rates have remained unchanged among African American women during this period (Figure 2.6).

Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Newman *et al*., 2000, Newman *et al*., 2003; Butler, 2004; Balunas *et al*., 2005).
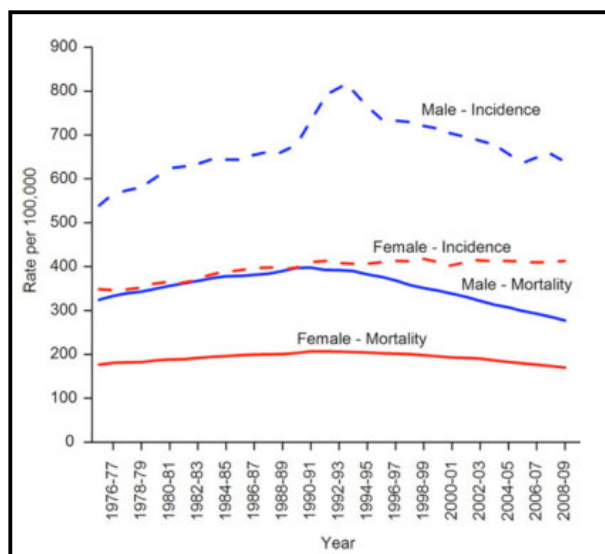
Figure 2.6. Age-Adjusted Cancer Incidence and Mortality Rates Among African Americans by Sex, 1975 to 2009 (Desantis *et al.*, 2013)

1.8.2. Secondary metabolites as anticancer agents

Large-scale anticancer drug discovery and screening programs such as those promoted by the National Cancer Institute (NCI) have played an important role in the development of anticancer natural compounds. Of all available anticancer drugs between 1940 and 2002, 40% were natural products per se or natural product-derived with another and 8% was considered natural product mimics (Newman *et al.*, 2003). The most famous anticancer natural product is Taxol, which was originally isolated from *Taxus brevifolia Nutt.* (*Taxaceae*) and later was demonstrated that it was produced by endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia* (Strobel *et al.*, 1993; Aly, 2007). Paclitaxel (taxol®) was clinically introduced to the U.S. market in the early 1990s (Wall and Wani, 1996; Oberlies and Kroll, 2004). The taxanes, including paclitaxel and derivatives, act by binding tubulin without allowing depolymerization or interfering with tubulin assembly (Balunas, 2005). Taxol is the world's first billion dollar anticancer drug and is used to treat a number of other human tissue proliferating diseases as well (Strobel, 2002).

Moreover, the cytotoxic plant alkaloid, camptothecin, originally described from *Camptotheca acuminate* and *Nothapodytes foetida*, showed unacceptable myelosuppression (Wall and Wani, 1996; Balunas, 2005). Interest in camptothecin was revived when it was found to act by selective inhibition of topoisomerase I, involved in cleavage and reassembly of DNA (Cragg and Newman, 2004). Clinical trials of camptothecin, which was identified in cultures of

Entrophospora infrequens endophytic in *Nothapodytes foetida,* are undergoing since 1992 as anticancer drug (Amna *et al.*, 2006).

Together, the taxanes and the camptothecins accounted for approximately one-third of the global anticancer market in 2002, over 2.75 billion dollars (Oberlies and Kroll, 2004).

Another anticancer drug, which has been given in chemotherapy treatment for some types of cancer including leukemia, lymphoma, breast and lung cancer for many years, is the indole derivative vincristine. This drug, available under the trade names Oncovin®, Vincasar®, and Vincrex®, was originally obtained from *Catharanthus roseus*. Very recently, a Chinese group reported preliminary evidence that vincristine might be produced by *Fusarium oxysporum* endophytic in the same plant (Zhang *et al.*, 2006). Since then fungal microorganisms became a hunting ground for novel drug leads (Strobel and Daisy, 2003; Larsen *et al.*, 2005). This stimulated pharmaceutical companies to sample and screen large collections of fungal strains (Butler, 2004). Consequently, promising novel natural product leads were isolated. Many of these compounds can be produced in large quantities and at a reasonable cost by fermentation employing wild type or genetically altered fungi. The search for new drug candidates is still pressing to find the adequate cures for not only cancer but also newly emerging diseases, such as drug-resistant pathogenic microbes, or parasitic protozoans, are urgently needed. As microorganisms occupy almost every niche on earth (Strobel, 2002), scientists speculate that many undescribed species exist in unexplored habitats, where the incidence of finding microorganisms that produce novel bioactive constituents is high (Hawksworth and Rossman, 1997).

2.9. Natural products as inhibitors of Hsp 90 chaperon machinery
2.9.1. Hsp 90 chaperone machinery

The 90-kDa heat shock protein Hsp90 has an important role in the maintenance of malignant transformation and is required to keep the folded and functionally active conformation of several aberrantly functioning onco-proteins. In this respect, Hsp90 regulates (in a transformation-specific manner) signaling pathways necessary for the growth, survival and limitless replicative potential of most tumors. Whereas in normal cells Hsp90 interacts in a low-affinity 1-2%, dynamic fashion with a plethora of proteins to help them fold and mature, in malignant cells tight association of Hsp90 with onco-client proteins maintains their ability to function in the dysregulated state and seems to be essential for their transforming, aberrant

activity which increases its affinity to 4-6%. This is most likely because cancer cells must cope with numerous external and internal stressors that are not experienced by normal cells, such as elevated temperature and oxygen or nutrient deprivation or mutation of important regulatory proteins (Figure 2.7) (Rodina *et al*., 2007; Chadli *et al*., 2008).
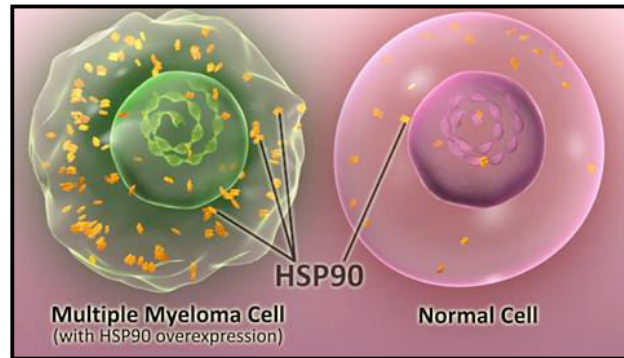
Figure 2.7. The expression of Hsp90 in normal and multiple myeloma cell
(@cancer.gov.com)

Hsp90 was originally identified by chance in the early 1960s as one of a group of so-called 'heat shock proteins' (Hsps) that were induced at elevated temperatures in the salivary gland of the fruit fly *Drosophila melanogaster*, providing protection against hyperthermia (thermotolerance) and other cellular stresses (Workman *et al*., 2007). Hsp90 works in concert with a number of chaperones and co-chaperones to modulate the conformation of Hsp90 and its client proteins. Over 20 co-chaperone proteins, such as p23, Cdc37, HIP, HOP, PP5, UNC45A, and immunophilins (FKBP51, FKBP52, and Cyp40), have been shown to regulate the function of the Hsp90 protein-folding machine (Chadli *et al*., 2008).

Hsp90's clientele is dominated by kinases, hormone receptors and transcription factors, many of which are oncogenic and have a key role in the hallmark traits of cancer. In addition to cancer, the Hsp90 machine has also been implicated as a target to treat neurodegenerative and cardiovascular diseases.

2.9.2. Hsp 90 inhibitors

Heat shock protein 90 "Hsp90" is a promising therapeutic target. The inactivation of Hsp90 delivers a combinatorial attack on multiple signaling pathways leading to a more efficient killing of cancer cells and reducing resistance to chemotherapy (Workman, 2004). Extensive

research efforts in the last two decades have resulted in over 40 clinical trials in phases I-III with 13 small molecule inhibitors of Hsp90 ongoing worldwide. Hsp90 inhibitors can be classified as those that target the N-terminal domain, the C-terminal domain, Hsp90 co-chaperones or client-protein/Hsp90 interactions (Neckers *et al.*, 2012).

Hsp90 inhibitors, initially natural products and later synthetic small molecules, have proved invaluable as chemical tools to investigate Hsp90 biology; the initial chemical probes have now morphed into drugs showing therapeutic activity in cancer patients.
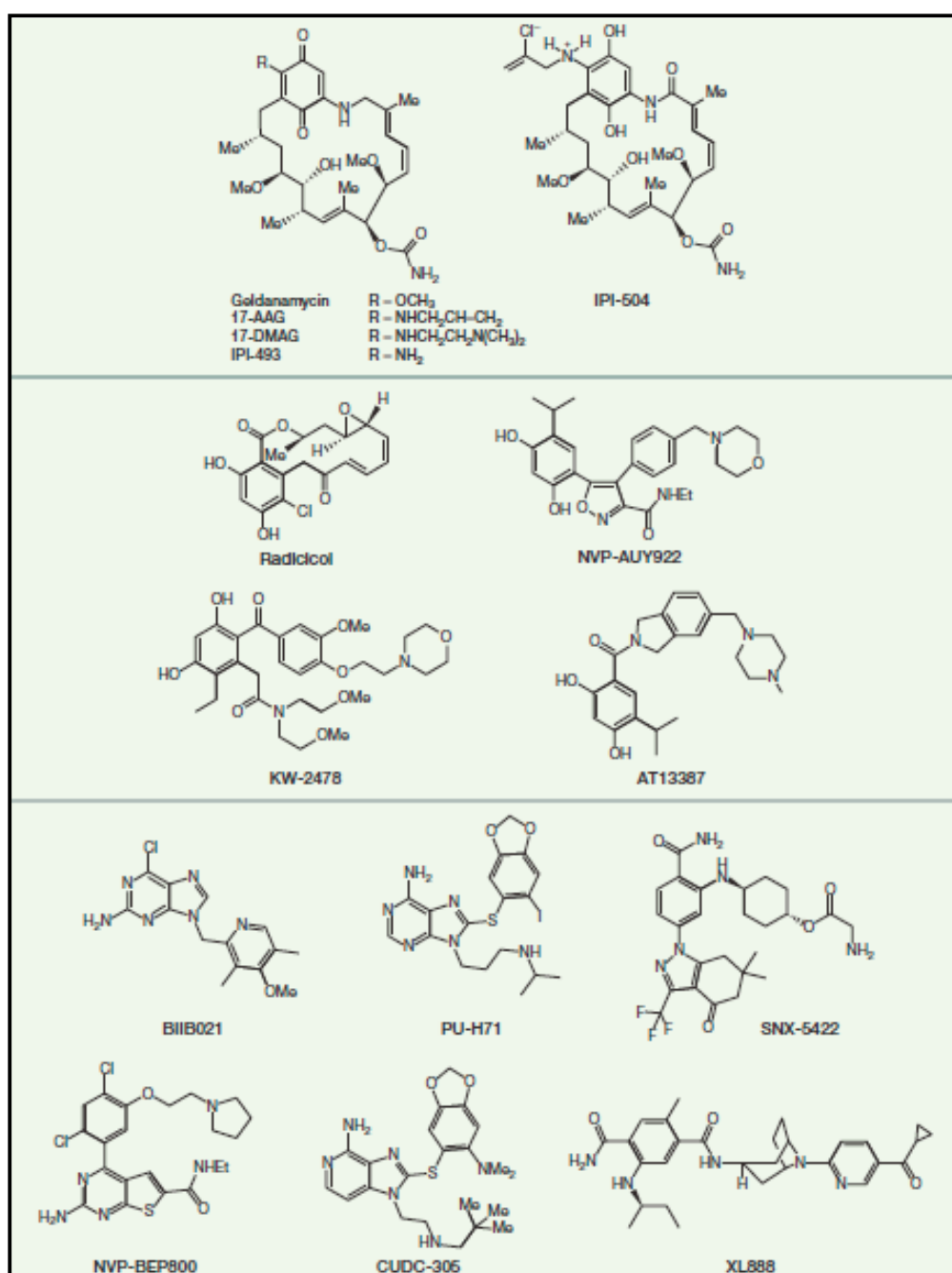
Figure 2.8. Chemical structures of selected Hsp90 inhibitors (Neckers, 2012)

The natural products geldanamycin and radicicol as well as semisynthetic derivatives 17-N-allylamino-17-demethoxygeldanamycin (17AAG) (Figure 2.8) target the ATP-binding site of the N-terminal domain to destabilize Hsp90 client complexes, leading to their cellular degradation through the proteasome pathway.

Recently, a Chadli team reported that the natural product gedunin inactivates Hsp90 chaperoning machinery by targeting the co-chaperone p23 *in vivo* and *in vitro*. Gedunin is a tetranortriterpenoid natural product that is isolated from the *Meliacae* family of medicinal plants and has been used for the treatment of malaria and other infectious diseases in traditional Indian medicine (Chadli, 2008).

2.9.3. Example of the inhibition of Hsp 90 in small-cell lung cancer

Hsp90 operates with a number of co-chaperones, including Hsp70 and Hop (Hsp90 organizing protein, also known as p60), to orchestrate the ATP-dependent maturation of many oncogenic client proteins (CP). Inhibition of Hsp90 function leads to ubiquitin proteasome–dependent client protein degradation and inhibition of multiple aspects of the malignant phenotype, including evasion of apoptosis in SCLC. During apoptosis, cytochrome c (cyt c) is released from the mitochondria to associate with Apaf-1. This triggers the activation of proteolytic caspases, which induce apoptosis. Mitochondrial permeabilization is controlled by a balance of antiapoptotic (for example, BCL2) and proapoptotic (for example, BAX and BAD) members of the BCL2 family.

Activation of the kinase AKT (an Hsp90 client protein) shifts the balance in favor of cell survival by phosphorylating BAD, inducing its inactivation by association with 14-3-3 protein. Hsp90 and Hsp70 are both antiapoptotic, acting at several points in the apoptotic cascade. Rodina group have shown Hsp90 to be a critical dual regulator of apoptosis in SCLC by supporting AKT activation, which in turn prevents cyt c release, and also by negatively regulating the association of Apaf-1 with caspase 9 (Rodina *et al*., 2007).

Hsp90 inhibition reverses these effects, leading to extensive apoptosis. Several compounds that modulate the activity of Hsp90 (17-AAG, radicicol, PU24FCl) and Hsp70 (MAL3-101) have been described, along with compounds that can alter the expression of Hsp70 and other heat shock proteins in response to stress (for example, KNK437) (Paul Workman *et al*., 2007).
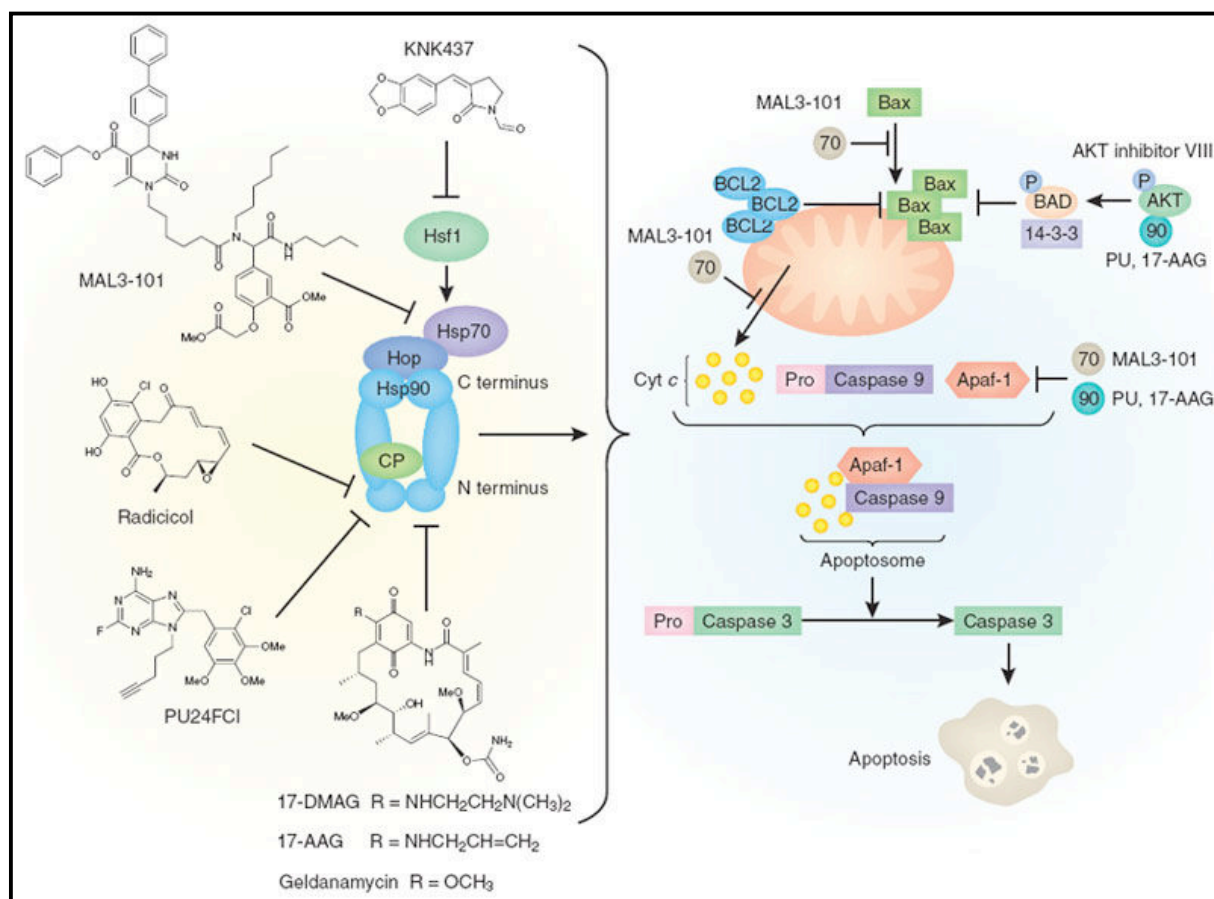
Figure 2.9. Inhibition of Hsp90 leads to apoptosis in small-cell lung cancer (SCLC).
(Paul Workman *et al.*, 2007)

Since then, natural drug discovery has focused on the inhibitors of chaperone Hsp90 (heat shock protein 90) because it plays a key role in assisting mutated proteins, making it an attractive cancer drug target.

3. Materials and Methods

3.1. Biological material

3.1.1. Plant material

3.1.1.1. Ethnopharmacological study

Study design

The work consists of an ethnopharmacological, prospective and randomized investigation. It was carried out on a period of seven months (from September 2009 to March 2010) in two departments at the National Institute of Oncology in Rabat (Department of Chemotherapy and Department of Radiotherapy). The National Institute of Oncology (NIO) is a center where patients come from all over Morocco. The patients are taken randomly, to have a heterogeneous point of view: origin, sex, age, socio-professional class, type and stage of cancer. This study has been carried out with the permission of NIO director. All patients have been informed of the study objective and gave their consent.

Selection of parameters

An exhaustive questionnaire was established including:

- Information related to the identification of the patient [registration number, sex, age, origin, province region (rural or urban), and socio-professional class];

- Information on pathology [nature, localization, stage and degree of tumor extension as well as protocol and treatment follow-up];

- Information on the use of traditional medicine [use or non use of TM, its use along with modern medicine];

- Information on the anti-cancer plants used [vernacular name and the part of the plant used, the method of preparation, dosage, treatment duration and observance during phytotherapy];

After collecting all the information, a table was prepared to gather different information on the plants used with their vernacular name and their scientific name.

Statistical analysis

Statistical analysis of data was carried out by Graphpad program (for Windows version 5.01. Graphpad, San Diego, CA, USA) and statistical methodology was based on two axes, which includes descriptive statistics and statistical analysis. Descriptive statistics reveal the

frequencies and characteristics of each parameter (average/mean, minimum, maximum). Results are expressed in raw values for qualitative parameters and in mean +/- standard deviation for quantitative parameters. The number of valid data (n active) of each variable has been mentioned in results section. Statistical analysis was based on associated tests such as the Khi 2 test which measures the gap between the observed frequencies and theoretical frequencies. We have used this test to compare the two sexes. We have also used one factor variance analysis (ANOVA), which estimates intergroup variation (report F). The results are considered significant where p is below 0.05, very significant when $p < 0.01$ and highly significant when $p < 0.001$.

On the other hand, calculation of relative risk (RR) for each age interval as well as sex variable concerning the use of TM has allowed us to estimate the degree of association between two given parameters. If value 1 is included in the confidence interval (CI) of RR, we deduce that there is no association between these two parameters. However, if value 1 is excluded of the CI of RR, we deduce the existence of association between them.

3.1.1.2. Plant worked on during the study

Thymelaea lythroides was selected according to an ethnopharmacological study of traditional plants used in Morocco by cancer patients as herbal therapeutics (Kabbaj *et al.*, 2012). *Thymelaea lythroides*, an ibero-endemic plant in Morocco, is popularly used as medicinal plant to treat otitis, diabetes, rheumatism, inflammation of the prostate and uterus cancer (Gmira *et al.*, 2007).
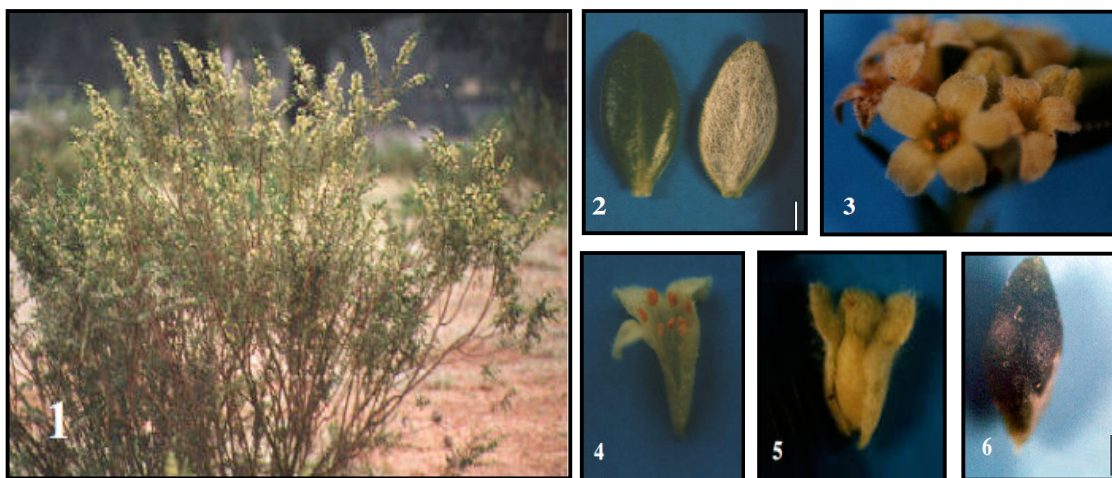
Figure 3.1. *Thymelaea lythroides*. (**1**: Arial part of *T. lythroides*. **2**: Leaf. **3**: Flower. **4**: Flower male. **5**: Flower female. **6**: Seed) (Dohou, 2003)

3.1.1.3. Field trip and sample collection

Fresh, healthy areal parts of *Thymelaea lythroides* have been collected from the Maâmora forest (NI29-08) in Rabat, Morocco. Small stem, leaf and flower pieces were directly placed in paper bags and stored dark and at 4°C until the isolation procedure of the fungi was performed two days later.
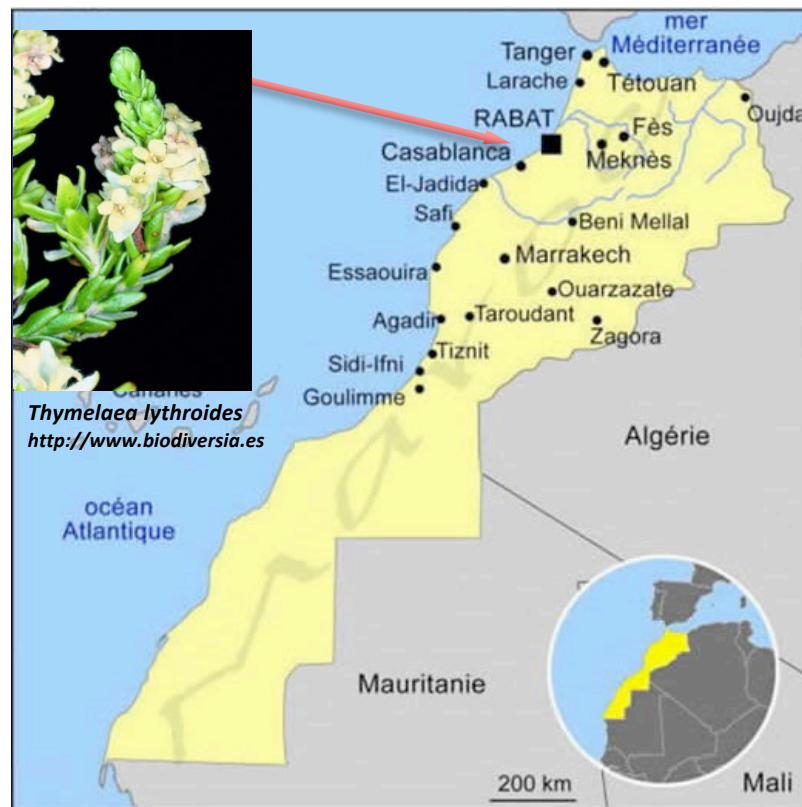
Figure 3.2 Collection area of *Thymelaea lythroides*.

3.1.1.4. Taxonomy of collected plant sample

*Phylum: **Plantae***
  *Class: **Magnoliopsida***
    *Order: **Myrtales***
      *Family: **Thymelaeaceae***
        *Genus: **Mentha***
          Specie: ***Thymelaea lythroides***

3.1.2. Fungal Material

3.1.2.1. Pure fungal strain isolated from the selected plant

Fresh, healthy parts of *Thymelaea lythroides* (leaf, stem and flower) were rinsed twice with sterilized distilled water. Surface sterilization was achieved by immersing the parts of the

plant in 70% ethanol for 2 min (twice) followed by rinsing in sterilized distilled water. Then, they were cleaved aseptically into small segments (approx.1 cm in length). The material was placed onto a Petri dish with malt agar medium containing chloramphenicol and streptomycin to suppress bacterial growth. After one-week incubation at room temperature, when fungal mycelium tips were observable, each hyphal tip was transferred onto a fresh malt agar dish. For purification of the fungal strains this step was repeated several times until the colony was deemed uniform.
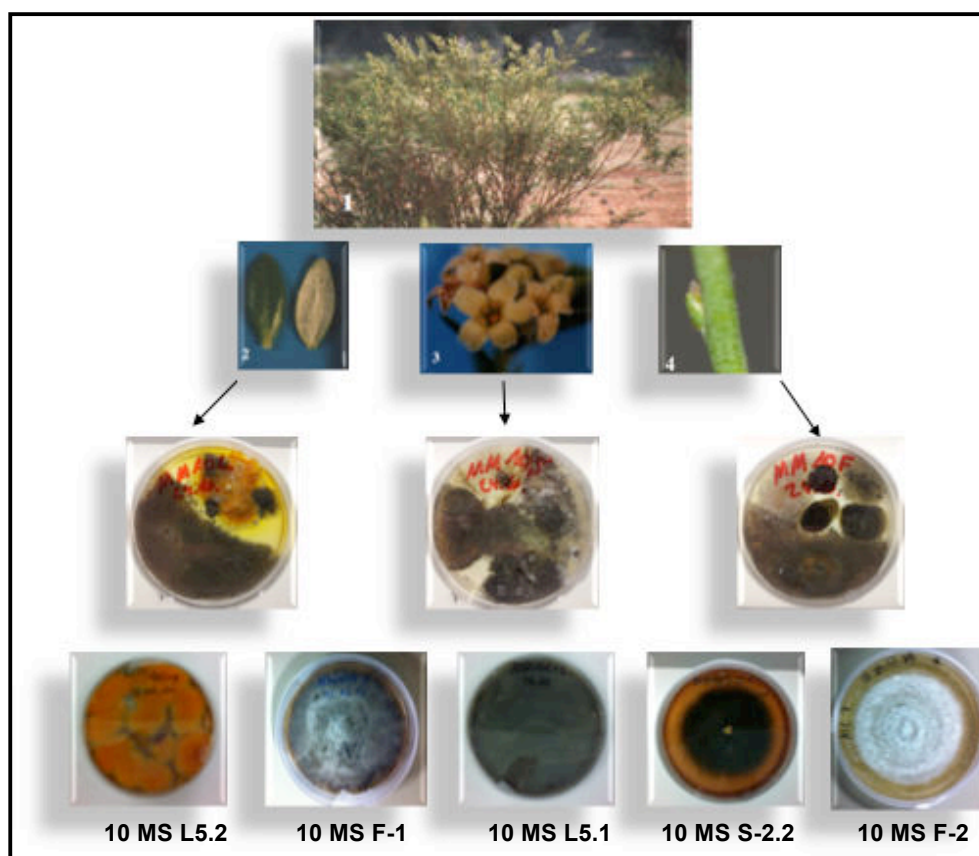
Figure 3.3. Pure fungal strain from *Thymelaea lythroides*.

3.1.2.2. Taxonomy of isolated fungi

Table 3.1. Taxonomy of isolated fungi.

Fungal code	Plant part	Fungal species	Family
10 MS L-5.2	Leaf	*Epicoccum nigrum*	Incertae sedis
10 MS F-1	Flower	*Altenaria sp.*	Pleosporaceae
10 MS L.5.1	Leaf	*Cladosporium sp.*	Davidiellaceae
10 MS S-2.2	Stem	*Chaetomium aureum*	Chaetomiaceae
10 MS F-2	Flower	*Pleospora sp.*	Pleosporaceae

3.1.2.3. Endophytic fungi worked on during the study

After conducting biological activity screening test toward the five fungal strains, derived from the plant *Thymelaea lythroides*, originated from Morocco, one fungal strain was eventually selected for further scrutinizing of their secondary metabolite contents, which is *Chaetomium aureum* (Figure 3.4).
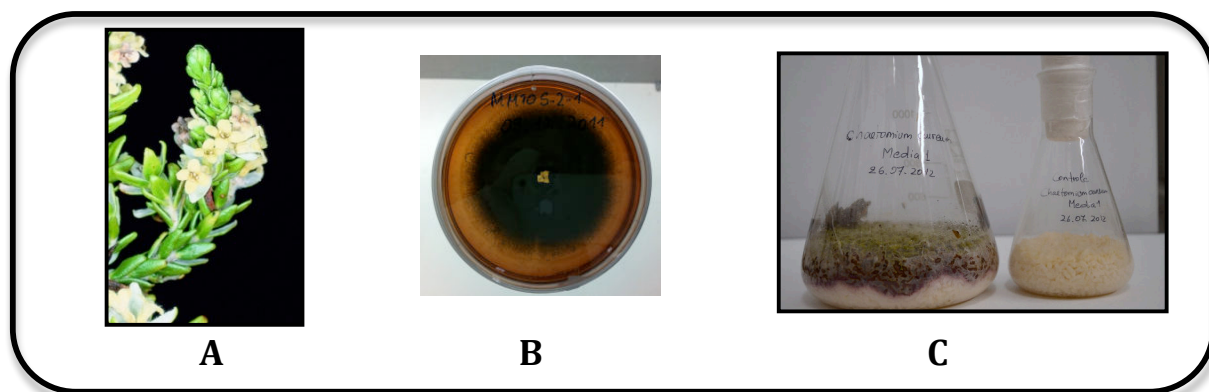
Figure 3.4. *Chaetomium aureum* (**A**: *Thymelaea lythroides*; **B**: pure strain in plate agar; **C**: Culture on solide rice medium)

3.2. Isolation and cultivation of endophytic fungi

3.2.1. Composition of media

Medium for isolation of fungal strains from the plant or purification and short term storage of the pure fungal strain

Bacto agar (Galke)	15.0 g
Malt extract (Merck)	15.0 g
Artificial sea salt (Sera)	10.0 g
pH	7.4-7.8 (adjusted with NaOH/HCl)
Dem. Water	ad 1000 mL

For the isolation of endophytic fungi from plant tissues chloramphenicol or streptomycin (0.2 or 0.1 g, respectively) were added to the medium to suppress bacterial growth.

MexA medium for long term storage

Malt extract (Merck)	20.0 g
Yeast extract (Sigma)	0.1 g
Glycerin (Roth)	50.0 g
Artificial sea salt (Sera)	10.0 g
Bacto Agar (BD)	13.0 g
Dem. water	ad 1000 mL

Wickerham medium for liquid cultures

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Distilled water	to 1000 mL
pH	7.2 - 7.4 (adjusted with NaOH/HCl)

Solid rice medium

Rice	100 g
Dem. Water	110 mL

Solid red bean medium

Bean	100 g
Dem. Water	110 mL

Solid chickpeas medium

Bean	100 g
Dem. Water	110 mL

Solid corn medium

Bean	100 g
Dem. Water	110 mL

Solid peas medium

Bean	100 g
Dem. Water	110 mL

Solid Beans M medium

Bean	100 g
Dem. Water	110 mL

Solid black bean medium

Bean	100 g
Dem. Water	110 mL

Solid lentils medium

Bean	100 g
Dem. Water	110 mL

Solid carrots medium

Bean	100 g
Dem. Water	110 mL

Solid potaetos medium

Bean	100 g
Dem. Water	110 mL

3.2.2. Short term storage of pure strains

For short storage, pure fungal strains in malt agar plate were stored at 4°C for a maximum period of 6 months, and then re-inoculated onto fresh malt agar plate.

3.2.3. Long term storage of pure fungal strains

For long term storage, pieces of pure fungal strains were transferred to 10 mL BD Falcon® tubes containing approx. 5 mL MexA medium. After approx. from three to seven days, the growths strain was placed in a deep freezer at -80°C.

3.2.4. Cultivation for screening and isolation

Pure fungal strain was inoculated in a 1000 mL Erlenmeyer flask containing 100 g of solid rice medium and 110 ml of distilled water, which were autoclaved. Small pieces were cut from a Petri dish containing the purified fungus, and then transferred under sterile conditions to the sterilised rice medium. Cultivation was performed at room temperature under static conditions for 4-6 weeks.

Small scale fermentation was carried out in one Erlenmeyer flask to gain enough extract for first bioactivity screening. For mass growth to gain enough fungal extract for isolation of secondary metabolites, 20 flasks of rice medium were inoculated.

3.3. Identification of plant sample and pure fungal strain

3.3.1. Plant identification

A voucher specimen of the plant was identified by Prof. FZ. ALAOUI, Department of Botany, Faculty of Sciences, Mohamed V University and Prof. Dr. M. IBN TATOU, Scientific Institute of Rabat, Morocco. Reference samples are deposited as a herbarium at the Scientific Institute of Rabat, in which a collection number (RAB 77777) has been assigned.

3.3.2. Fungal identification

Fungal strains were identified based on the analysis of the DNA sequences of the internal transcribed spacer regions (ITS) of its ribosomal RNA gene. The identification was performed according to a molecular biologic protocol previously described (Kjer *et al.*, 2010).

3.3.2.1. DNA extraction

The extraction of fungus DNA was carried out with ZR Fungal/Bacterial DNA Kit™ (ZYMO RESEARCH, D6005), following the manufacturer protocol:

Step 1- Add 50-200 mg of fungal cells to a ZR BashingBead™ Lysis Tube. Add 750 µl Lysis Solution to the tube.

Step 2- Vortex for 5 minutes. Processing times may be as little as 40 seconds when using high-speed cell disrupters.

Step 3- Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at 10,000 x g for 1 minute.

Step 4- Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 rpm (~7,000 x g) for 1 minute. (Snap off the base of the Zymo-Spin IV™ Spin Filter prior to use).

Step 5- Add 1,200 µl of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.

Step 6- Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

Step 7- Discard the flow through from the Collection Tube and repeat Step 6.

Step 8- Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new collection Tube and centrifuge at 10,000 x g for 1 minute.

Step 9- Add 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute.

Step 10- Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml micro-centrifuge tube and add 100 µl (25 µl minimum) DNA Elution Buffer directly to the column matrix or sterile water.

Step 11- Centrifuge at 10,000 x g for 30 seconds to elute the DNA.

After isolation of the genomic DNA, quantification was done after agarose Gel Electrophoresis for DNA with the Software GelQuant.NET, where the fluorescent of the samples, were compared to that of a known amount of the latter.

3.3.2.2. DNA amplification

DNA amplification by PCR was performed using Hot StarTaq Master Mix Taq polymerase (Qiagen) and the primers (Invitrogen):

ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	White *et al.* 1990
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White *et al.* 1990
NS1	5'-GTAGTCATATGCTTGTCTC-3'	White *et al.* 1990
Fung	5'-ATTCCCCGTTACCCGTTG-3'	May *et al.* 2001

PCR program:

<u>ITS1\ITS4:</u>

Initial denaturation	95,0 °C	15:00	min.	
Denaturation	95,0 °C	1:00	min.	
Annealing	56,0 °C	0:30	sec.	35 times
Extension	72,0 °C	1:00	min.	
Final extension	72,0 °C	10:00	min.	

3.3.2.3. Purification of PCR products

DNA purification from Agarose Gel was carried out with QiaQuick Gel Extraction Kit (Quiagen) following the manufacturer instructions:

Step 1- Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

Step 2- Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

Step 3- Add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

Step 4- Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.
IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

Step 5- After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

Step 6- Add 1 gel volume of isopropanol to the sample and mix. For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

Step 7- Place a QIAquick spin column in a provided 2 ml collection tube.

Step 8- To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

Step 9- Discard flow-through and place QIAquick column back in the same collection tube. Collection tubes are re-used to reduce plastic waste.

Step 10- Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.

Step 11- To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

Step 12- Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x g (~13,000 rpm).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

Step 13- Place QIAquick column into a clean 1.5 ml micro-centrifuge tube.

Step 14- To elute DNA, add 50 µl of Buffer EB (10 mM Tris•Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. The purified PCR products can be stored at -20 °C before sequencing.

3.3.2.4. Fungi Identification

DNA sequencing was performed using primers ITS1 or NS1 and performed by BMBF, Heinrich Heine Universität Düsseldorf or GATC Biotech AG. Nucleotide sequences of ITS rDNA of each taxon were compared using a Blast search in the nucleotide database in GenBank.

3.4. Extraction of plant and fungal cultures

3.4.1 Extraction and fractionation of *Thymelaea lythroides* plant material

The dried aerial part of *Thymelaea lythroides* was extracted extensively with EtOAc. The EtOAc extract was washed with water, taken to dryness, and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH soluble fraction was fractionated over Silica using *n*-Hexane:EtOAc and DCM:MeOH gradient elution and the obtained fractions were analysed by HPLC and LC/MS.

3.4.2. Extraction and fractionation of fungi grown on solid rice medium

To each 1L Erlenmeyer about 250 mL EtOAc were added and culture media were then cut into small pieces to allow exhaustive extraction with EtOAc. The extraction left 24h under agitation. The contents were filtered under vacuum using a Buchner funnel. The extraction repeated three times with EtOAc until exhaustion. The combined EtOAc phases were washed with 300 mL demineralised water to eliminate remaining sugar and starch.

All obtained extracts were taken to dryness under reduced pressure at 40°C and partitioned between 90% MeOH and *n*-hexane to remove the fatty acids. After evaporating the solvent, these crude extracts were submitted to TLC, analytical HPLC, LC-MS and also to bioactivity screening including cytotoxicity and antimicrobial assays. The extraction scheme is described in Figure 3.4.
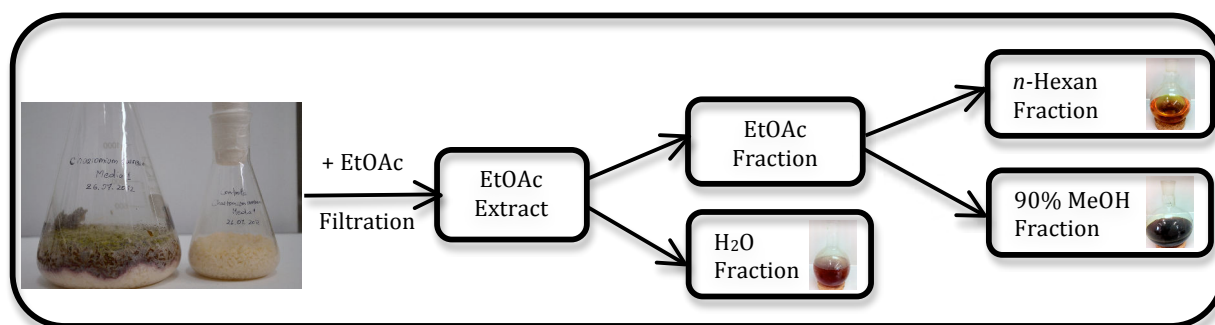
Figure 3.5. Extraction and fractionation of solid rice culture

3.5. Isolation of secondary natural products

For the isolation of natural products different chromatographic methods depending on the nature of the product have been used. A chromatographic system comprises two phases, a stationary phase absorbing the compounds according to their physical properties, e. g. polarity (silica gel, normal or reversed phase), size (Sephadex LH-20) or charge (Diaion), whereas the mobile phase moves through the stationary phase and gradually elutes the compounds according to their affinity from it.

3.5.1. Thin layer chromatography (TLC)

Thin-layer chromatography is, in essence, liquid chromatography performed on a stationary phase present as a sheet or layer of solid particles immobilized on a planar support, or a layer of polymerized substance (Yrjönen, 2004). TLC is a mature and very established technique, frequently used in many fields of applications ranging from natural product analysis to chemistry or pharmaceutical applications.

For rapid characterization and testing of extracts, fractions and isolated compounds the thin layer chromatography was used. After development of the TLC plate in a saturated fluid chamber, the band separation was observed under a UV lamp at 254 or 366 nm, followed by spraying TLC plates with anisaldehyde-sulphuric acid or ninhydrin spraying reagent and

heating plates at 110°C for 10 min in an oven heated.

During this work, TLC was performed on pre-coated TLC plates using the following systems:

TLC on silica gel 60 F254, layer thickness 0.2 mm (Merck):
  ➢ *n*-hexane:EtOAc [90:10, 80:20 and 70:30 (V/V)] and *n*-hexane:MeOH [95:5 and 90:10 (V/V)] for non-polar compounds
  ➢ DCM:MeOH [95:5, 90:10, 85:15, 80:20 and 70:30 (V/V)] for semi-polar compounds
  ➢ EtOAc:MeOH:H2O[30:5:4, 30:65 and 30:7:6 (V/V)] for polar compounds TLC on Diol F254S, layer thickness 0.25 mm (Merck):
  ➢ *n*-hexane:EtOAc [90:10, 80:20 and 70:30 (v/v)] EtOAc:acetone [95:5 and 90:10 (V/V)]

TLC on reversed phase RP-18, F254 S, layer thickness 0.25 mm (Merck):
  ➢ MeOH:H2O (90:10, 80:20, 70:30 and 60:40 (V/V).

The different compounds could be compared and identified due to their specific retention factors in defined chromatographic systems. These so-called Rf-values can be calculated as migration distance of compound migration distance of solvent front.

With ninhydrin spray reagent (0.2 % in MeOH, m/V) amino acids, amines and amino sugars can be detected, and the anisaldehyde/sulphuric acid spray reagent was used for the detection of phenols, steroids, sugars and terpenes.

The composition of anisaldehyde/sulphuric acid spray reagent was:
  ➢ Methanol   85 mL
  ➢ Glacial acetic acid   10 mL
  ➢ Conc. sulphuric aci   5 mL
  ➢ Anisaldehyde   0.5 mL

3.5.2. Vacuum liquid chromatography (VLC)

Vacuum liquid chromatography (VLC) is a useful method for the initial isolation procedure for large amounts of a sample. The apparatus consists of a 500 mL sintered glass Büchner filter funnel with an inner diameter of 12 cm. Fractions were collected in Erlenmeyer flasks. Silica gel 60 was packed to a hard cake at a height of 5 cm under applied vacuum. The sample

used was covered onto a small amount of silica gel using volatile solvents. The resulting sample mixture was then packed onto the top of the column. Step gradient elution with a non-polar solvent (Hexane) and increasing the gradient of a polar solvents (EtOAc, MeOH) gave successive fractions. The flow was produced by vacuum and the column was allowed to run dry after each fraction collected.

3.5.3. Flash chromatography

Flash chromatography is a preparative column chromatography based on optimized prepacked columns and an air pressure driven eluent at a high flow rate. It is a simple and quick technique widely used to separate a variety of organic compounds. Normally, the columns are dry Silica Gel 60 GF254 pre-packed, of 18 cm height, vertically clamped and assembled in the system. The column is filled and saturated with the desired mobile phase just prior to sample loading. Samples are dissolved in a small volume of the initial solvent used and the resulting mixture was then packed onto the top of the column using special syringe. The mobile phase (isocratic or gradient elution) is then pumped through the column with the help of air pressure resulting in sample separation. This technique is considered as a low to medium pressure technique and is applied to samples from few milligrams to some gram of sample.

3.5.4. Low pressure liquid chromatography (LC)

Open column chromatography plays a vital role in the separation of compounds from natural product extracts. The separation takes place through selective distribution of the components between a mobile phase and a stationary phase. Different choice of packing material and mobile phase can be applied depending on the class of compounds or fractions. Fractions derived from VLC were subjected to repeated separation through column chromatography using appropriate stationary and mobile phase solvent systems previously determined by TLC.

The following types of separation systems were used in this study:

• **Normal phase chromatography** using a polar stationary phase, typically silica gel (Silica Gel 60, 0.04 – 0.063 mm mesh size, Merck) or Diol (LiChroprep® Diol (40 – 63 μm) for liquid chromatography, Merck), in conjunction with a non- polar mobile phase (e.g. n-hexane,

DCM) or with a gradually increasing amount of a polar solvent (e.g. EtOAc or MeOH). Hydrophobic compounds elute quicker than hydrophilic compounds.

• **Reversed phase (RP) chromatography** using a non-polar stationary phase and a polar mobile phase (e.g. H_2O or MeOH). The stationary phase consists of reversed phase silica material. For instance, RP-18 stands for an octadecyl ligand in the matrix of the RP-18, 0.04 – 0.0.63 mm mesh size (Merck) material that was used. The more hydrophobic the matrix, the greater the tendency of the column to retain hydrophobic compounds.

Thus, hydrophilic compounds elute more quickly than do hydrophobic compounds. Elution was performed using H_2O with gradually increasing amounts of MeOH or acetonitrile.

• **Size exclusion chromatography** involves separations based on molecular sizes of analyzed compounds. The stationary phase consists of porous beads (Sephadex LH-20, 0.25 – 0.1 mm mesh size, GE Healthcare). Compounds having larger molecular diameter are excluded from the interior of the bead and thus elute firstly, while compounds with smaller molecular diameters enter the beads and elute according to their ability to exit from the small sized pores where they are trapped. Elution was performed using MeOH or MeOH:DCM [1:1 (V/V)] as mobile phases.

• **Ion exclusion chromatography** uses ion exchange resin beds (Diaion HP-20, Supelco) that act as a charged solid separation medium. The components of the processed sample have different electrical affinities to this medium and consequently are differently retained by the resin according to their different affinities.

The eluted fractions were collected by an automate fraction collector and combined according to TLC results.

3.5.5. High pressure liquid chromatography (HPLC)

High pressure liquid chromatography (HPLC) is a high resolution chromatographic technique, where the mobile phase is forced through the column containing the stationary phase by a pump, resulting in a fast separation. The high resolution is achieved by the use of small particle size of the absorbent material, which mostly is RP silica material. Adjacent to the HPLC a UV detector is connected for monitoring the separation of the eluted compounds

shown in a chromatogram. The method can be used for analytical purposes or semi preparative.

3.5.5.1. Analytical HPLC

Analytical HPLC was used to identify interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds. The gradient used started with 10:90 [MeOH: nanopure H_2O (0.1% *o*-phosphoric acid)] to 100% MeOH in 35 minutes; then 10 minutes with 100% MeOH to wash the column and finely 15 min with 10:90 [MeOH: nanopure H_2O (0.1% *o*-phosphoric acid)] to stabilize the column for the next injection. Peaks are detected by UV-VIS diode array detector.

Parameters:

Pump	Dionex P580A LPG
Detector	Dionex Photodiode Array Detector UVD 340S
Column	Knauer (125 × 4 mm, ID), pre-packed with Eurosphere 100-5 C18, with integrated pre-column. (Knauer, Berlin, Germany).
Column thermostat	STH 585
Autosampler	ASI-100T
HPLC Program	Chromeleon (V. 6.3)
Run time	60 min
Injection volume	20 µL
Flow rate	1,0 mL/min
Detection	235 nm; 254 nm; 280 nm and 340 nm

In case of insufficient separation the gradient was adjusted adequate to the substances to be separated.

3.5.5.2. Semi preparative HPLC

The semipreparative HPLC was used for the isolation of pure compounds from fractions previously separated using column chromatography. Each injection consists of 1-3 mg of the fraction dissolved in 1 mL of the solvent system. The most appropriate solvent system was determined before running the HPLC separation. The solvent system, which is composed of MeOH and Nanopure water with or without 0.1% TFA, was pumped through the column at a rate of 5 mL/min. The eluted peaks, which were detected by the online UV detector, were collected separately in glass tubes.

Semi-preparative HPLC system specifications are described as follows:

Pump	Merck Hitachi L-7100
Detector	Merck Hitachi UV detector L-7400
Column	Knauer (300 × 8 mm, ID), prepacked with Eurosphere 100-10 C18, with integrated pre-column.

3.5.6. Isolation and purification of secondary metabolites

3.5.6.1. Isolation and purification of plant extract *Thymelaea lythroides*
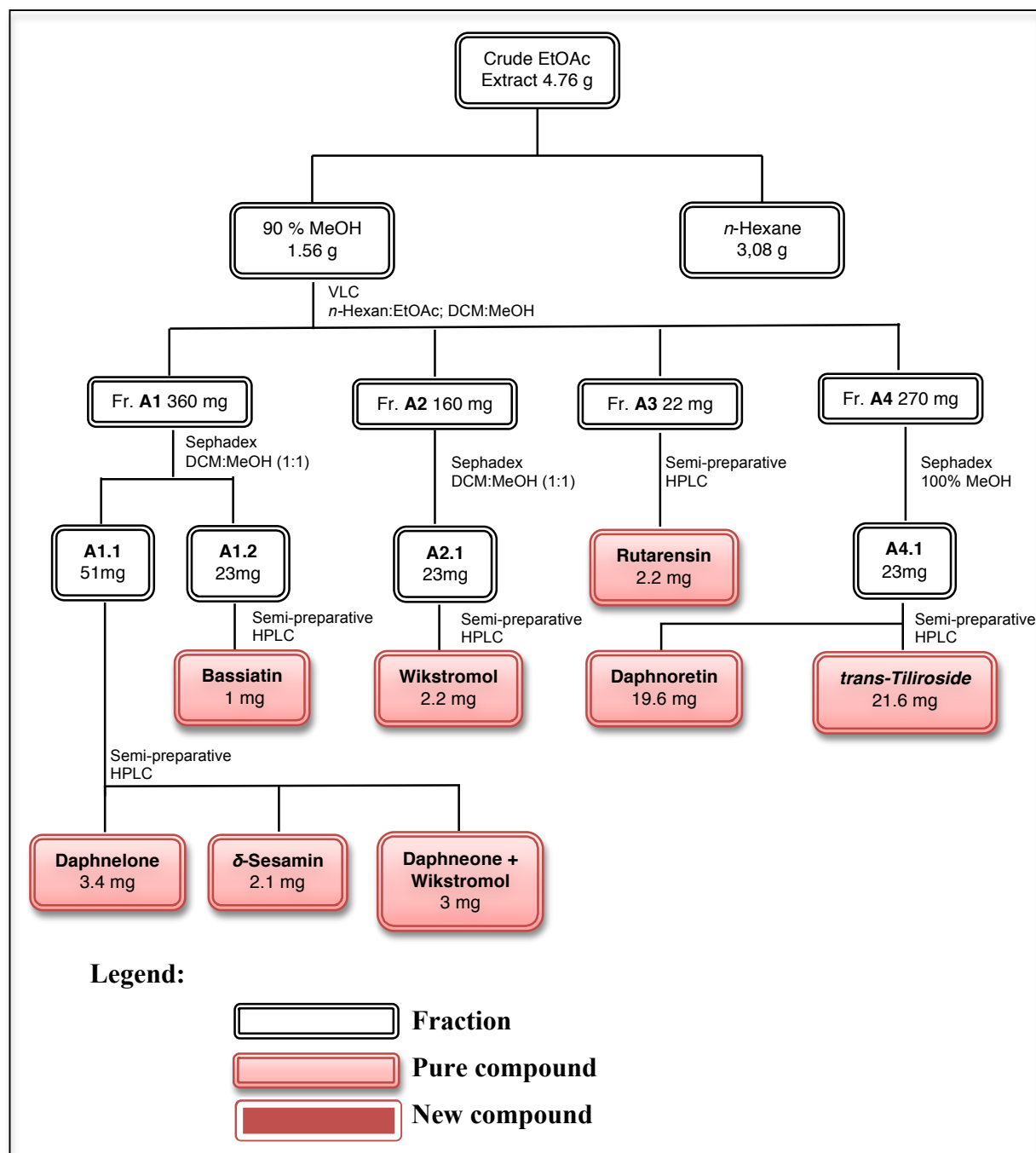
Scheme 3.1. Isolation and purification of plant extract *Thymelaea lythroides*

3.5.5.2 Isolation and purification of endophyte extract *Chaetomium aureum*

3.5.5.2.1 Isolated secondary metabolites from fermentation of *Chaetomium aureum* on solid rice
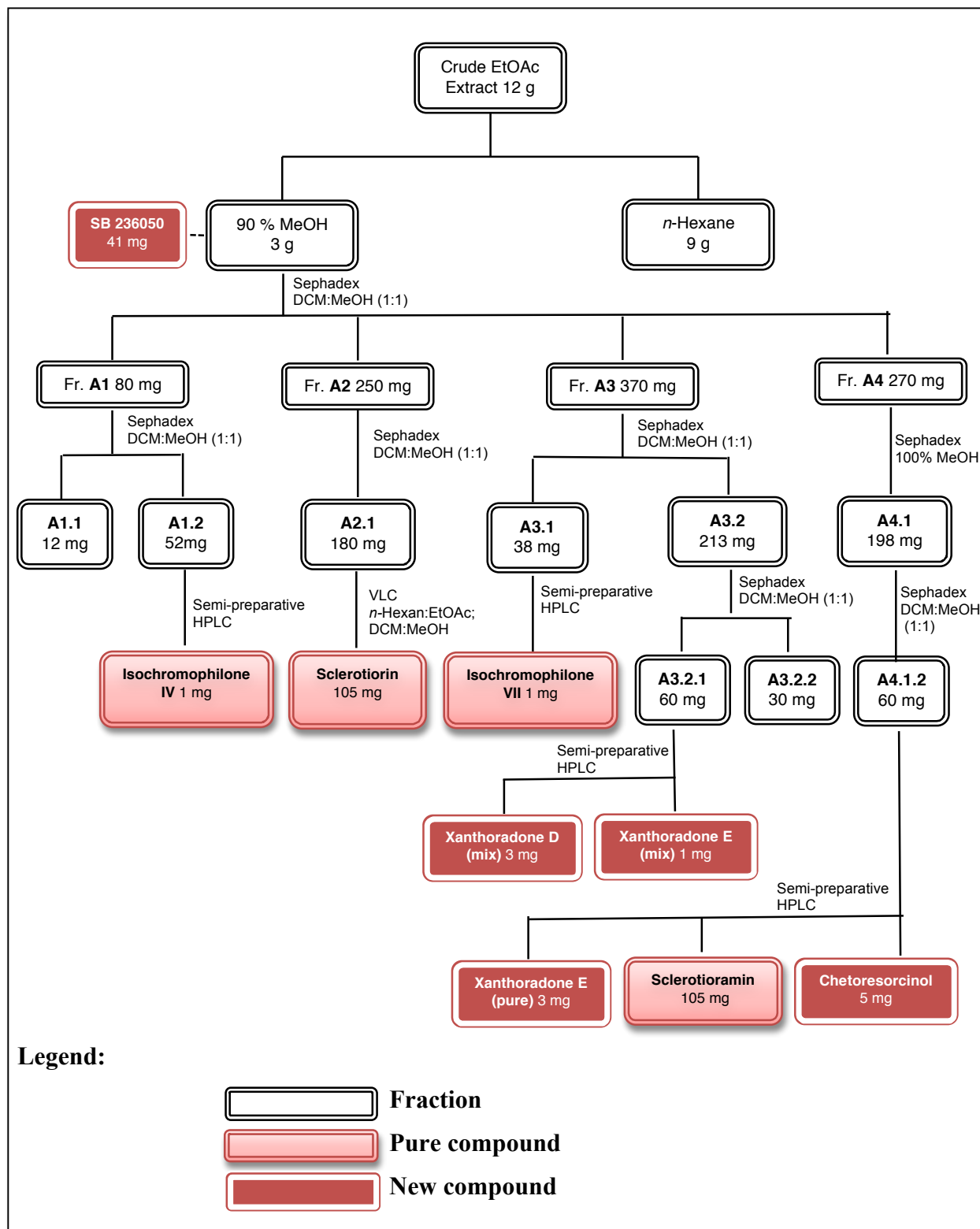
Scheme 3.2. Isolation and purification of plant extract *Chaetomium aureum*

3.6. Structure elucidation of the isolated metabolites

The structure elucidation of the isolated compounds followed a standard scheme. Firstly, the data obtained from analytical HPLC were compared with the in-house substance library regarding their retention times at the standard gradient program and their UV spectra. Comparable hits indicated the class to which the compound belongs. From LCMS measurement the mass of the compound and from 1H-NMR measurement substructures could be compiled. With this information, together with the identity of the fungus, a literature search using the latest versions of Dictionary of Natural Products (DNP, Chapman and Hall, 2005-2009), Antibase (2002-2007), Antimarin 0512, 2012 and SciFinder was performed. In some cases these data were insufficient and additional measurements, especially one and two-dimensional NMR experiments were necessary to finally identify the secondary metabolite.

3.6.1. Mass spectrometry (MS)

Mass spectroscopy (MS) is an analytical technique used to determine the molecular weight, the elemental composition of a molecule and for the chemical structures elucidation of the molecules. It is a very sensitive technique and even from micro gram amounts good spectra can be obtained. Technically, mass spectrometers consist of three parts: ionization source, analyzer, and detector, which should be maintained under high vacuum conditions so as to maintain the ions flight through the instrument without any hindrance from air molecules. The sample is ionized in the ionization source and the rising ions are sorted and separated according to their mass (m) charge (z) ratio (m/z) in the mass analyzer. Both negative and positive charged ions can be observed. Once the separated ions reach the detector, the signals are transmitted to the data system where the mass spectrum is recorded. The molecular ion (parent ion) has to be identified giving the molecular weight of the compound. Therefore, mass spectrometry is used to determine the molecular weights of pure compounds or compounds in a mixture. From the fragmentation patterns of the compound information about substructures can be attained.

Many different method of ionization are used in mass spectrometry and the selection of the method depends on the type of samples to be analyzed. Some known ionization methods include:

  ➢ Electron Impact (EI)
  ➢ Electro-Spray Ionization (ESI)
  ➢ Fast Atom Bombardment (FAB)

➢ Chemical Ionization (CI)

➢ Atmospheric Pressure Chemical Ionization (APCI)

➢ Matrix Assisted Laser Desorption Ionization (MALDI)

➢ Field Desorption / Field Ionization (FD/FI)

➢ Thermo-Spray Ionization (TSI)

Low resolution mass spectra were measured by ESI-, EI-, CI- and FAB-MS on a Bruker micrOTOF with Liquid chromatography Agilent 1100 Serie mass spectrometer.

3.6.1.1. Electron Impact Mass Spectrometry (EI-MS)

Analysis involves vaporizing a compound in an evacuated chamber and then bombarding it with electrons having 25.80 eV (2.4-7.6 MJ/mol) of energy. The high-energy electron stream not only ionizes an organic molecule (requiring about 7-10 eV) but also causes extensive fragmentation (the strongest single bonds in organic molecules have strengths of about 4 eV). The advantage is that fragmentation is extensive, giving rise to a pattern of fragment ions, which can help to characterize the compound. The disadvantage is the frequent absence of a molecular ion.

3.6.1.2. Electron spray ionization mass spectrometry (ESIMS)

ESI-MS is a gentle ionization method where the sample is passed through a high voltage metal capillary. At the end of this capillary it is sprayed by a flow of nitrogen gas at atmospheric pressure to form an aerosol. Together with heating, the nitrogen evaporates the emerging droplets forcing the ions in each droplet together until repulsion causes them to eject from the surface. The ions are extracted into the vacuum of the mass analyzer where they are detected. Additionally to the molecular ion peaks [M+H]+ or [M-H]- fragments of these can detected.

⇒ **Liquid chromatography mass spectrometry (LC/MS)**

High pressure liquid chromatography is a powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilitated. Usually, ESI-MS is interfaced with LC to make an effective on-line LC/MS. HPLC/ESI-MS was carried out using a Finnigan LCQ-DECA mass spectrometer connected to a UV detector. The samples were dissolved in

water/MeOH mixtures and injected to HPLC/ESI-MS set-up. For standard MS/MS measurements, a solvent gradient that started with acetonitrile:nanopure H2O (10:90), adjusted with 0.1 % HCOOH, and reached to 100 % acetonitrile in 35 minutes was used.

LC/UV/MS system specifications are described as follows:

HPLC system	Agilent 1100 series (pump, detector and autosampler) Finnigan LC Q-DECA
MS spectrometer	Knauer, (250 × 2 mm, ID), prepacked with Eurosphere 100-5
Column	C18, with integrated pre-column

3.6.1.3. Fast atom bombardment mass spectrometry (FAB-MS)

This was the first widely accepted method that employs energy sudden ionization. FAB is useful for compounds, especially polar molecules, unresponsive to either EI or CI mass spectrometry. It enables both non-volatile and high molecular weight compounds to be analyzed. In this technique, a sample is dissolved or dispersed in a polar and relatively non-volatile liquid matrix, introduced into the source on a copper probe tip. Then, this matrix is bombarded with a beam of atoms of about 8 Kev. It uses a beam of neutral gas (Ar or Xe atoms) and both positive and negative ion FAB spectra can be obtained.

3.6.1.4. High-resolution mass spectrometry (HR-MS)

High resolution is achieved by passing the ion beam through an electrostatic analyzer before it enters the magnetic sector. In such a double focusing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With measurement of this accuracy, the atomic composition of the molecular ions can be determined.

After the determination of molecular weight, pure compounds are then submitted to the measurement of nuclear magnetic resonance (NMR) spectra.

3.6.2. Nuclear magnetic resonance spectroscopy (NMR)

First observed in 1946, nuclear magnetic resonance leads to the development of a technic, called NMR spectroscopy, which is now almost indispensable for structure elucidation. NMR spectroscopy allows determining chemical and physical proprieties of those nuclei of atoms having magnetic properties. Some nuclei experience this phenomenon, and others do not, depending upon whether they possess a property called spin. NMR spectroscopy is nowadays

routinely used to study chemical structure by giving not only the constituent of a molecule but also its relative stereochemistry.

This technique utilizes the atomic nuclei spinning behavior of atoms with an odd number of nucleons, e. g. ^{1}H and ^{13}C. A NMR spectrum is acquired by varying the magnetic field that is applied to the sample dissolved in a deuterated solvent over a small range while observing the resonance signal from the sample. Depending on the electron density around each proton they obtain different shielding and deshielding effects appearing in different parts of the resulting NMR spectrum and thus provide information about the environment of each proton. The resulting frequency where the nuclei resonate, the so-called chemical shift, is given in ppm and the coupling constants between adjacent nuclei in Hertz (Hz). NMR experiments can be conducted in a one (1D) or two (2D) dimensional manner. 2D NMR spectra can be either measured between two equal (H, H-COSY, correlated spectroscopy) or two different (H, C-COSY) frequency axes. For the H-H 2D experiments the connection between two adjacent protons (COSY), between two protons through space (NOESY, nuclear Over-hauser enhancement spectroscopy; ROESY, rotating- frame enhancement spectroscopy) or between all protons in one spin system (TOCSY, total correlation spectroscopy) are given. H-C 2D experiments measure the direct correlation between a proton and a carbon (HMQC, heteronuclear multiple quantum correlation) or the connection of protons over two, three and even four bonds to carbon atoms, so-called long range coupling (HMBC, heteronuclear multiple bond correlation). Correlations are shown as cross peaks in the plane between two axes containing the 1D NMR shifts.

Most NMR spectra were measured at the University Wuppertal with BRUKER AVANCE III 600 (1H - Messfrequenz 600.13 MHz) by Andreas Siebert and Ilka Polanz.

3.6.3. Optical activity

Optical rotation or optical activity is a phenomenon observed in the 1811 in quartz by the French physicist Dominique F.J. Arago. Polarized light changes its polarization after passing through a chiral molecule; this can be measured with a polarimeter. This equipment consists of a light source, two polarizing filters and a cell that contains a solution of the analyzed compound.

Optical activity is a macroscopic property of a molecule and differs between enantiomers. Samples containing two enantiomers in the same ratio are optically inactive. In a solution the measured optical rotation depends on concentration (c) and light path length (l) of the sample. The specific rotation, [α], expresses the optical rotation degree after correction of concentration and path length. Thus the specific rotation is a specific quantity for a chiral molecule at certain temperature (T) and at certain wavelength (λ).

$$[\alpha]_{\lambda}^{T} = 100\ \alpha\ /c.l$$

where:

$[\alpha]_{\lambda}^{T}$ is specific rotation at certain temperature T and wavelength λ

a = the angle of rotation (^{o}),

l = optical path length in (dm)

λ = wavelength

T = temperature

c = concentration (g/100 mL)

The measurement of optical rotation presented in this study, was recorded on Perkin-Elmer 241 MC polarimeter and using a 0.5 mL cuvette with 1 dm length.

$[\alpha]_{D}^{20}$ is the specific optical rotation of Sodium-D-line at the wavelength, 589 nm and at a temperature of 20 °C.

Pure substances were dissolved in appropriate spectroscopic grade solvent. The optical rotation of known compounds was compared to literature data.

3.6.4. Circular dichroism (CD) spectroscopy

Circular dichroism is based on the difference between the absorption of left-handed circularly polarized light (L-CPL) and right-handed circularly polarized light (R-CPL). It occurs when a molecule contains one or more chiral chromophores (light-absorbing groups).

$$\text{Circular dichroism} = \Delta A(\lambda) = A(\lambda)LCPL - A(\lambda)RCPL$$

where λ is the wavelength.

Circular dichroism (CD) spectroscopy is a spectroscopic method. The CD of molecules is recorded over a range of wavelengths. CD spectroscopy is widely used to study chirality of molecules.

Measurements are carried out in the visible and ultra-violet region of the electro-magnetic spectrum to monitor electronic transitions. If the molecule under investigation contains chiral chromophores, one CPL state will be absorbed to a greater extent than the other and the CD signal over the corresponding wavelengths will be non-zero. A circular dichroism signal can be positive or negative, depending on whether L-CPL is absorbed to a greater extent than R-CPL (CD signal positive) or to a lesser extent (CD signal negative).

Circular dichroism spectra are measured using a circular dichroism spectrometer, which is a highly specialized descendant of an ordinary absorption spectrometer. CD spectrometers alternately measure the absorption of L- and R-CPL, usually at a frequency of 50 KHz, and then calculate the circular dichroism signal.

In this study, the CD spectra were recorded on a J-810 CD spectropolarimeter. Conformational searches were carried out by means of the Macromodel 9.7.211 (MacroModel *et al.*, 2009) software using Merck Molecular Force Field (MMFF) with implicit solvent model for chloroform. Geometry reoptimizations at B3LYP/6-31G(d) level of theory followed by TDDFT calculations using various functionals (B3LYP, BH&HLYP, PBE0) and TZVP basis set were performed by the Gaussian 03 (Frisch *et al.*, 2004) package. Boltzman distributions were estimated from the ZPVE corrected B3LYP/6-31G(d) energies. CD spectra were generated as the sum of Gaussians (Stephens and Harada, 2010) with 3000 cm–1 half-height width (corresponding to ca. 16 nm at 230 nm), using dipole-velocity computed rotational strengths for conformers above 5%. The MOLEKEL (Varetto, 2009) software package was used for visualization of the results.

3.7. Testing the biological activity

A bioassay-guided separation can lead to the discovery of compounds with interesting activity. Samples from crude extracts and from different fractions, resulting of different separation were submitted to biological activity tests.

3.7.1. Antimicrobial assay

Agar diffusion assay

This method was used to detect the capability of a substance to inhibit the growth of microorganisms by measuring the diameter of inhibition zone around a tested compound on an agar plate. The agar diffusion assay was performed according to the Bauer-Kirby-Test (Bauer et al., 1966).

Culture preparation

The agar diffusion assay was performed according to the Bauer-Kirby-Test (DIN 58940, Bauer et al, 1966). Prior to testing, a few colonies (3 to 10) of the organism to be tested, were subcultured in 4 ml of tryptose-soy broth (Sigma, FRG) and incubated for 2 to 5 h to produce a bacterial suspension of moderate cloudiness. The suspension was diluted with sterile saline solution to a density visually equivalent to that of a $BaSO_4$ standards. The standards were prepared by adding 0.5 ml of 1 % $BaCL_2$ to 99.5 ml of 1% H2SO4 (0.36 N). The prepared bacterial broth is inoculated onto Müller-Hinton-Agar plates (Difco, USA) and dispersed by means of sterile beads.

Microorganisms

Crude extracts and isolated pure compounds were tested for activity against the following standard strains:

- Gram-positive bacteria *Bacillus subtilis*
- Gram-negative bacteria *Escherichia coli*
- Yeast *Saccharomyces cerevisiae*
- Fungi *Cladosporium cucumerinum*
- Fungi *Cladosporium herbarum*.

3.7.2. Cytotoxicity tests

3.7.2.1.Microculture tetrazolium (MTT) assay

Cytotoxicity tests were carried out by Prof. Dr. W. E. G. Müller, 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 compared to that of untreated controls (Carmichael, 1987).

Cell cultures

L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were maintained in a humified atmosphere at 37° C with 5% CO_2.

MTT colorimetric assay

Of the test samples, stock solutions in ethanol 96% (v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 μL containing 3750 cells were pipetted into 96-well microliter plates. Subsequently, 50 μL of a solution of the test samples containing the appropriate concentration was added to each well. The concentration range was 3 and 10 μg/mL. The small amount of ethanol present in the wells did not affect the experiments. The test plates were incubated at 37° C with 5% CO_2 for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 μL was pipetted into each well.

The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3h 45 min at 37° C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min, 20 °C, 210 x g) with 200 μL DMSO, the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microliter-well spectrophotometer. The color intensity is correlated with the number of healthy living cells. Cell survival was calculated using the formula:

$$\text{Survival \%} = 100 \, \frac{\text{Absorbance of treated cells} - \text{absorbance of culture}}{\text{Absorbance of untreated cells} - \text{absorbance of culture}}$$

All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

3.7.2.2. MTT cell viability assays

Cytotoxicity tests were carried out by Prof. Dr. M. U. Kassack, Institut für Pharmazeutische und Medizinische Chemie, Heinrich-Heine University, Düsseldorf.

Materials, cell lines and cell culture

The human ovarian carcinoma cell line A2780 (A2780 sens) was obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). A2780 cells were exposed to weekly cycles of 2 μmol/L cisplatin over a period of 24 weeks.

Cisplatin-resistant cells were denoted A2780 *Cis*R. The human chronic myelogenous leukemia cell line K562 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). All other reagents were supplied by Sigma Chemicals unless otherwise stated. All cell lines were grown at 37 °C under humidified air supplemented with 5% CO_2 in RPMI 1640 (PAN Biotech, Germany) containing 10% fetal calf serum (PAN Biotech, Germany), 100 IU/mL penicillin and 100 μg/mL streptomycin. The cells were grown to 80% confluency before using them for the MTT cell viability assay.

MTT cell viability assays

The rate of cell-survival under the action of test substances was evaluated by an improved MTT assay (Müller *et al*., 2004). The assay is based on the ability of viable cells to metabolize yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Applichem, Germany) to violet formazane crystals that can be detected spectrophotometrically. In brief, A2780 cells were seeded at a density of 8.000 cells/well and K562 at a density of 30,000 cells/well in 96 well plates (Corning, Germany). After 24 h, cells were exposed to the test compounds at concentrations of 10^{-5} M and 10^{-4} M. Incubation was stopped after 72 h and cell survival was determined by addition of MTT solution (5 mg/mL in phosphate buffered saline). The formazan precipitate was dissolved in DMSO. Absorbance was measured at 544 nm and 620 nm in a FLUO starmicroplate-reader (BMG LabTech, Offenburg, Germany). The absorbance of untreated control cells was taken as 100% viability. All tests were performed in triplicate.

3.7.2.3. Protein kinase assay

Protein kinase assays were carried out by Dr. Michael Kubbutat (ProQinase GmbH, Freiburg, Germany).

Protein kinase enzymes are integral components of numerous signal transduction pathways involved in the regulation of cell growth, differentiation, and response to changes in the extracellular environment. Consequently, kinases are major targets for potentially developing novel drugs to treat diseases such as cancer and various inflammatory disorders.

The inhibitory potency of the samples was determined using 24 protein kinases (see Table 3.2). The IC_{50} profile of compounds/fractions showing an inhibitory potency of $\geq 40\%$ with at least one of the 24 kinases at an assay concentration of 1×10^{-06} g/mL was determined. IC_{50} values were measured by testing 10 concentrations of each sample in singlicate (n=1).

Sample preparation

The compounds/fractions were provided as 1×10^{-03} g/mL stock solutions in 100% DMSO (1000 or 500 µL) in micronic boxes. The boxes were stored at -20° C. Prior to the assays, 100 µL of the stock solutions were transferred into separate microtiter plates. Subsequently, they were subjected to serial, semi-logarithmic dilution using 100% DMSO as a solvent resulting in 10 different concentrations. 100% DMSO was used as control. Subsequently, 7×5 µL of each concentration were aliquoted and diluted with 45 µL H_2O only a few minutes before the transfer into the assay plate to minimize precipitation. The plates were shaken thoroughly and then used for the transfer of 5 µL compound solution into the assay plates.

Recombinant protein kinases

All protein kinases were expressed in Sf9 insect cells as human recombinant GST-fusion proteins or His-tagged proteins by means of the baculovirus expression system. Kinases were purified by affinity chromatography using either GSH-agarose (Sigma) or NiNTH-agarose (Qiagen). Purity was checked by SDS-PAGE/silver staining and the identity of each kinase was verified by western blot analysis with kinase specific antibodies or by mass spectrometry.

Protein kinase assay

A proprietary protein kinase assay (33PanQinase® Activity Assay) was used for measuring the kinase activity of the protein kinases. All kinase assays were performed in 96-well Flash PlatesTM from Perkin Elmer/NEN (Boston, MA, USA) in a 50 µL reaction volume. The reaction mixture was pipetted in the following order: 20 µL assay buffer, 5 µL ATP solution in H_2O, 5 µL test compound in 10% DMSO and 10 µL substrate/10 µL enzyme solution

(premixed). The assay for all enzymes contained 60 mM HEPES-NaOH (pH 7.5), 3 mM MgCl$_2$, 3mM MnCl$_2$, 3 pM Na-orthovanadate, 1.2 mM DTT, 50 pg/mL PEG20000, 1 pM [y-33P]-ATP. The reaction mixtures were incubated at 30°C for 80 minutes and stopped with 50 µL 2% (v/v) H$_3$PO$_4$. The plates were aspirated and washed two times with 200 µL of 0.9% (w/v) NaCl or 200 pL H$_2$O. Incorporation of 33Pi was determined with a microplate scintillation counter (Microbeta Trilux, Wallac). All assays were performed with a Beckman Coulter/Sagian robotic system.
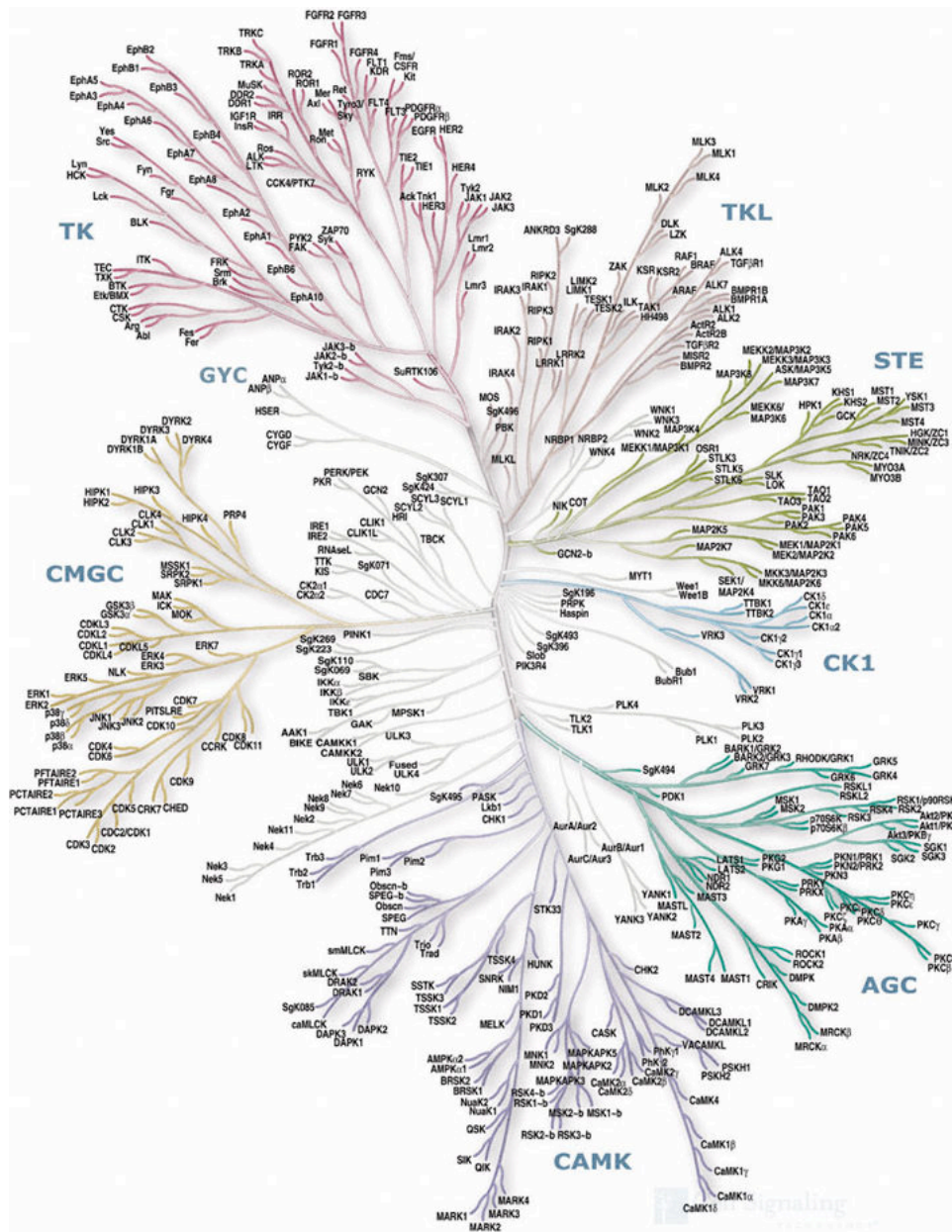
Group Names AGC Containing PKA, PKG, PKC families; CAMK Calcium/calmodulin-dependent protein kinase; CK1 Casein kinase 1; CMGC Containing CDK, MAPK, GSK3, CLK families; STE Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases; TK Tyrosine kinase; TKL Tyrosine kinase-like (@ cellsignal.com).

Table 3.2: List of Protein kinases and their substrates

Family	Kinase	Substrate	Oncologically relevant mechanism	Disease
Serine/threonine kinases	AKT1/PKB Alpha	GSC3 (14-27)	Apoptosis	Gastric cancer (Staal, 1987)
	ARK5	Autophos.	Apoptosis	Colorectal cancer (Kusakai *et al.*, 2004)
	Aurora B	Tetra(LRRWSLG)	Proliferation	Breast cancer (Keen and Tylor 2004)
	PLK-1	Casein	Proliferation	Prostate cancer (Weichert *et al.*, 2004)
	MEK1 wt	ERK2-KR	Apoptosis	Multiple cancers (Ryan *et al.*, 2000)
	NEK2	RB-CTF	Apoptosis	Ewing's tumors & B cell lymphoma (Schultz *et al.*, 1994)
	NEK6	GSK3(14-27)	Apoptosis	Multiple cancers (Li *et al.*, 2003)
	PIM1	GSK3(14-27)	Apoptosis	prostate cancer (Dhanasekaran *et al.*, 2001)
	PRK1	RBER-CHKtide	Proliferation	Prostate cancer (Manser *et al.*, 1994)
Receptor tyrosine kinase	IGF1-R	Poly(glu,Tyr)$_{4:1}$	Apoptosis	Braest cancer (Zhang and Yee 2000)
	MET wt	Poly(Ala,glu,Lys,Tyr)$_{6:2:4:1}$	Metastasis	Lung cancer (Qiao *et al.*, 2002)
	VEGF-R2	Poly(glu,Tyr)$_{4:1}$	Angiogenesis	Pancreatic cancer (Li *et al.*, 2003)
	ALK	poly(Glu,Tyr)$_{4:1}$	Apoptosis	anaplastic large-cell lymphoma (Morris *et al.*, 1994)
	AXL	poly(Glu,Tyr)$_{4:1}$	Proliferation	Ovarian, gastric and breast cancer (*Liu et al.*, 1988)
Soluble tyrosine kinase	FAK	Poly(glu,Tyr)$_{4:1}$	Metastasis	Breast cancer (Schmitz *et al.*, 2005)
	SRC	Poly(glu,Tyr)$_{4:1}$	Metastasis	Colon cancer (Dehm *et al.*, 2001)

3.8. Inhibiting the hsp90 chaperone pathway

3.8.1. Heat shock protein Hsp 90

Heat shock proteins (HSPs) are chaperone proteins that become up-regulated in response to cellular environmental stresses, such as elevated temperature and oxygen or nutrient deprivation. Hsps are chaperones that facilitate the proper folding and repair of other cellular proteins, referred to as "client proteins", and also aid the refolding of misfolded proteins. Of the several families of Hsps, the Hsp90 family is one of the most abundant, representing approximately 1-2% of the total protein content in non-stressed cells and 4-6% of the protein content of cells that are stressed.

The N-terminal domain of Hsp90 comprises an ATP-binding site that is central to the chaperone function. The C-terminal domain of Hsp90 mediates constitutive Hsp90 dimerization. Conformational changes of Hsp90 are orchestrated with the hydrolysis of ATP. Hsp90 is highly conserved and facilitates the folding and maturation of over 200 client proteins, which are involved in a broad range of critical cellular pathways and processes. In non-stressed cells Hsp90 participates in low affinity interactions to facilitate protein folding and maturation. In stressed cells Hsp90 can assist the folding of dysregulated proteins, and is known to be involved in the development and maintenance of multiple diseases.

Hsp90 maintains the conformation and stability of many oncogenic proteins, transcription factors, steroid receptors, metalloproteases and nitric oxide synthases that are essential for survival and proliferation of cancer cells (Whitesell *et al.*, 2005). Thus, Hsp90 client proteins have been associated with the development and progression of cancer. Furthermore, Hsp90 is thought to contribute to maintenance of multiple neurodegenerative diseases that are associated with protein degradation and misfolding (proteinopathy), such as Alzheimer's disease, Huntingdon's disease and Parkinson's disease, through the mis-folding or stabilization of aberrant (neurotoxic) client- proteins.

Inhibition of Hsp90 function results in the misfolding of client proteins, which are subsequently ubiquitinated and degraded through proteasome-dependent pathways. Hence, inactivation of the Hsp90 pathway represents a combinatorial attack on multiple signaling pathways and Hsp90 inhibitors have been developed as therapeutics with efficacy in a broad variety of human diseases.

3.8.2. Progesterone receptor PR reconstitution assay

An in vitro progesterone receptor PR reconstitution assay as a model system was used to identify novel compounds that may inhibit the Hsp90 chaperoning machine. This assay uses RRL as a source of molecular chaperones and it has been fundamental in further understanding of how geldanamycin and related compounds such as 17-AAG inhibit Hsp90-dependent chaperoning (Pratt *et al*., 2003). The assay directly measures the ability of molecular chaperones to refold the heat denatured PR to its hormone binding state. Hormone binding activity of the PR therefore reflects the functional integrity of molecular chaperones. Whether compounds isolated from endophyte affected the recovery of hormone binding activity of PR after mild heat denaturation was tested. Compounds with diverse chemical structures from Moroccan medicinal plants and microorganisms living in these plants were screened.

For the progesterone receptor (PR) reconstitution assay, purified PR was adsorbed onto PR22 antibody-protein A-sepharose resin beads and was assembled into complexes as described previously (Kosano *et al*., 1998). Briefly, about 0.05 µM PR was incubated with RRL diluted twice in reaction buffer (20 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 4 mM DTT, 0.02% NP-40, 100 mM KCl and 10 mM ATP). After incubation for 30 min at 30°C, 0.1 µM [3H]-progesterone (American Radiolabeled Chemicals, Inc #ART 0063) was added. Samples were incubated on ice for 3 h at 4°C. Complexes were then washed three times with 1ml of reaction buffer and assessed for bound progesterone by liquid scintillation using PerkinElmer Microbeta plate reader.

3.8.3. Cytotoxicity of Sclerotiorin to breast cancer Hs578T cells

Cell Culture

Cell lines were purchased from American type culture collection (ATCC). Cell culture medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco. Penicillin/streptomycin, and tissue culture grade trypsin were bought from Sigma Chemical Co. 3.103.

Cytotoxicity Assay

Cells of breast cancer triple negative cell lines Hs578T and MDA-MB-231 and prostate cancer cell line LNCaP were seeded in 6-well plates (Corning #3516). The next day, cells were treated with various concentrations of sclerotiorin and deacetyl-sclerotiorin for 48h.

Every 24h, the old media was substituted with new media containing new drug at the same concentration. Cells were harvested at 48 h and cell lysate were made.

3.8.4. Comparison of deacetylsclerotiorin and sclerotiorin by inhibiting the Hsp90 chaperoning of progesterone receptor (PR) *in vitro*

Production of deacetylsclerotiorin

Sclerotiorin was chemically modified by deacetylation. Deacetylsclerotiorin was obtained by deacetylation of sclerotiorin, according to the procedure described previously by Isaka *et al.*, 2001.

Isaka describes a method for deacetylation, including adding concentrated H_2SO_4 (0.2 mL) to a compound (9.0 mg) suspended in dry MeOH (2.0 mL) and stirring the mixture for 45 h. The resulting homogeneous solution is partially concentrated under reduced pressure, and the residue is diluted with EtOAc (30 mL). The EtOAc solution was washed with H_2O (3 x 10 mL) and concentrated in vacuo to yield the deacetylated compound as a powder that is subsequently purified by preparative HPLC (MeCN/H_2O)

3.9. General laboratory equipments

Autoclave	Varioklav, H&P
Balances	Mettler 200, Mettler AT 250,
	Mettler PE 1600, Sartorious MC1 AC210S
Centrifuge	Biofuge pico, Heraeus
Cleanbench	HERAsafe, Heraeus
Digital pH meter	420Aplus, Orion
Drying Ovens	Kelvitron t, Heraeus
Fraction collector	Cygnet, ISCO
Freeze dryer	Lyovac GT2, Steris
- 80 °C Freezer	Forma Scientific, 86-Freezer
Hot plate	Camag
Magnetic stirrer	Combi Mag, IKA
Rotary evaporator	Vacuubrand, IKA
Sonicator	Sonorex RK 102, Bandelin
Syringes	Hamilton
Ultra Turrax	T18 basic, IKA

UV Lamp   Camag (254 and 366)

Vacuum centrifuge   SpeedVac SPD 111V, Savant

3.10. Solvents

3.10.1. General solvents

Acetone, acetonitrile, dichloromethane, ethanol, ethyl acetate, n-hexane and methanol were used. They were distilled before using and special grades were used for spectroscopic measurements.

3.10.2. Solvents for HPLC

Acetonitrile   LiChroSolv HPLC grade (Merck)

Methanol   LiChroSolv HPLC grade (Merck)

Nanopure water   distilled and heavy metals free water obtained by passing distilled water through nano- and ionexchange filter cells (Barnstead, France)

3.10.3. Solvents for optical rotation

Chloroform   Spectral grade (Sigma)

Methanol   Spectral grade (Sigma)

Water   Spectral grade (Fluka)

3.10.4. Solvents for NMR

Acetone-d6   Uvasol, Merck

Chloroform-d   Uvasol, Merck

DMF-d7   Uvasol, Merck

DMSO-d6   Uvasol, Merck

Methanol-d4   Uvasol, Merck

Pyridine-d5   Uvasol, Merck

3. Results

4.1. Ethnopharmacological study

4.1.1. Frequency of Cancer according to gender

During our investigation, we found that the incidence of cancer in the patients attending the National Institute of Oncology in Rabat is higher among women than men. Of 691 patients, 422 were women (61.1 %), and 301 were men (43.5%). This data coincides with that of NIO register during the period of our investigation. Out of a total of 2693 patients hospitalized or treated, 1677 were women (62.2%) and 1016 were men (37.7%).

This may be explained by the high frequencies of gyneco-mammary cancers that present 57% of all cancers affecting women in Morocco (RCRC, 2007). According to cancers register of Casablanca of 2004, the incidence of global standardized cancers in Morocco is of 10.171 new cases per 100.000 inhabitants per year. Cancer affects more frequently women (raw incidence = 100.1) than man (raw incidence = 84.3), while in other developed countries, cancer is more frequent in man than women (RCRC, 2007).

4.1.2. Frequency of cancer according to the localization

Breast cancer in women is the most frequent cause of mortality and represents 16% of death in adult women (The World Health Statistics, 2008). In our study it was found that the incidence of breast cancer comes in the first place with a percentage of 34%, and according to the register of Rabat region in 2005, one cancer out of three is breast cancer with a risk cumulated 0-74 years of 3.8%. The number of new cases expected yearly would be 4660. However, the incidence of breast cancer in Morocco (396 for 100000) remains clearly inferior to incidences found in western countries (more than 80 for 100000) (RCR, 2009). In men broncho-pulmonary cancer comes in first place with percentage of 11%.

RECRAB (2005) estimates that the number of new cases expected yearly in Morocco is 3000 with a risk cumulated 0-74 years, that is 3.0% (RCR, 2009). In developed countries, smoking causes over 80% of such cancers and generally, heavy smoking increases the risk by around 30-fold making lung cancer a major problem in developing countries where the consumption of tobacco is flourishing (Boutayeb A and Boutayeb S, 2005) Non Hodgkin Lymphoma is ranked third, 7%, and it is the most frequent of malignant homeopathies. The incidence of cancers of the lung, colon and rectum, breast and prostate generally increases in parallel with economic development, while the incidence of stomach cancer usually declines with

development (The World Health Statistics, 2008). In developing countries, around 60% of such cancers are thought to be a result of micronutrient deficiencies related to a restricted diet that is low in fruit and vegetables and animal products. There is also consistent evidence that consuming drinks and foods at a very high temperature increases the risk for these cancers (The World Health Statistics, 2008) (Table 4.1).

Table 4.1. The incidence of cancer according to its localization (RECRAB, 2009)

Localization	Number of patients		Frequency (%)		Frequencies of 2005 in Rabat (%)	
	W	M	W	M	W	M
Tonsil	3	2	0,43	0,28	0,26	0,26
Oral cavity	5	1	0,72	0,14	0	0,26
Pharynx	11	26	1,60	3,76	0	0
Colon	11	14	1,60	2,02	1,58	2,60
Cervical	7	-	1,01	0	13,46	-
Stomach	17	17	2,46	2,46	3,17	4,69
Liver	2	2	0,28	0,28	1,06	2,34
Intestine	9	4	1,30	0,57	0,26	0
Tongue	0	1	0	0,14	1,32	0,26
Larynx	0	3	0	0,43	0	3,65
Naso-pharynx	1	2	0,14	0,28	1,58	2,60
Oro-pharynx	0	1	0	0,14	0	0
Lips	0	2	0	0,28	0	0
Mediastinal	0	1	0	0,14	0,26	0,52
Esophagus	1	3	0,14	0,43	0,53	0,78
Bone	6	12	0,86	1,73	1,06	0,78
Orbit	0	2	0	0,28	0	0
Pancreas	0	4	0	0,57	1,85	2,86
Ovaries	27	-	4,00	0	4,49	-
Skin	3	7	0,43	1,01	0,26	0,26
Peritoneum	4	0	0,57	0	0,26	0
Pleura	1	0	0,14	0	0,26	0,52
Lung	8	64	1,15	9,26	2,9	19,89
Prostate	-	3	0	0,43	-	16,67
Rectum	12	13	1,73	1,88	2,64	3,13
Breast	229	2	33,14	0,28	33,51	0,78
Central nervous system	2	3	0,28	0,43	2,37	2,60
Testicle	-	8	0	1,15	-	0,52
Gallbladder	3	1	0,43	0,14	1,58	1,04
Uterus	5	-	0,72	0	3,43	-
Bladder	1	5	0,14	0,72	0,79	8,07
Vulva	1	0	0,14	0	0,53	-
Hodgkin lymphoma	22	21	3,18	3,03	1,06	1,04
Non Hodgkin Lymphoma	28	27	4,05	3,90	2,64	5,73
Leukemia	0	2	0	0,28	0,26	1,04

Globally, many of the risk factors are due to lifestyle and can be prevented. Physical inactivity, western diet and smoking are prominent causes (Alberti, 2001). It should be

remembered that the patients surveyed were under treatment with either chemotherapy or radiotherapy.

4.1.3. Frequency of cancer according to the age

According to cancer register of NIO, the age interval mostly affected by cancer in the course of our investigation is that between 41 and 60 years. This data is similar to that found during our study (Table 4.2)

Table 4.2. The incidence of cancer according to age

Ages	Frequency data of study	Overall frequency between September 2009 and March 2010
Between 1 and 20 years	25 patients (3,6%)	116 patients (4,3%)
Between 21 and 40 years	180 patients (26%)	499 patients (18,5%)
Between 41 and 60 years	363 patients (52,5%)	1342 patients (50%)
More than 61 years old	117 patients (17%)	742 patients (27,5%)

This shows that the incidence of cancer increases with age, while its decrease from 61 years on may be explained only by the high rate of deaths.

4.1.4. Frequency of using traditional medicine by NIO patients

Among the total 691 questioned patients, 272 patients (39%) were identified to regularly use medicinal plants along with medical treatment, while 159 patients have used traditional medicine before using medical treatment. This data show that phytotherapy is still being practiced in Morocco.

4.1.5. Use of Traditional Medicine according to the gender

We have found that women (22.5%) use medicinal plants more frequently than men (16,4%); wich confirms results from previous studies (Hamdani S.E, 1984; El Beghdadi M, 1991; Jaouad L, 1992; Nabih M, 1992; Ziyyat A and al., 1997). This may be explained by the high rate of illiteracy among women in relation to men, as well as the transmission of information from mothers to daughters. When we compare the intragroup variation, the use of medicinal plant was markedly increased 60.60 ± 7.98 and 45.20 ± 5.58 respectively, when comparing women to men. The difference was significant ($p<0.05$). The age interval between 41 years and 60 years use more frequently TM compared to other age intervals.

In some ethnopharmacological studies (El Beghdadi M, 1991; Nabih M, 1992) the same results have been found.

4.1.6. Use of Traditional Medicine according to the origin

The origin and the educational level were also a variable, because in traditional medicine the statements of an illiterate or of an expert are not the same.
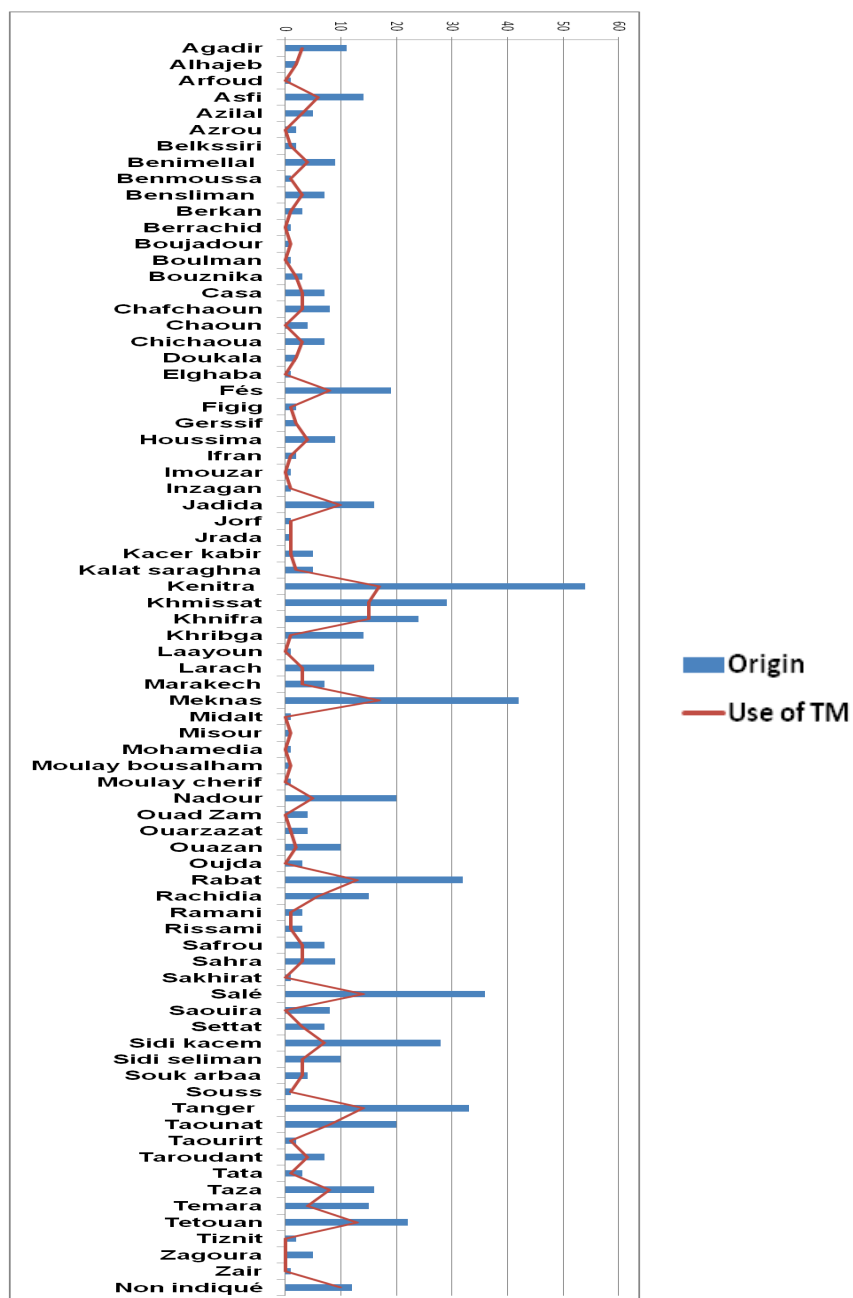

Figure 4.1. Use of Traditional Medicine according to the origin

4.1.7. Certainty and error biases

Data collection relative to origin, place of residence, age as well as type, stage and localization of tumor have been established with the help of the person responsible of hospital register of NIO, and with the study of medical files of each questioned patient. The accuracy of information on the use of traditional medicine as well as the socio-professional class remains underestimated since it depends on the degree of the goodwill of participation.

During the investigation, there was some reluctance from some patients about clearly and honestly answering questions concerning their use of medicinal plants, either out of fear of their clinician, or fear of the consequences of our investigation. The variable of sex has never been missing in our data.

4.1.8. Medicinal plants used in traditional medicine by the patients of the National Institute of Oncology in Rabat

Fiftyfive plants have been cited during this investigation of which *Aristolochia longa*, *Trigonella foenum-graecum*, *Cassia absus* and *Nigella sativa* are the most used (Table 3.3). Among these plants, 28 are proven to have anticancer activity (Table 3.4).

4.1.9. The toxicity and side effects of medicinal plants

The effect of the action of a drug results in the risk benefit ratio. It depends on the drug itself, dose, disease, other drugs consumed in parallel and the patient him self. Only the clinician can find the balance between the toxic doses and the therapeutic doses expected. However, the role of the clinician is lacking in Traditional Medicine, which explains the high rate of poisoning caused by this treatment.

Moreover, there are plants that are proven to be toxic such as *Pinus halepinsis*, *Peganum harmala*, *Aristolochia longa* and *Euphorbia resinifera*, regardless of their toxicity these plants are used by patients in the INO, but have a degree of toxicity or side effects. Nevertheless, despite the existence of the modern medicine for cancer treatment, traditional medicine continues to be a viable health alternative for the large underprivileged section of the Moroccan population.

Table 4.3. Medicinal plants used in traditional medicine by the patients of the National Institute of Oncology in Rabat

Scientific name	Vernacular name		Ecological distribution	Part used	Preparation	Administration	Types of Cancer	Number of citation
Anacardiaceae								
Pistacia lentiscus L.	Drou	ضرو	Mediterranean region	Leaf	Brut, Decoction	Oral	Digestive	5
Apiaceae								
Apium graveolens L	Krafess	كرافـس	Littoral, Mediterranean et Asia	Leaf	Decoction	Oral	Digestive, Kidney	1
Ammodaucus leucotrichus	Kamoun sooufi	كامون الصوفي		Seed	Grind with honey	Oral	Lung	4
Carum carvi L.	Karwiya	كروية	Europe, Asia occidental-Orient	Seed	Grind with honey	Oral	Lung	2
Coriandrum sativum L.	Qezbour	قزبور	Africa	Aerial parts	Grind with honey	Oral	Digestive, Kidney	3
Cuminum cyminum L.	Kamoun	كامون		Seed	Grind with honey	Oral	Lung	1
Daucus carota L.	Khizzou	خيزو	Asia	Root	Decoction	Oral	Digestive, Kidney	5
Foeniculum vulgare Mill.	Nafaâ	نافع	Mediterranean Basin	Seed	Decoction	Oral	Digestive	6
Petroselinum crispum Mill.	Maâdanous	معدنوس	Europe central, Macedonia.	Aerial parts	Decoction	Oral	Kidney	3
Pimpinella anisum L.	Habbat hlawa	حبة حلاوة	Chine et East	Seed	Decoction	Oral	Digestive, Kidney	1
Apocynaceae								
Nerium oleander L.	Defla	دفلة	Africa du Nord, Asia (South West)	Leaf	Decoction	Mouthwash	Gingival	1
Araliacées								
Panax ginseng C.A. Meyes.	Jinsin	جنسن	Nord-East of china and of Korea	Leaf	Grind with honey	Oral	Lung	1
Arécacées								
Phoenix dactylifera	Tamer	الثمر	Saudi and Gulf Country Nord Africa,	Fruit	Brut	Oral	Lymphoma	2
Aristelochiaceae								
Aristolochia longa L.	Berraztam	برزطم		Root	Grind with honey	Oral	General	98
Boraginacées								
Borago officinalis	Hoboub allikaah	حبـوب اللقاح		Stamen	Grind with honey	Oral	General	2
Cruciferae								
Lepidium sativum L.	Hebb rchad	حب الرشاد		Seed	Grind with honey	Oral	Lung,, Digestive	9
Capparaceae								
Capparis spinosa L.	Kebbar	الكبار		Fruit	Grind with honey	Oral	Lymphoma	2
Caryophyllaceae								
Corrigiola telephiifolia	Sarghina	صرغينة		Root	Decoction	Oral	Digestive, Liver	1
Herniaria glabra L	Hrasset lehjer	هراست الحجر		Aerial parts			Digestive, Renal	1

Chenopodiaceae								
Chenopodium ambrosioides	Mkhinza	مخينزة	Mexico	Leaf	Decoction	Oral	Amygdale	1
Haloxylon scoparium Pomel	Eremt	الرمت		Leaf, Fruit	Decoction	Oral	Liver	1
Compositae								
Artemisia absinthium L.	Chiba	الشيبا		Leaf	Infusion	Oral	Digestive	1
Artemisia vulgaris	Chih	الشيح	Orient, Occident	Aerial parts	Infusion	Oral	Digestive	2
Artemisia herba-alba	Chih	الشيح الخرساني		Aerial parts	Infusion	Oral	Digestive	1
Inula viscosa (L.) Ait.	elkhorrassani Bagraman	بگرمان	Tlemcen	Leaf, Flower	Grind with honey	Oral	Breast	22
Euphorbiaceae								
Euphorbia resinifera	Daghmous	الدغموس	Morocco	Aerial parts	Grind with honey	Oral	General	8
Fabaceae								
Cassia absus	Habat albaraka	الحبة البركة	Saudi	Seed	Grind with honey	Oral	General	58
Cicer arietinum	Homos	الحُمص	India and Mediterranean Basin	Seed	Grind with honey	Oral	Lung	1
Vicia faba	Foul	الفـول	Asia	Seed	Grind with honey	Oral	Lung	1
Iridacées								
Crocus sativus	Zâafran	الزعفران		Stamen	Decoction	Oral	General	3
Lamiaceae								
Ajuga iva L.	Chendgoura	الشندگّورة		Rod, Leaf	Grind with honey	Oral	Breast	6
Lavandula officinalis L.	Khzama	خزامة	Mediterranean region	Leaf	Infusion	Oral	The urinary and	9
Marrubium vulgare L.	Marrîwet	مريوت	Europe méridionale	Rod, Leaf	Decoction	Oral	genital system	19
Mentha pulegium L.	Fliyou	فـليو	Zarifet, El-Meffrouche, Hafir	Rod, Leaf	Infusion	Oral	Digestive, Gingival	7
Origanum compactum Beneth	Zaâtar	زعتر		Rod, Leaf	Infusion	Oral	Digestive, Gingival	44
Rosmarinus officinalis L.	Azîr	آزير	Mediterranean	Leaf	Decoction	Oral	Digestive	4
Salvia officinalis L.	Salmiya	سلمية		Leaf	Infusion	Oral	Intestine, Lung	2
Thymus ssp.	Zîtra	زِعترة	Mediterranean	Rod, Leaf	Infusion	Oral	Digestive	1
Leguminosae								
Trigonella foenum-graecum	Halba	حلبة		Seed	Grind with honey	Oral	Digestive	60
Liliacaea								
Allium cepa L.	El Bassla	بصل		Bulb	Brut	Oral	General	1
Allium sativum L	Touma	الثوم		Bulb	Brut	Oral	General	14
Linaceae								
Linum usitatissimum	Zariat alkhatan	زريعة الكتان	Morocco, Argentina and Egypt	Seed	Grind with honey	Oral	Lymphoma	13
Liliacées								
Aloe ferox Mill.	Siber	الصبر	Oriental Africa et méridionale	Leaf	Extraction	Oral	Digestive	4
Lythraceae								
Lawsonia inermis	Henna	الحناء	La carie région de l'asie mineure	Flower	Grind with water	Cataplasm	Skin	5

Moracées								
Ficus carica	Karmous	كرموس	Mediterranean Region	Fruit	Brut	Oral	Digestive	2
Myrtaceae								
Myrtus communis L.	Rihan	الريحان	Ouest d'Asie sud, Europe	Leaf	Decoction	Oral	Digestive	3
Oléacées								
Olea europaea	Zaytoun	الزيتون	Nord de l'Asie.	Fruit	Extraction	Oral	Lung	2
Pinacées								
Pinus halepinsis mill	Katran	قطران	Mediterranean Region	Seed	Extraction	Oral	Esophagi	1
Punicaceae								
Punica granatum L.	Rouman	رمان	Afrique orientale et méridionale	Rind	Decoction	Oral	Skin	2
Ranunculaceae								
Nigella sativa L	Samouj, Haba Saoudaâ	سانوج الحبة السوداء	Bassin méditerranéen	Seed	Grind with honey	Oral	General	54
Sapotaceae								
Argania spinosa	Argan	أركَان	Endémique marocaine	Seed	Extraction	Oral	Skin	1
Thymelaeaceae								
Thymelaea lhytroides	Ftiticha , matnan	متنان , فتيتشة	Endémique ibéro-marocaine	Aerial parts	Decoction	Oral	Uterus	2
Verbenaceae								
Verbena officinalis	Louiza	اللويزة	Espèce cultivée dans les jardins des maisons	Leaf	Infusion	Oral	Gallbladder	1
Zingiberacées							General	
Zingiber officinale roscoe	Sknjbir	سكنجبير	Sud de l'Asie ; Pays tropicaux	Root	Grind with honey	Oral		1
Zygophylaceae							General	
Peganum harmala L.	alharmal	الحرمل		Seed	Grind with honey	Oral		1

Table 4.4. List of plants reported to have anticancer activity

Species	Family	Reference
Pistacia lentiscus L.	*Anacardiaceae*	Balan K.V, 2007
Petroselinum crispum Mill.	*Apiaceae*	Hui Z and al., 2006
Nerium oleander	*Apocynaceae*	Luay J.R and al., 2001
Panax ginseng C.A. Meyes	*Araliaceae*	Shi S and al., 2011
Phoenix dactylifera	*Arécaceae*	Biglari F and al., 2011
Capparis spinosa L.	*Cruciferae*	Sze-Kwan L and al., 2009
Chenopodium ambrosioides	*Chenopodiaceae*	Ruffa M. J and al., 2002
Artimesia vulgaris	*Compositae*	Nibret E and al., 2010
Artemisia herba-alba	*Compositae*	Nibret E and al., 2010
Inula viscosa (L.) Ait.	*Compositae*	Danino O and al., 2009
Euphorbia resinifera	*Euphorbiaceae*	Lavie D and al., 1963
Cicer arietinum	*Fabaceae*	Ajiaikebaier A and al., 2011
Crocus sativus	*Iridaceae*	Akshi H. A and al., 2009
Rosmarinus officinalis L.	*Lamiaceae*	Shuwen C and al., 2001
Salvia officinalis L.	*Lamiaceae*	Toshiya M and al., 2002
Trigonella foenum-graecum	*Leguminosae*	Jayadev R and al., 2004
Allium cepa L.	*Liliacaea*	Jun Y and al., 2004
Allium sativum L.	*Liliacaea*	Yoshiyuki M and al., 2010
Linum usitatissimum	*Linaceae Moraceae*	Abarzua S and al., 2007
Ficus carica	*Myrtaceae*	Sarfaraz Khan M and al., 2011
Olea europaea	*Oléaceae*	Mijatovic S.A and al., 1955
Pinus halepinsis mill	*Pinaceae*	Volker M-S and al., 2011
Punica granatum L.	*Punicaceae*	Oliveira L.P and al., 2010
Nigella sativa L.	*Ranunculaceae*	Worthen D.R and al., 1998
Argania spinosa	*Sapotaceae*	El Babili F and al., 2010
Verbena officinalis	*Verbenaceae*	Ucar Turker A and al., 2010
Zingiber officinale roscoe	*Zingiberaceae*	Kim E-C and al., 2005
Peganum harmala L.	*Zygophylaceae*	Changhong W and al., 2005

4.2. Compounds isolated from the plant *Thymelaea lythroides*

The genus *Thymelaea* Mill. (Thymlaeaceae) comprises 31 species of xerophyllous shrubs and herbs (Galicia-Herbada, 2006). *Thymelaea lythroides*, an ibero-endemic plant in Morocco, is popularly used as medicinal plant to treat otitis, diabetes, rheumatism, inflammation of the prostate and uterus cancer (Gmira *et al.*, 2007). *Thymelaea lythroides* was selected according to an ethnopharmacological study of traditional plants used in Morocco by cancer patients as herbal therapeutics (Kabbaj *et al.*, 2012).

Fresh, healthy parts of *Thymelaea lythroides* have been collected from Maâmora forest in Rabat, Morocco. Voucher specimens were identified by Prof. FZ. ALAOUI, Department of Botany, Faculty of Sciences, Mohamed V University and Prof. Dr. M. IBN TATOU,

Scientific Institute of Rabat, Morocco. Reference samples are deposited as the herbarium of the Scientific Institute of Rabat, and a collection number (RAB 77777) has been assigned.
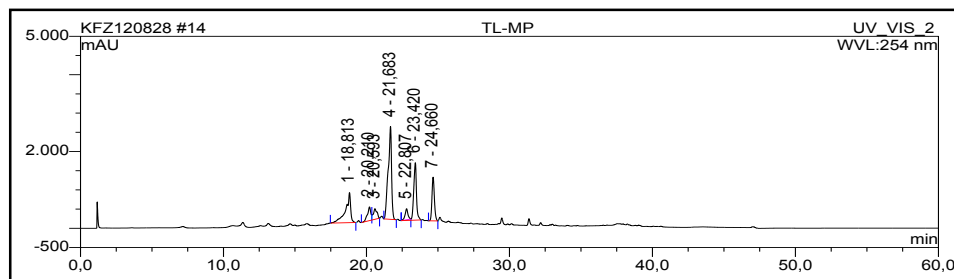
Figure 4.5. HPLC chromatogram of methanol fraction of *Thymelaea lythroides*

Comparison of the HPLC chromatograms of the different fractions of *Thymelaea lythroides* and the preliminary biological screening assays, i.e. antibacterial, antifungal, and cytotoxicity showed that the methanol fraction had the best range of secondary metabolites and the most active one (see Table 4.5 and Figure 4.2).

Table 4.1. Preliminary biological screening assays of the different fractions of *Thymelaea lythroides*

Fungal strain	L5178Y	MRSA	*Strep. pneumonia*	*Entero. Faecalis*
Ethylacetate Fraction	99.7%	>62,5µg/ml	>62.5µg/ml	n.a
Hexane Fraction	125%	n.a	>3.2µg/ml	n.a
Methanol Fraction	99.7%	>31.25µg/ml	>62.2µg/ml	>61.5µg/ml
Water Fraction	99,7%	>31.25µg/ml	> 31.25µg/ml	>61.5µg/ml

Compound isolated from the plant *Thymelaea lythroides*

Bassiatin (**1**, Known)

Bassiatin	
Synonym(s)	(3S,6R)-4-Methyl-6-(l-methylethyl)-3 phenylmethyl-l,4-perhydrooxazine-2,5-dione, (3S, 6R) Bassitin
CAS Registry Number	173008-70-3
Biological source	*Thymelaea lythroides*
Sample amount	1 mg
Physical Description	white powder
Molecular Formula	$C_{15}H_{19}NO_3$
Molecular Weight	261,14 g/mol
Retention time HPLC	33.54 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	+ 176.09 (c 0.02, CHCl$_3$)
UV spectrum λ_{max}	213.2 and 356.2 nm

No ionisation in the negative mode

1 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH$_2$Cl$_2$/MeOH). The fraction was subjected to a sephadex LH-20 column using CH$_2$Cl$_2$/MeOH (1:1) as eluent system, then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded white powder (yield, 1 mg). It showed UV absorbance at λ_{max} (MeOH) 213.2 and 356.2 nm. Positive ESI-MS of **1** exhibited a prominent peak at m/z 261.9 [M+H]$^+$ (base peak) indicating a molecular weight of 261 g/mol, which established a molecular formula of C$_{15}$H$_{19}$NO$_3$. The ^{1}H NMR spectrum showed the presence of 19 protons signals, two methyl protons at (δ_H 0.26 ppm) and (δ_H 0.85 ppm) assigned to H-8 and H-9; one N-methyl protons at (δ_H 3.14 ppm) assigned to 4-CH$_3$; three methine protons at (δ_H 5.79 ppm) (δ_H 4.84 ppm) (δ_H 1.81 ppm) assigned to H-3, H-6 and H-7; one pair of methylene protons (δ_H 3.03-3.39 ppm) assigned to H-10 and five aromatic protons, four of them are overlapping at (δ_H 7.24-7.25 ppm) assigned to H-12, H-13, H-15 and H-16, the H-14 appears at (δ_H 7.18 ppm).
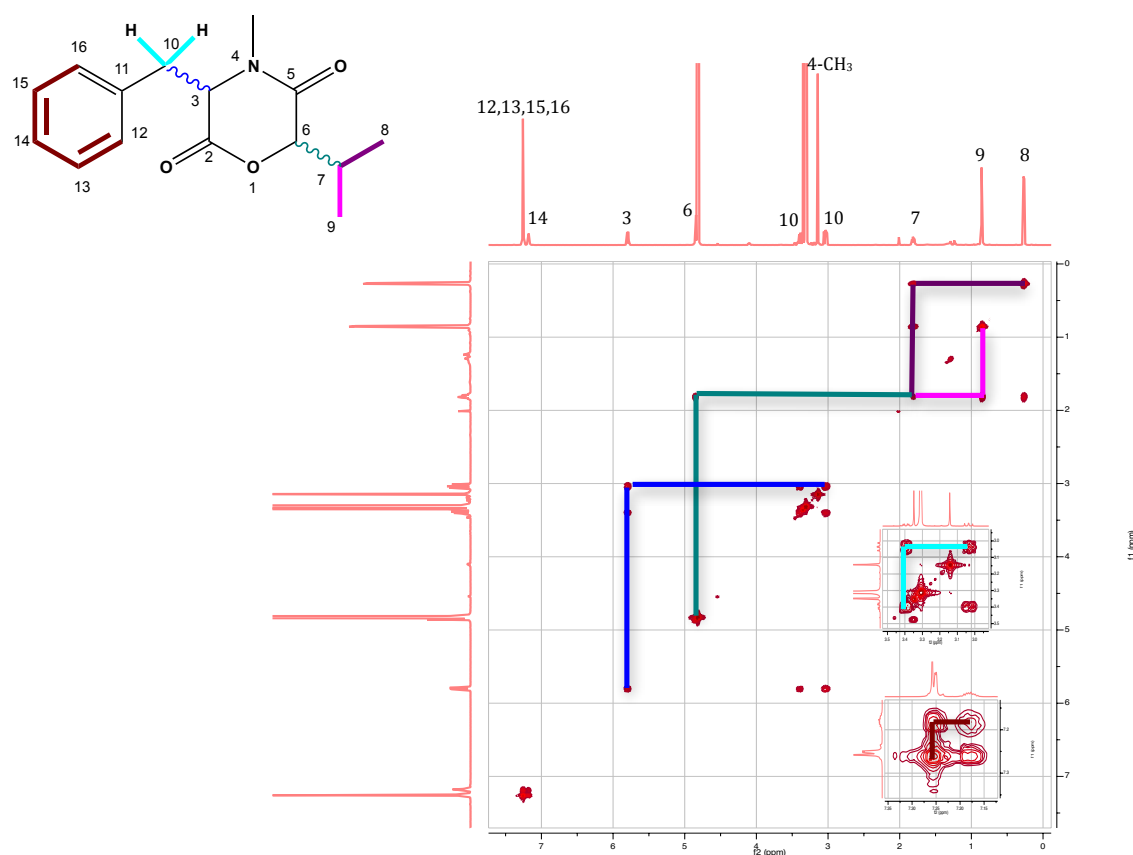
Figure 4.3. ^{1}H-^{1}H NMR COSY

The ^{1}H-^{1}H NMR COSY showed the presence of four spin systems assigned H(3)H(10), H(6)→H(8), from H(7)H(9) and the aromatic ring system H(12)H(13)H(14)H(15)H(16), whereas the connection of these spin systems was established by inspection of ^2J and ^3J HMBC correlations (Figure 4.3 and 4.4).

4-CH$_3$ showed a correlation to the carbon C-3 (δ_C 58.0 ppm) and C-5 (δ_C 170.3 ppm), H-3 showed a correlation to the carbon C-2 and C-5 appearing at (δ_C 172.3 ppm) and (δ_C 170.3 ppm), the same H-6 showed a correlation to the two carbonyl carbons C-2 and C-5. On the other hand, H-10 showed correlations to the overlapping aromatic carbons, which were attributed to C-12, C-13, C-15 and C-16 (δ_C 129.8-130.0 ppm), as well as to an isolated aromatic carbon assigned to C-11 (δ_C 138.2 ppm), hence establishing the connection between the identified spin systems (Figure 4.3). Thus the structure of **1** was elucidated as 4-methyl-6-(l-methylethyl)-3-phenylmethyl-l,4-perhydrooxazine-2,5-dione, consisting of one mole each of *N*-methylphenylalanine and 2-hydroxy-3-methylbutyric acid.
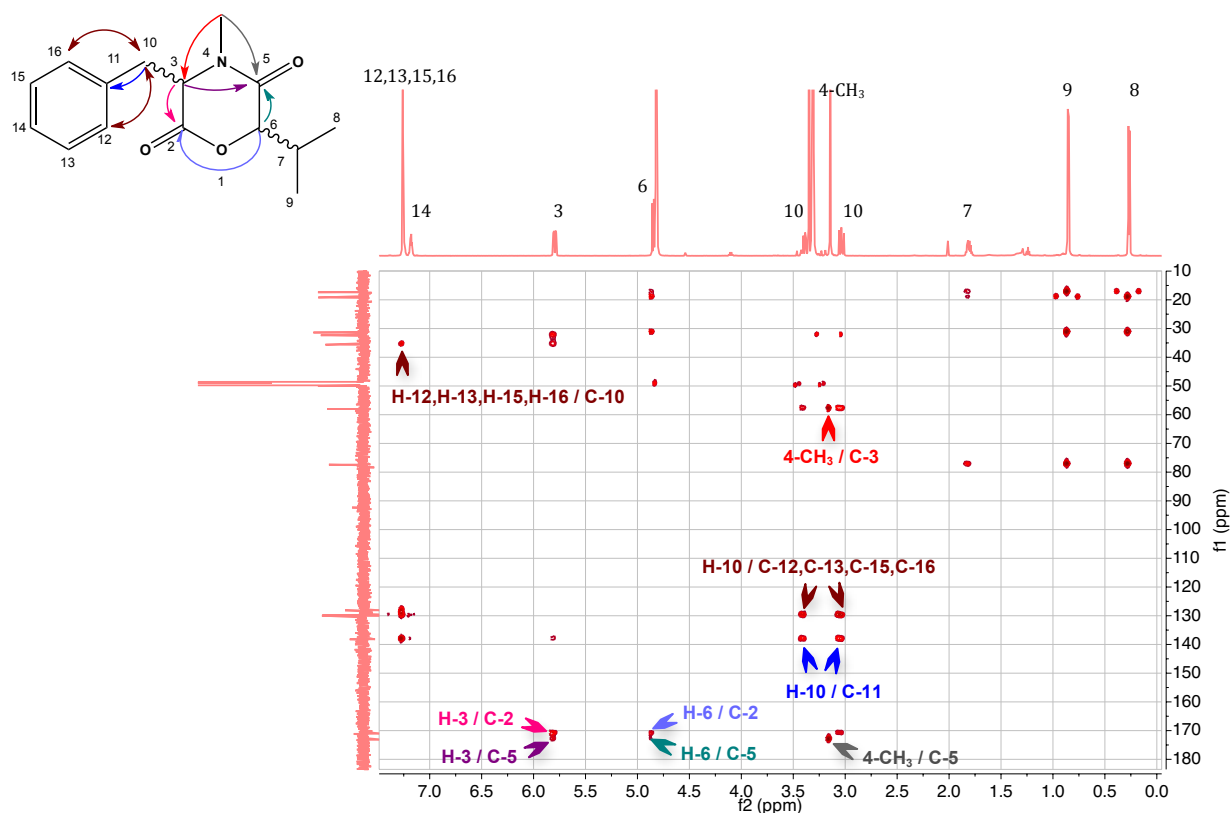
Figure 4.4. Selected ^{13}C-^{1}H NMR HMBC correlations

The optical rotations of 4 ([α]$_D$ + 176.09° (c 0.02, CH$_3$Cl)) was compared with that of bassiatin   [(3S,6R)-4-methyl-6-(1-methylethyl)-3-phenylmethyl-1,4-perhydrooxazine-2,5-di-

one] ($[\alpha]_D$ + 181.05° (c 0.024, CH$_3$Cl)) and to the other three isomeric - (3R,6S)-, (3R,6R)-, (3S,6S)- compounds (Kagamizono *et al.*, 1995). The structure of **1** was determined to be (3S,6R)-4-methyl-6-(l-methylethyl)-3-phenylmethyl-l ,4-perhydrooxazine-2,5-dione, (3S,6R) Bassitin.

Table 4.6. Comparison of ^{1}H and ^{13}C NMR chemical shifts of **1** with Bassiatin (Kagamizono *et al.*, 1995)

Position	1 δ_H (mult., *J* in Hz)	δ_C	Bassiatin δ_H (mult., *J* in Hz)	δ_C
2	-	172.3	-	170.2
3	5.79, 1H (dd, *J*= 12.6, 4.5 Hz)	58.0	5.47 (br d)	57.6
4-CH$_3$	3.14, 3H (s)	32.3	3.00 (s)	32.6
5	-	170.3	-	169.5
6	4.84, 1H (d, *J*= 8.9 Hz)	77.3	4.92 (d, 8.7 Hz)	75.7
7	1.81, 1H (m)	31.3	2.02 (m)	29.9
8	0.26, 3H (d, *J*= 6.9 Hz)	17.3	0.42 (d, *J*= 6.9 Hz)	17.8
9	0.85, 3H (d, *J*= 6.6 Hz)	19.1	0.80 (d, *J*= 6.9 Hz)	18.5
10	3.39, 1H (dd, *J*= 14.7, 4.6 Hz)	35.6	3.36 (dd, *J*= 14.5, 5.1 Hz)	35.0
	3.03, 1H(dd, *J*= 14.7, 12.7 Hz)		2.98 (dd, *J*= 14.5, 11.9 Hz)	
11	-	138.2	-	136.9
12, 16	7.25 - 7.24, 2H (m)	130.0	7.23 – 7.23 (m)	129.1
13, 15	7.25 - 7.24, 2H (m)	129.8	7.23 – 7.23 (m)	128.8
14	7.18, 1H (m)	128.1	7.17 (m)	127.0

Bassiatin is an unusual secondary metabolite to be obtained from plants. To the best of our knowledge, bassiatin was only described as natural product from the cultured broth of *Beauveria bassiana* (Kagamaizono *et al.*, 1995), from the insect-body portions of *Cordyceps cicadae* (Kuo *et al.*, 2002) and later isolated from the endophytic fungus *Fusarium oxysporum J8-1-2* (Meng *et al.*, 2011). This is the first report of this compound from the plant *Thymelaea lythroides*. This can possibly be explained by the presence of an endophyte, which produced bassiatin in large quantity to be detected in the host plant.

Compound isolated from the plant *Thymelaea lythroides*

Daphneone (**2**, Known)
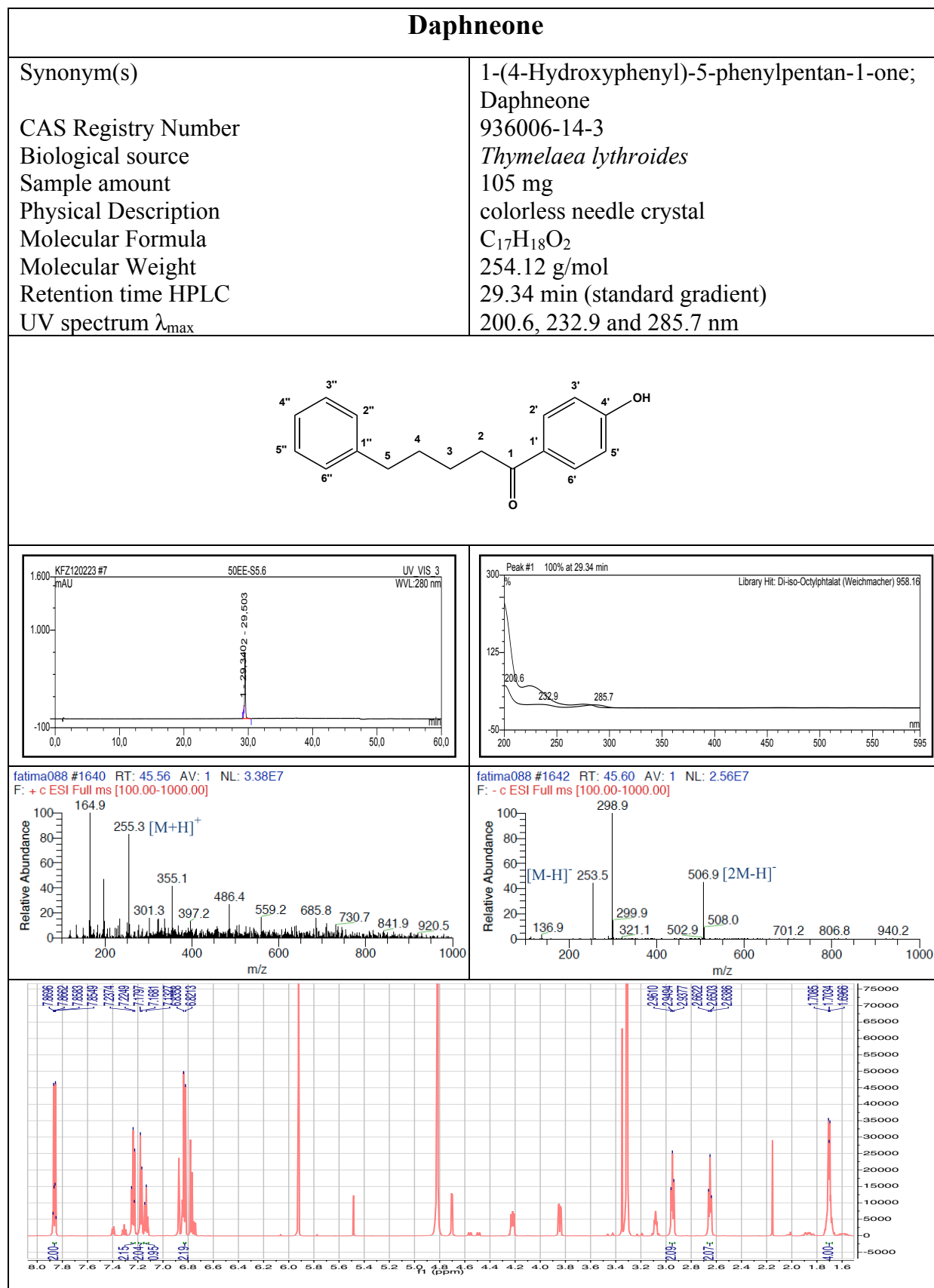
Daphneone	
Synonym(s)	1-(4-Hydroxyphenyl)-5-phenylpentan-1-one; Daphneone
CAS Registry Number	936006-14-3
Biological source	*Thymelaea lythroides*
Sample amount	105 mg
Physical Description	colorless needle crystal
Molecular Formula	$C_{17}H_{18}O_2$
Molecular Weight	254.12 g/mol
Retention time HPLC	29.34 min (standard gradient)
UV spectrum λ_{max}	200.6, 232.9 and 285.7 nm

2 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH_2Cl_2/MeOH). The fraction was subjected to a sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent system, then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH. Compound **2** was obtained as a mixture with compound **6** forming colorless needle crystals with colorless oil (yield, 3 mg). It showed UV absorbance at λ_{max} (MeOH) 200.6, 232.9 and 285.7 nm. Positive and negative ESI-MS of **2** exhibited a prominent peak at m/z 255.3 $[M+H]^+$ (base peak) and m/z 253.5 $[M-H]^-$ (base peak) indicating a molecular weight of 254 g/mol. The molecular formula of compound **2** was determined to be $C_{17}H_{18}O_2$ based on the analysis of the molecular weight and ^{13}C NMR data. The ^1H NMR spectrum (MeOD) (Table 4.7) displayed signals of a *p*-substituted aromatic protons at (δ_H 6.82 and 7.86 ppm; each 2H) assigned to H-3', H-5', H-2' and H-6' respectively and mono-substituted aromatic protons at (δ_H 7.16 and 7.23 ppm; each 2H) with (δ_H 7.13 ppm; 1H) assigned to H-2", H-6", H-3", H-5" and H-4" respectively, indicated that there are two aromatic rings in the structure. In addition, four methylene protons at (δ_H 1.70 ppm; 4H), (δ_H 2.65 ppm; 2H) and (δ_H 2.95 ppm; 2H) assigned to H-3, H-4, H-5 and H-2 respectively. The ^{13}C NMR spectrum indicated the presence of 17 carbon signals displayed of four aliphatic methylene carbons, nine aromatic methine carbons and four quaternary carbons, one carbonyl carbon and one oxygenated quaternary carbon. The ^1H-^1H COSY NMR spectrum (Figure 4.5.A) showed 4 spin systems assigned for H(2')H(3'), H(5')H(6'), from H(2) to H(5) and the aromatic ring from H(2")to H(6"), whereas the connection of these spin systems was established by inspection of ^2J and ^3J HMBC correlations (Figure 4.5.B).
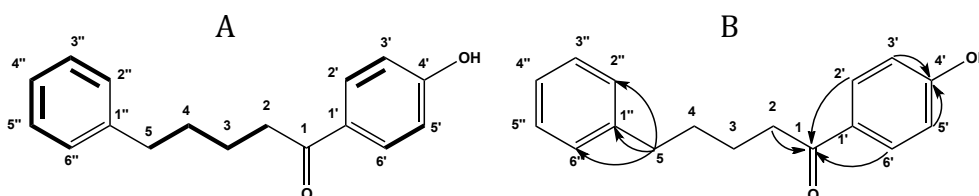
Figure 4.5 A) ^1H-^1H COSY correlations **B)** selected ^{13}C-^1H NMR correlations of **2**

H-5 showed a correlations to the overlapping aromatic carbons, which were attributed to C-2" and C-6" (δ_C 129.9 ppm), as well as to an isolated aromatic carbon assigned to C-1" (δ_C 144.1

ppm), these correlations indicated that the mono-substituted aromatic ring is connected to the side chain of four methylene groups at C-1". On the other hand, the second *p*-substituted aromatic ring is connected to a hydroxyl group at C-4' (δ_C 164.30 ppm) and to carbonyl group at C-1' (δ_C 130.6 ppm), these connection were confirmed with the correlations between the overlapping aromatic protons H-3' and H-5' to the C-4' and from the overlapping aromatic protons H-2' and H-6' to the carbon C-1 (δ_C 202.28 ppm). Furthermore, the carbonyl carbon C-1 (δ_C 202.28 ppm) was connected to side chain of four methylene groups at C-2 (δ_C 39.43 ppm), which was confirmed with the correlation between H-2 to C-1, thus, establishing the connection between the identified spin systems.

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as $[\alpha]_D$ value of **2** with those previously published data for Daphenone (Zhang *et al.*, 2006).

Daphenone was previously isolated from some species of Daphne including, *Daphne odora*, which is the first biological source report (Zhang *et al.*, 2006), from *Daphne giraldii Nitsche* (Sun *et al.*, 2006) and from *Daphne acutiloba* (Huang *et al.*, 2012). This the first report of Daphenone from the species *Thymelaea lythroides*.

Compound isolated from the plant *Thymelaea lythroides*

(+)-Daphneolone (**3**, Known)
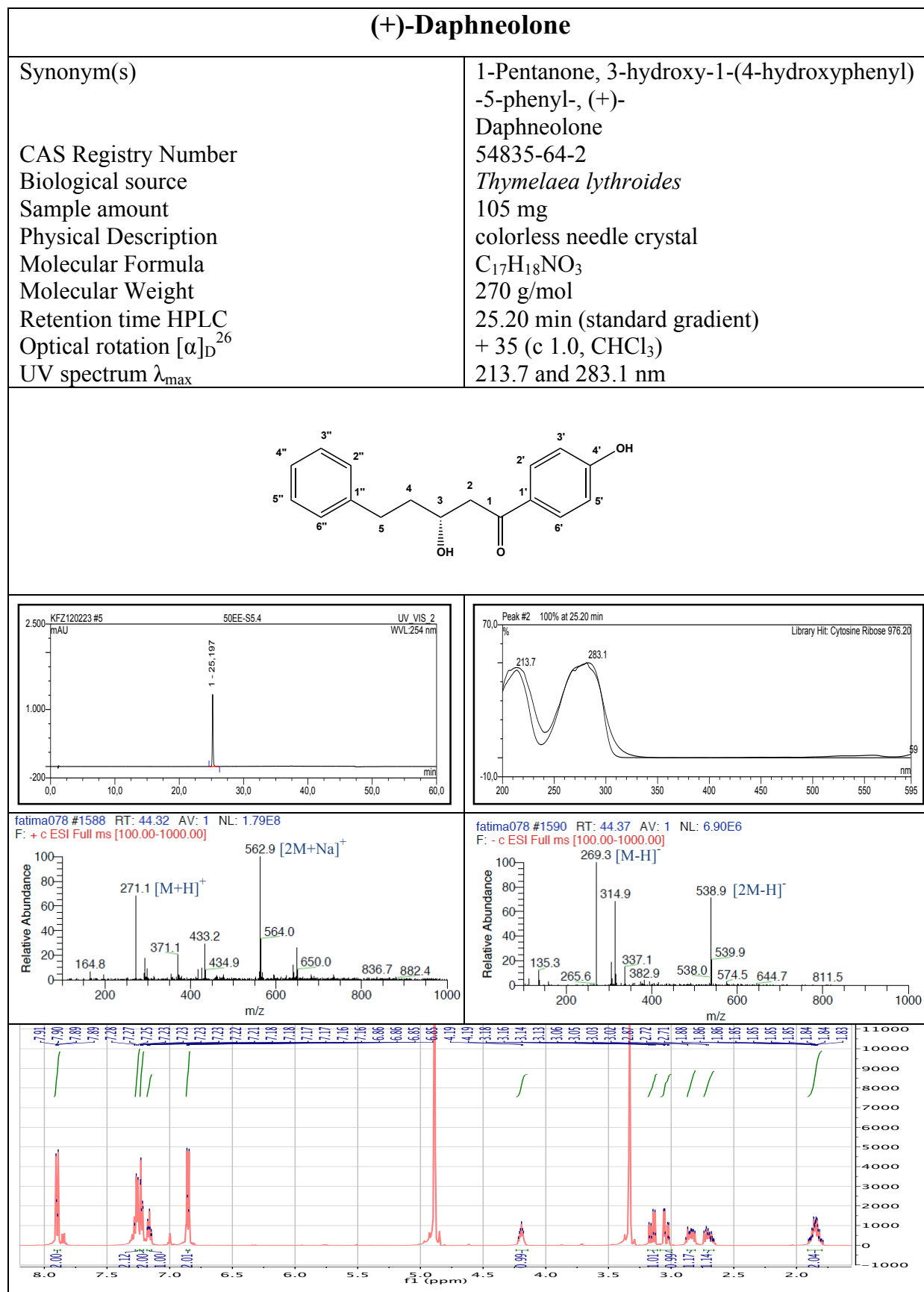
(+)-Daphneolone	
Synonym(s)	1-Pentanone, 3-hydroxy-1-(4-hydroxyphenyl)-5-phenyl-, (+)-Daphneolone
CAS Registry Number	54835-64-2
Biological source	*Thymelaea lythroides*
Sample amount	105 mg
Physical Description	colorless needle crystal
Molecular Formula	$C_{17}H_{18}NO_3$
Molecular Weight	270 g/mol
Retention time HPLC	25.20 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	+ 35 (c 1.0, CHCl$_3$)
UV spectrum λ_{max}	213.7 and 283.1 nm

3 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH$_2$Cl$_2$/MeOH). The fraction was subjected to a sephadex LH-20 column using CH$_2$Cl$_2$/MeOH (1:1) as eluent system, then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H$_2$O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded colorless needle crystal (yield, 3 mg). It showed UV absorbance at λ_{max} (MeOH) 213.7 and 283.1 nm. Positive and negative ESI-MS of **3** exhibited a prominent peak at m/z 271.1 [M+H]$^+$ (base peak) and m/z 269.3 [M-H]$^-$ (base peak) indicating a molecular weight of 270 g/mol with a 16 mass unit decrease compared to **2**. To fulfill the molecular weight, the presence of one hydroxyl group was supposed. The molecular formula of compound **3** was determined to be C$_{17}$H$_{18}$NO$_3$ based on the analysis of the molecular weight and ^{13}C NMR data.

The ^{1}H NMR spectrum (MeOD) (Table 4.3) displayed the same aromatic protons signals as compound **2**, indicating that there are also two aromatic rings in the structure. In addition, two typical AMX system at (δ_H 3.02 ppm ; 1H, dd, *J*= 17.6, 8.5 Hz, assigned to H-2$_A$), (δ_H 3.13 ppm ; 1H, dd, *J*= 17.6, 2.6 Hz, assigned to H-2$_M$) and (δ_H 4.21 ppm ; 1H, m, assigned to H-3$_X$) together with the second AMX$_2$ system at (δ_H 2.75 ppm ; 1H, m, H-5$_A$), (δ_H 2.89 ppm ; 1H, m, H-5$_M$) and (δ_H 1.81 ppm ; 2H, m, H-4$_{X1}$ and H-4$_{X2}$) indicate that there are three methylene protons and one methine proton with hydroxyl group substitution. The ^{1}H-^{1}H COSY NMR spectrum showed the same spin systems as daphenone (Figure 4.6).
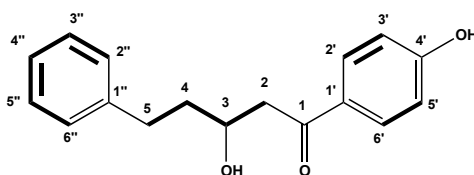
Figure 4.6. ^{1}H-^{1}H COSY correlations of **3**

The optical rotation of **3** was close to the rotation reported in the literature for (+)-daphneolone (Zhuang *et al.*, 1982).

The identification was further corroborated by comparison of UV, NMR, MS data as well as [α]$_D$ value of **3** and those previously published data for daphneolone (Zhuang *et al*., 1982). Daphnelone was previously isolated from some species of *Daphne* including, *Daphne odora*, which is the first biological source report (Kogiso *et al*., 1974), from *Daphne tangutica* (Zhuang *et al*., 1982), from *Daphne mezereum* (Kreher *et al*., 1990), from *Daphne pedunculata* (Xu *et al*., 2008), from *Daphne giraldii* (Wu *et al*., 2009), from *Daphne bholua* (Chen *et al*., 2009), from *Daphne retusa* (Hu *et al*., 2011), from *Daphne papyracea var. crassiuscula* (Wei *et al*., 2012) and from *Daphne genkwa* (Chen *et al*., 2013). This the first report of Daphnelone from the species *Thymelaea lythroides*.

Table 4.7. The ^{1}H and ^{13}C chemical shifts of **2** and **3**

Position	2		3	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	-	202.28	-	202.28
2	2.95 (2H, t, *J*= 6.96 Hz)	39.43	3.02 (1H, dd, *J*= 17.6, 8.5 Hz)	44.3
			3.13 (1H, dd, *J*= 17.6, 2.6 Hz)	
3	1.70 (2H, m)	26.17	4.21 (1H, m)	67.2
4	1.70 (2H, m)	32.85	1.81 (2H, m)	38.1
5	2.65 (2H, t, *J*= 7.14 Hz)	37.29	2.75 (1H, m)	31.9
			2.89 (1H, m)	
1'	-	130.66	-	130.66
2'	7.86 (2H, d, *J*=8.82 Hz)	132.40	7.90 (2H, d, *J*=8.82 Hz)	132.40
3'	7.23 (2H, t, *J*= 7.56 Hz)	116.80	6.86 (1H, d, *J*=8.82 Hz)	116.80
4'	-	164.30	-	164.30
5'	7.23 (2H, t, *J*= 7.56 Hz)	116.80	6.86 (1H, d, *J*=8.82 Hz)	116.80
6'	7.86 (2H, d, *J*=8.82 Hz)	132.40	7.90 (2H, d, *J*=8.82 Hz)	132.40
1"	-	144.17	-	144.17
2"	7.16 (2H, d, *J*= 6.96 Hz)	129.99	7.24 (2H, d, *J*= 6.96 Hz)	129.99
3"	6.82 (1H, d, *J*=8.82 Hz)	127.29	7.25 (2H, t, *J*= 7.56 Hz)	127.29
4"	7.13 (1H, t, *J*= 7.32 Hz)	129.87	7.19 (1H, t, *J*= 7.32 Hz)	129.87
5"	6.82 (1H, d, *J*=8.82 Hz)	127.29	7.25 (2H, t, *J*= 7.56 Hz)	127.29
6"	7.16 (2H, d, *J*= 6.96 Hz)	129.99	7.24 (2H, d, *J*= 6.96 Hz)	129.99

Compound isolated from the plant *Thymelaea lythroides*

(-)-Wikstromol (**4**, Known)

(-)-Wikstromol	
Synonym(s)	2(3H)-Furanone,dihydro-3-hydroxy-3,4bis[(4-hydroxy-3-methoxyphenyl)methyl]-,(3S-cis); (-)-Nortrachelogenin;(-)-Wikstromol;
CAS Registry Number	34444-37-6
Biological source	*Thymelaea lythroides*
Sample amount	2 mg
Physical Description	colorless powder
Molecular Formula	$C_{20}H_{22}O_7$
Molecular Weight	374 g/mol
Retention time HPLC	19.6 min (standard gradient)
Optical rotation $[\alpha]_D^{25}$	- 38.5 (c 1.0, CHCl$_3$)
UV spectrum λ_{max}	280.9 nm

4 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH_2Cl_2/MeOH). The fraction was subjected to a sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent system, then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded colorless powder (yield, 2 mg). It showed UV absorbance at λ_{max} (MeOH) 280.9 nm. Positive and negative ESI-MS of **4** exhibited a prominent peak at m/z 375.0 $[M+H]^+$ (base peak) and m/z 373.3 $[M-H]^-$ (base peak) indicating a molecular weight of 374 g/mol. The molecular formula of compound **4** was determined to be $C_{20}H_{22}O_7$ based on the analysis of the molecular weight and ^{13}C NMR data.

The ^1H NMR spectrum (MeOD) (Table 4.8) displayed signals of two- tri-substituted aromatic systems at (δ_H 6.70 ppm ; 4H) assigned to H-1, H-1', H-4 and H-4' with (δ_H 6.59 ppm ; 2H) assigned to H-5 and H-5', in addition, two methoxy protons at (δ_H 3.78 ppm and 3.81 ppm) assigned to H-10 and H-10', three methylene protons at (δ_H 2.48-2.79 ppm), (δ_H 2.85-3.12 ppm) and (δ_H 3.98 ppm) assigned to H-7', H-7 and H-9' and one methine proton at (δ_H 2.44 ppm) assigned to H-8'.

^{13}C NMR and DEPT NMR spectrum showed the presence of two oxygenated methyl carbons, two aliphatic methylene carbons, one oxygenated methylene carbon, one aliphatic methine carbon, six aromatic methine carbons, four oxygenated aromatic quaternary carbons, two aromatic quaternary carbons, one oxygenated aliphatic quaternary carbon and one carbonyl carbon.

The ^1H-^1H COSY showed the presence of four spin systems assigned for H(4-4')H(5-5'), a continuous spin system H(7')→H(9') and a spin system between the protons of the three methylene groups as showed in the Figure 4.7.A.

The HMBC-NMR spectra (Figure 4.7.B) showed the connection between the previously mentioned spin systems. The cross peak of the proton H-7' to the aromatic carbons, which were attributed to C-1', C-5' and C-6', indicated that the aromatic ring is connected to the aliphatic side at the C-6'. In opposite, from the protons H-7 to the aromatic carbons assigned

to C-1, C-5 and C-6, indicating that the aromatic ring is connected to the aliphatic side at the C-6. Furthermore, the cross peak from the proton H-7 to the carbonyl carbons C-9 and C-8 and in the opposite, from the H-7' to the carbons C-9' and C-8' showed that the partial structure 1 and 2 (Figure 4.8) was connected at C-8 and at the carbonyl carbon C-9, these connections were confirmed with the correlations between the proton H-8' and the carbon C-8 and from H-9' to the carbonyl carbon C-9, thus establishing the connection between the identified spin systems.
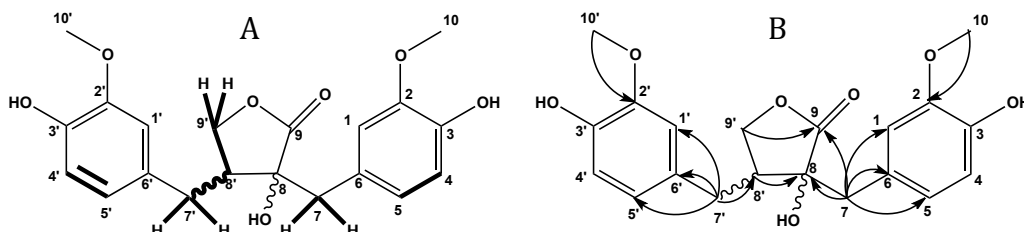
Figure 4.7. A) ^{1}H-^{1}H COSY correlations **B)** Selected ^{13}C-^{1}H NMR correlations of **4**

Table 4.8. ^{1}H and ^{13}C NMR chemical shifts of (-)-wikstromol (**4**)

N°	4	
	δ_H	δ_C
1	6.70, 1H (m)	113.9[a]
2	-	146.3[e]
3	-	149.1[f]
4	6.59, 1H (ddd, J= 1.92, 8.01, 12.39 Hz)	116.3[b]
5	6.70, 1H (m)	122.6[c]
6	-	128.5
7	3.98, 2H (d, J= 13.8 Hz)	42.1
8	-	77.7
9	-	77.6
10	3.81, 3H (s)[x]	56.6[d]
1'	6.70, 1H (m)	115.3[a]
2'	-	146.3[e]
3'	-	149.1[f]
4'	6.59, 1H (ddd, J= 1.92, 8.01, 12.39 Hz)	116.4[b]
5'	6.70, 1H (m)	124.2[c]
6'	-	122.1
7'	2.48, 1H (dd, J= 9.37, 13.35 Hz)	32.3
	2.79, 1H (dd, J= 4.77, 13.35 Hz)	
8'	2.44, 2H (qd, J= 4.72, 8.13, 8.60, 8.60 Hz)	44.7
9'	3.98, 2H (d, J= 8.13 Hz)	72.1
10'	3.78, 3H (s)[x]	56.7[d]

[a, b, c, d, f, x] could be opposite
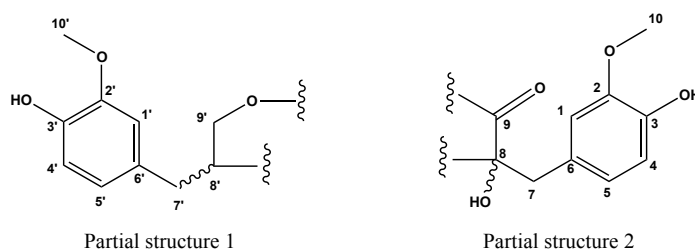
Figure 4.8. The partial structure 1 and 2
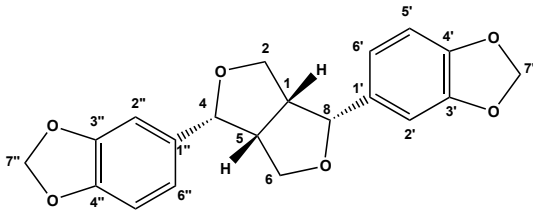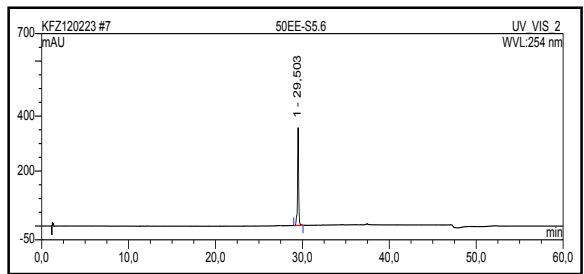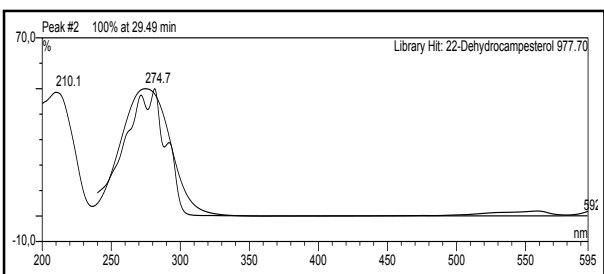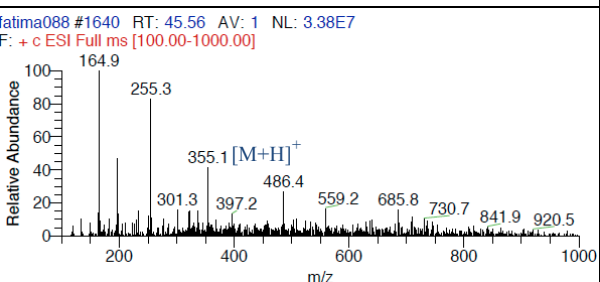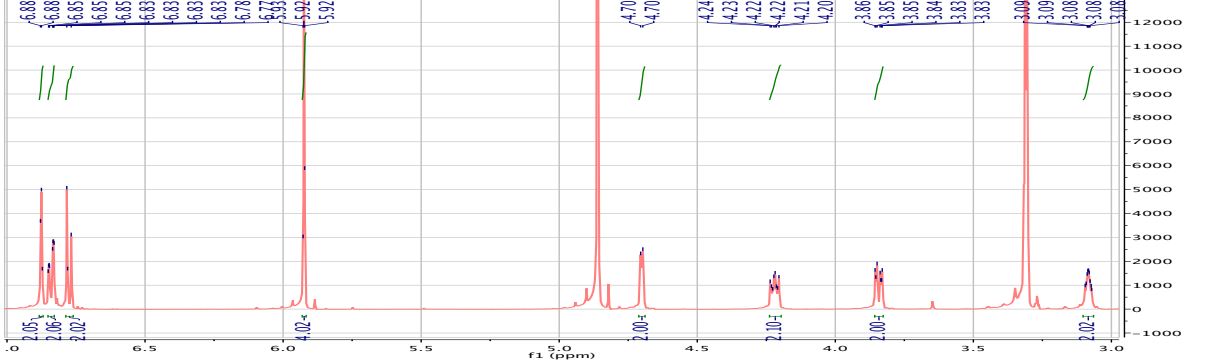
The optical rotation was found to be $[\alpha]_D^{25}$ - 38.5 (c 1.0, CHCl$_3$).

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as $[\alpha]_D$ value of **2** and those previously published data for (-)-wikstromol (Sefkow, 2001).

(-)-Wikstromol was reported for the first time from *Wikstroemia viridiflora* (Tandon and Rasiiig, 1976). Then from *Cedrus deodara* (*Piniaceae*) (Rao *et al*., 2002) and from *Didymochlaena truncatula* (*Hypodematiaceae*) (Cao *et al*., 2006). We report here the presence of wikstromol in *T. lythroides* for the first time.

Compound isolated from the plant *Thymelaea lythroides*

δ-Sesamin (**5**, Known)

δ-Sesamin	
Synonym(s)	Tetrahydro-1,4-bis[3,4 (methylenedioxy)phenyl]-1H,3H-furo[3,4-c]furan; δ-Sesamin
CAS Registry Number	607-80-7
Biological source	*Thymelaea lythroides*
Sample amount	2 mg
Physical Description	colorless oil
Molecular Formula	$C_{20}H_{18}O_6$
Molecular Weight	382 g/mol
Retention time HPLC	29.50 min (standard gradient)
Optical rotation $[\alpha]_D^{25}$	- 60 (c 1.0, CHCl$_3$)
UV spectrum λ_{max}	210.1 and 274.7 nm

No ionisation in the negative mode

5 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH_2Cl_2/MeOH). The fraction was subjected to a sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent system, then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded colorless powder (yield, 2 mg). It showed UV absorbance at λ_{max} (MeOH) 280.9 nm. Positive ESI-MS of **5** exhibited a prominent peak at m/z 355.1 $[M+H]^+$ (base peak) and m/z 253.5 $[M-H]^-$ (base peak) indicating a molecular weight of 354 g/mol. The molecular formula of compound **5** was determined to be $C_{20}H_{18}O_6$ based on the analysis of the molecular weight and ^{13}C NMR data.

Table 4.9. Comparison of ^1H and ^{13}C NMR chemical shifts of (+)-sesamin (**5**)

N°	5	
	δ_H	δ_C
1	3.08, 1H (m)	137.0
2	4.70, 1H (d, *J*= 3.95 Hz)	87.8
3	-	149.9
4$_A$	3.84, 1H (dd; *J*= 3.77, 9.10 Hz)	73.2
4$_M$	4.22, 1H, dd; *J*= 6.80, 9.10 Hz	73.2
5	3.08, 1H (m)	109.5
6	4.70, 1H (d, J= 3.95 Hz)	121.1
7	-	-
8$_A$	3.84, 1H (dd; *J*= 3.77, 9.10 Hz)	73.2
8$_M$	4.22, 1H, dd; *J*= 6.80, 9.10 Hz	73.2
1'	-	137.0
2'	6.88, 1H (d; *J*= 1.72 Hz)	108.1
3'	-	149.9
4'	-	149.1
5'	6.78, 1H (d; *J*= 7.76 Hz)	109.5
6'	6.84, 1H (dd; *J*= 1.05, 7.92 Hz)	121.1
7'	5.92, 2H (d; *J*= 1.46 Hz)	-
1"	-	137.0
2"	6.88, 1H (d; *J*= 1.72 Hz)	108.1
3"	-	149.9
4"	-	149.1
5"	6.78,1H (dd, *J*= 7.76 Hz)	109.5
6"	6.84, 1H (dd; *J*= 1.05, 7.92 Hz)	121.1
7"	5.92, 2H (d; *J*= 1.46 Hz)	-

The ^1H NMR spectrum (MeOD) (Table 4.9) displayed 18 protons signals: a typical AMX system at (δ_H 3.84 ppm; 2H, dd; *J*= 3.77, 9.10 Hz, H-4$_A$ and H-8$_A$), (δ_H 4.22 ppm; 2H, dd; *J*= 6.80, 9.10 Hz, H-4$_M$ and H-8$_M$) and (δ_H 3.08 ppm; 2H m, H-1$_X$ and H-5$_X$) indicating the presence of two overlapping methylene protons, each one was connected to one methine proton; 2 overlapping -O-CH_2-O- at (δ_H 6.78 ppm) assigned to H-7' and H-7"; two overlapped

methine protons at (δ_H 4.70 ppm) assigned to H-1 and H-5; six aromatic protons of two tri-substituted benzene moieties at (δ_H 6.78 ppm) assigned to H-2' and H-2", (δ_H 6.84 ppm) assigned to H-6' and H-6" and (δ_H 6.88 ppm) assigned to H-5' and H-5".

The ^{1}H-^{1}H COSY showed the presence of four spin systems assigned for H(5-5')H(6-6'), a continuous spin system H(4)→H(6), H(1)H(2)H(8) and spin system between the protons H(1)H(5) as shown in the Figure 4.9.A.
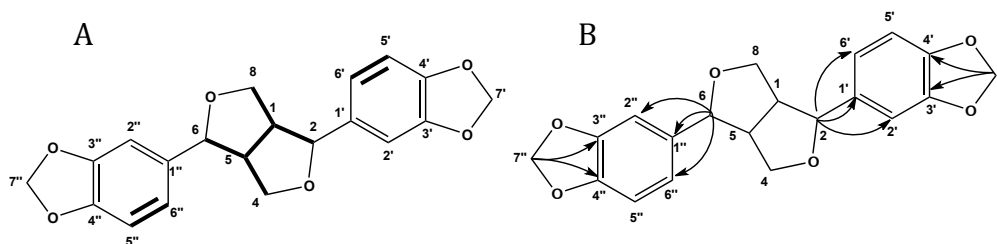
Figure 4.9. A) ^{1}H-^{1}H COSY correlations **B)** selected ^{13}C-^{1}H NMR correlations of **5**

The ^{1}H-^{13}C MBC NMR spectra showed the connection between the previously mentioned spin systems. H-4 showed correlations to the overlapping aromatic carbons, which were attributed to C-2" and C-6" (δ_C 129.9 ppm), as well as to an isolated aromatic carbon assigned to C-1" (δ_C 144.1 ppm), these correlations indicated that the tri-substituted aromatic ring is connected to the 3,7-O-bicyclooctane moieties at C-1". The same correlations appeared for the other tri-substituted aromatic ring, from H-8 to the overlapping aromatic carbons C-2' and C-6', together with C-1', to confirm that the second tri-substituted aromatic ring was connected to the 3,7-O-bicyclooctane moieties at C-1'. On the other hand, the two overlapping -O-CH$_2$-O- showed correlations to the overlapped aromatic carbons C-3'-C-3" and to C-4'-C-4", to prove that -O-CH$_2$-O- was connected to the tri-substituted aromatic ring at C-3' and C-4' for the first one, and at C-3" and C4" for the second aromatic ring, thus establishing the connection between the identified spin systems.

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as [α]$_D$ value (Lin et al., 2004) of **5** with those published data for (+)-sesamin.

(+)-Sesamin is a natural product, isolated for the first time from *Piper sylvaticum* (Banerji and Dhara, 1974), then from *Artemisia argentea* (El-Emary and Attia, 1988). Later, **5** was reported from *Thymelaeaceae* family (Takaku *et al.*, 2001), from *Plectranthus mollis* (Kulkarni *et al.*, 2012), and from *Sesamum indicum* (Yoshida *et al.*, 2007). This is the first report of this compound from genus *Thymelaea*.

Compound isolated from the plant *Thymelaea lythroides*

Daphnoretin (**6**, Known)

Daphnoretin	
Synonym(s)	Coumarin, 7-hydroxy-6-methoxy-3,7'-oxydi- (7CI,8CI); Daphnoretin; NSC 291852; Thymelol
CAS Registry Number	2034-69-7
Biological source	*Thymelaea lythroides*
Sample amount	19 mg
Physical Description	faint yellow needle crystals
Molecular Formula	$C_{19}H_{12}O_7$
Molecular Weight	252 g/mol
Retention time HPLC	22.84 min (standard gradient)
UV spectrum λ_{max}	200.0, 259.9 and 344.9 nm

6 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH_2Cl_2/MeOH). The fraction was subjected to a sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent system, then purified by semipreparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded colorless powder (yield, 19 mg). It showed UV absorbance at λ_{max} (MeOH) 200.0, 259.9 and 344.9 nm. Positive and negative ESI-MS of **6** exhibited a prominent peak at m/z 353.1 $[M+H]^+$ (base peak) and m/z 351.3 $[M-H]^-$ (base peak) indicating a molecular weight of 352 g/mol. The molecular formula of compound **6** was determined to be $C_{19}H_{12}O_7$ based on the analysis of the molecular weight and ^{13}C NMR data.

The ^1H NMR spectrum of **6** (Table 4.10) displayed 12 proton signals, one methoxy group at (δ_H 3.82 ppm) assigned to 6-CH_3, one hydroxyl group signal at (δ_H 10.30 ppm) assigned to 7-OH, five aromatic protons at (δ_H 6.87 ppm), (δ_H 7.12 ppm), (δ_H 7.19 ppm), (δ_H 7.22 ppm), and (δ_H 7.72 ppm), which were assigned to H-8, H-6', H-8', H-5 and H-5', three heterocyclic protons at (δ_H 6.39 ppm), (δ_H 7.88 ppm) and (δ_H 8.05 ppm) assigned to H-3', H-4 and H-4'.

The ^1H-^1H COSY showed the presence of two spin systems assigned for H(5')H(6') and between the protons H(3')H(4') as shown in the Figure 4.10.A

Furthermore, ^{13}C-^1H long range couplings of ^2J and ^3J observed in ^{13}C-^1H heteronuclear multiple bond coherence (HMBC) spectrum gave the following linkages (Figure 4.10.B) :
(A) Cross peaks from H-4' to C-2', C-3', C-4a' and C-8a', From H-5' to C-4' and C-4a', from H-8' to C-8a' and C-7'. These correlations indicated that A is 1-benzopyran-2-one, the core of coumarin compounds, which is oxygenated at C-7'. On the other hand, the same correlations could be seen for the selected cross peaks of (B), from H-4 to C-2, C-3, C-4a and C-8a, from H-5 to C-4 and C-4a, from H-8 to C-8a and C-7, in addition, from 6-CH_3 to C-6. These correlations indicated that B is an other benzopyrone, which is tri-substituted with a methoxy group at carbon 6 and a hydroxygroup at C-7 and also an oxygenated carbon at C-3. Thus, the partial structure A and B were connected at the C-7'-O- and C-3-O-positions.
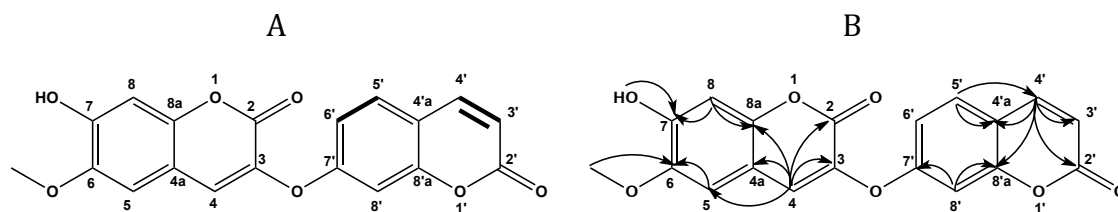
Figure 4.10. A) ^{1}H-^{1}H COSY correlations **B)** selected ^{13}C-^{1}H NMR correlations of **6**

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as [α]$_D$ value of **6** with those data published for Daphnoretin. (Ho *et al.*, 2010).

Daphnoretin has been previously isolated for first time from *Ladino clover* (*Fabaceae*) (Livingston *et al.*, 1964), then later in some genus of the *Thymelaeaceae* family *Daphne*, including *Wikstroemia, Daphnopsis, Edgeworthia, Thymelaea, Diarthron, Dirca, Enkleia* and *Peddiea* (Zhang *et al.*, 2008), However, this the first report of Daphnoretin from the species *Thymelaea lythroides*.

Table 4.10. Comparison of ^{1}H and ^{13}C NMR chemical shifts of **5** with Daphnoretin
(Wing *et al.*, 2010)

N°	5	Daphnoretin
	δ_H	δ_H
1	-	-
2	-	-
3	6.42 (1H, d, *J*= 9.6 Hz)	6.37 (1H, d, *J*= 9.6 Hz)
4	8.11 (1H, d, J= 9.5 Hz)	8.03 (1H, d, *J*= 9.5 Hz)
4a	-	-
5	7.78 (1H, d, J= 8.6 Hz)	7.71 (1H, d, *J*= 8.6 Hz)
6	7,18 (1H, dd, *J*= 8.6, 2.4 Hz)	7.11 (1H, dd, *J*= 8.6; 2.4 Hz)
6-OCH$_3$	3.89 (3H, s)	3.77 (3H, s)
7	-	-
7-OH	10.30 (1H, s)	10.23 (1H, s)
8	7.23 (1H, d, *J*= 2.4 Hz)	7.17 (1H, d, *J*= 2.4 Hz)
8a	-	-
1'	-	-
2'	-	-
3'	-	-
4'	7.91 (1H, s)	7.86 (1H, s)
4a'	-	-
5'	7.25 (1H, s)	7.21 (1H, s)
6'	-	-
7'	-	-
8'	6.96 (1H, s)	6.87 (1H, s)
8a'	-	-

Compound isolated from the plant *Thymelaea lythroides*

Rutarensin (**7**, Known)

Rutarensin	
Synonym(s)	6'-ester with 7-(β-D-glucopyranosyloxy)-6-methoxy-3-[(2-oxo-2H-1-benzopyran-7-yl)oxy]-2H-1-benzopyran-2-one; Rutarensin
CAS Registry Number	119179-04-3
Biological source	*Thymelaea lythroides*
Sample amount	5.7 mg
Physical Description	yellow needle crystal
Molecular Formula	$C_{31}H_{20}O_{16}$
Molecular Weight	658 g/mol
Retention time HPLC	21.51 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	- 2.0 (c 0.017, DMSO)
UV spectrum λ_{max}	201.2, 338.8 and 370 nm

7 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH_2Cl_2/MeOH). The fraction was subjected to a sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent system, then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded colorless powder (yield, 19 mg). It showed UV absorbance at λ_{max} (MeOH) 201.2, 338.8 and 370 nm. Positive and negative ESI-MS of **7** exhibited a prominent peak at m/z 658.9 [M+H]$^+$ (base peak) and m/z 657.1 [M-H]$^-$ (base peak) indicating a molecular weight of 658 g/mol. The prominent fragment ion peak of 352 (m/z 353.2 [M+H]$^+$ and 351.2 [M-H]$^-$) in EI-MS suggested the presence of a daphnoretin in the molecule. The molecular formula of compound **7** was determined to be $C_{31}H_{20}O_{16}$ based on the analysis of the molecular weight and ^{13}C NMR data.

From the ^1H NMR of **7** (Table 4.11) a daphnoretin system and hexopyranose was suggested with the presence of unassigned signals: two methylene (δ_H 2.62 ppm), (δ_H 2.72 ppm) and one methyl group (δ_H 1.33 ppm). Thus, the glucose moiety is bound at CO-1" (δ_C 102.0 ppm) to daphnoretin. A daphnoretin-7-ß-D-glucoside is already known as daphnorin (Tschesche *et al.*, 1963) (Figure 3.11).

The ^{13}C NMR spectrum of **7** showed all signals of daphnorin, whereas the signal for C-6" of glucose was found to be at lower field than expected (+ 3 ppm) and the signal for C-5" of glucose was shifted upfield (- 3 ppm), indicating that an acylation must be present at position C-6" of glucose. Further possibilities of acylation at C-2", C-3", and C-4" of glucose were excluded by comparison with data given in literature (Kashiwada *et al.*, 1986; Adegawa *et al.*, 1986).
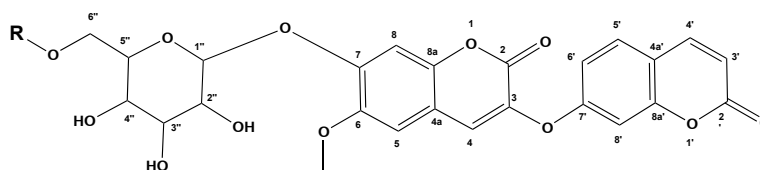
Figure 4.11. Structure of Daphnorin (R= H)

Table 4.11. Comparison of ^{1}H and ^{13}C NMR chemical shifts of **7** with Rutarensin

N°	7		Rutarensin	
	δ_H	δ_C	δ_H	δ_C
1	-	-	-	-
2	-	161.3	-	159.6
3	-	139.3	-	137.3
4	7.74 (1H, s)	131.3	7.87 (1H, s)	130.1
4a	-	114.5	-	112.4
5	7.22 (1H, s)	110.8	7.28 (1H, s)	109.8
6	-	148.4	-	146.5
6-OCH₃	3.90 (3H, s)	57.2	3.80 (3H, s)	56.1
7	-	150.8	-	149.0
8	7.20 (1H, s)	105.5	7.23 (1H, s)	103.1
8a	-	148.8	-	146.9
1'	-	-	-	-
2'	-	162.8	-	160.1
3'	6.32 (1H, d, *J*= 9.54 Hz)	116.4	6.39 (1H, d, *J*= 9.8 Hz)	114.1
4'	7.91 (1H, d, *J*= 9.6 Hz)	145.4	8.05 (1H, d, *J*= 9.8 Hz)	144.2
4a'	-	114.5	-	114.7
5'	7.61 (1H, d, *J*= 8.52 Hz)	131.0	7.72 (1H, d, *J*= 8.7 Hz)	129.9
6'	7.07 (1H, dd, *J*= 2.4 Hz)	115.2	7.15 (1H, dd, *J*= 8.7; 2.5 Hz)	113.8
7'	-	159.2	-	156.9
8'	7.04 (1H, d, *J*=2.34 Hz)	105.8	7.26 (1H, d, *J*= 2.5 Hz)	104.6
8a'	-	156.8	-	155.2
1"	5.05 (1H, d, *J*= 7.62 Hz)	102.0	5.16 (1H, d, *J*= 6.9 Hz)	99.6
2"	3.56 (1H, m)	74.8	3.10-3.50 (1H, m)	73.0
3"	3.51 (1H, m)	77.9	3.10-3.50 (1H, m)	76.5
4"	3.51 (1H, m)	71.8	3.10-3.50 (1H, m)	69.6
5"	3.76 (1H, m)	75.8	3.75 (1H, m)	73.9
6"	4.51 (1H, dd, *J*=2.1, 11.82 Hz)	64.7	4.25 (1H, dd, *J*= 11.5; 2.0 Hz)	62.8
	4.20 (1H, dd, *J*=7.32, 11.94 Hz)	64.7	4.03 (1H, dd, *J*= 11.5; 6.5 Hz)	62.8
1'''	-	172.6	-	170.8
2'''ₐ	2.61 (2H, s)	45.9	1.93 (1H, AB syst. *J*= 15.3 Hz)	47.0
2'''_b		45.9	2.20 (1H, AB syst. *J*= 15.3 Hz)	47.0
3'''	-	70.9	-	68.8
3'''-CH₃	1.32 (3H, s)	27.9	1.06 (1H, s)	28.3
4'''ₐ	2.75 (2H, m)	46.4	2.30 (1H, AB syst. *J*= 13.4 Hz)	47.0
4'''_b	2.75 (2H, m)	46.4	2.38 (1H, AB syst. *J*= 13.4 Hz)	47.0
5'''	-	177.7	-	176.0

Moreover, the ^{13}C NMR spectrum of **7** showed six further signals, which were characterized by DEPT-experiments as two carbonyl-carbons (δ_C 172.6 and 177.7 ppm) one quaternary carbon (δ_C 70.9 ppm), two methylene-carbons (δ_C 45.9 and 46.4 ppm) and one methyl group (δ_C 27.9 ppm). These data indicated that the glucose moieties is connected to a partial structure A at CO-6".

The COSY NMR spectrum showed the same correlations as Daphnoretin and those of glucose. [13]C-^{1}H long range couplings of ^2J and ^3J observed in a [13]C-^{1}H heteronuclear multiple bond coherence (HMBC) spectrum gave the same linkages as Daphnoretin as well as those of the glucose. Furthermore, the partial structure A showed the selected following linkages (Figure 4.12): From H-2''' to C-1''', C-3''', C-4''' and 3'''-CH₃; from H-4''' to C-2''', C-3''', 3'''-CH₃ and C-5''' and from the protons of 3'''-CH₃ to C-3''', C-2''' and C-4'''. From these results, it

was concluded that the substituent at C-6″ of the glucose was 3-hydroxy-3-methylglutaric acid.
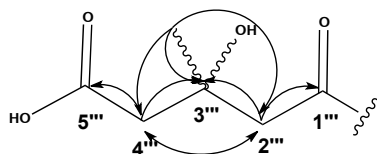
Figure 4.12. Selected correlations of partial structure A

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as [α]$_D$ value of **7** with those published for rutarensin (Fischer *et al.*, 1988).

Rutarensin was described for the first time as a constituent of cell cultures of *Ruta chalepensis L.* (*Rutaceae*), then from of *Edgeworthia chrysantha* (*Thymelaeacea*). Further acylglycosides esterified at C-6 of glucose with 3-hydroxy-3-methylglutaric acid are palustroside from *Ledurn palustre L.*, the tubeimosides from *Bolbostemma puniculatum*, one pyridoxin glucoside from *Pisum satioum L.* and the betacyanin glucosides from *Phyllocactus hybridus*, *Celosia cristata* and *Iresine herbstii* (Centrospermae). (Kreher *et al.*, 1990). This is the first report of Rutarensin from *Thymelaea lythroides*.

Compound isolated from the plant *Thymelaea lythroides*

trans-Tiliroside (**8**, Known)

trans-**Tiliroside**	
Synonym(s)	Kaempferol-3-O-β-D-(6"-O-(E)-p-coumaroyl)glucopyranoside; trans-Tiliroside
CAS Registry Number	20316-62-5
Biological source	*Thymelaea lythroides*
Sample amount	20 mg
Physical Description	yellowish powder
Molecular Formula	$C_{30}H_{26}O_{13}$
Molecular Weight	594 g/mol
Retention time HPLC	24.0 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	- 65 (C 1.0, $CHCl_3$)
UV spectrum λ_{max}	267.0 and 315.0 nm
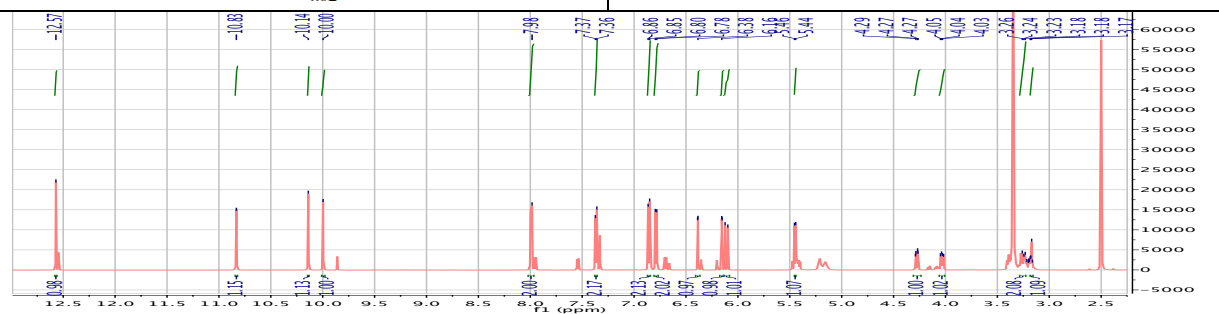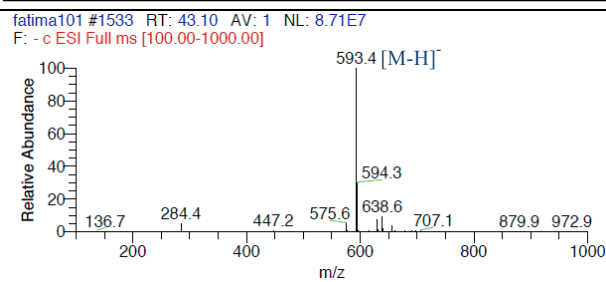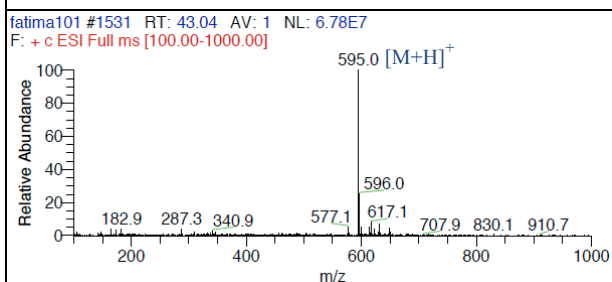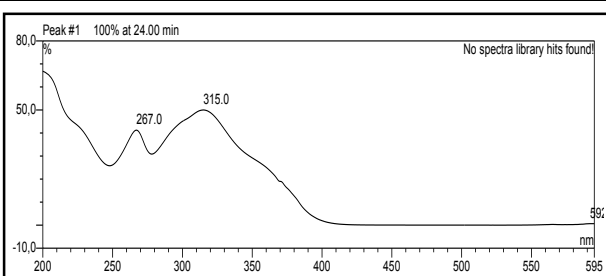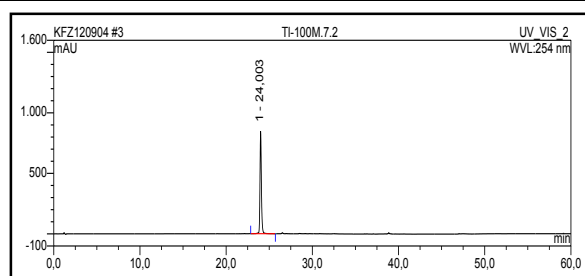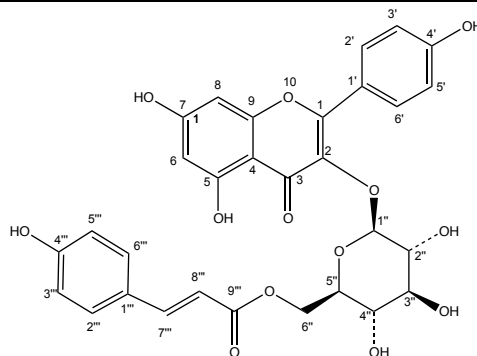
8 was isolated from *Thymelaea lythroides* extracted with EtOAc. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction was chromatographed over silica gel F254 using gradient elution (*n*-hexane/EtOAc/CH$_2$Cl$_2$/MeOH). The fraction was directly purified by semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H$_2$O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min 100% MeOH; 45 min, 100 % MeOH, which afforded colorless powder (yield, 19 mg). It showed UV absorbance at λ_{max} (MeOH) 267.0 and 315.0 nm. Positive and negative ESI-MS of **8** exhibited a prominent peak at m/z 695.0 [M+H]$^+$ (base peak) and m/z 695.3 [M-H]$^-$ (base peak) indicating a molecular weight of 694 g/mol. The molecular formula of compound **8** was determined to be C$_{30}$H$_{26}$O$_{13}$ based on the analysis of the molecular weight and ^{13}C NMR data.

The ^{1}H NMR of **7** (Table 4.12) displayed 26 proton signals, a kaempferol and hexopyranose was suggested with the presence of coumarin acid. The ^{13}C NMR of **7** showed the presence of 30 signals carbons (Table 4.12).

The ^{1}H-^{1}H COSY (Figure 4.13.A) spectra showed 6 spin systems, H(2")H(3"), H(5")H(6"), H(2''')H(3'''), H(5''')H(6'''), H(7''')H(8''') and a continuous spin system H(1")→(6").

The ^{1}H-^{13}C HMBC exhibited the selected following linkage: from H-8 to C-4 and C-9, from H-7-OH to C-7, C-6 and C-8, from H-4'-OH to C-4', C-5' and C-3', from H-2' to C-1' and C-1, from H-6' to C-1' and C-1, from H-4'''-OH to C-4''', C-3''' and C-5", from H-7''' to C-1''', C-2''' and C-6''', from H-8''' to C-1''' and from H-6" to C-9''' as shown in Figure 4.13. B.
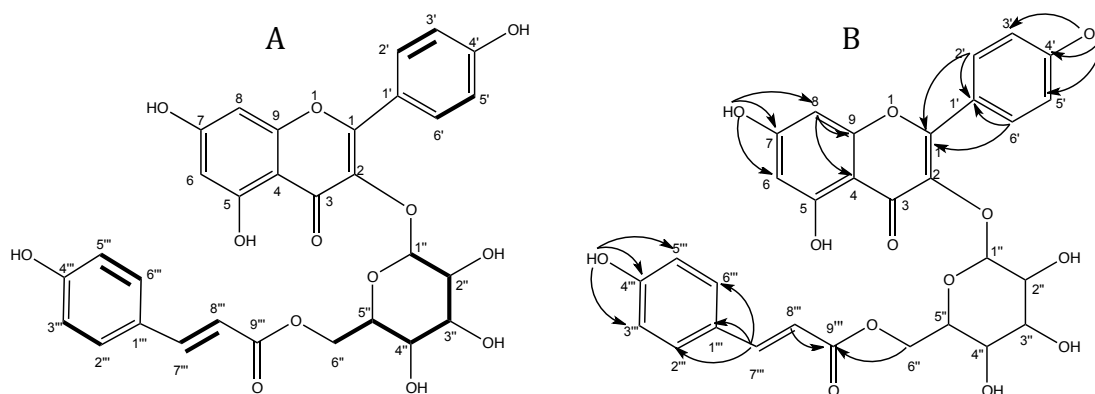
Figure 4.13. A) ^{1}H-^{1}H COSY correlations. **B)** Selected ^{1}H-^{13}C HMBC correlation of **8**

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as $[\alpha]_D$ value of **7** with those published for *trans*-Tiliroside (Budzianowski *et al.*, 1995).

trans-Tiliroside or kaempferol-3-*O*-β-D-(6''-*E*-p-coumaroyl)-glucopyranoside is an acylated glucoside of kaempferol, first isolated from *Rosa canina* and *Tilia argentea* and then from several *Tiliaceae* (Budzianowski *et al.*, 1995), later from *Daphne genkwa* (Song *et al.*, 2009), and from *Edgeworthia gardneri* (Xu *et al.*, 2012) of the *Thymeleaceae* family. This is the first report of *trans*-Tiliroside from *Thymelaea lythroides*.

Table 4.12. Comparison of ^{1}H and ^{13}C NMR chemical shifts of **8** with *trans*-Tiliroside

N°	**8**		*trans*-Tiliroside	
	δ_H	δ_C	δ_H	δ_C
1	-	-	-	-
2	-	156.4		156.4
3	-	133.0	-	133.0
4	-	177.3	-	177.3
5	-	161.1	-	161.1
5-OH	12.57 (1H, s)	-	12.60 (1H, s)	-
6	6.15 (1H, d, *J*= 2.06 Hz)	98.7	6.18 (1H, d, *J*= 2.1 Hz)	98.7
7	-	164.1	-	164.1
7-OH	10.83 (1H, s)	-	10.87 (1H, s)	-
8	6.39 (1H, d, *J*= 2.06 Hz)	93.6	6.41 (1H, d, *J*= 2.1 Hz)	93.6
9	-	156.3	-	156.3
10	-	103.8	-	103.8
1'	-	120.7	-	120.7
2'	7.98 (1H, d, *J*= 8.8 Hz)	130.1	8.02 (1H, d, *J*= 8.9 Hz)	130.0
3'	6.86 (1H, d, *J*= 8.8 Hz)	115.7	6.88 (1H, d, *J*= 8.9 Hz)	115.7
4'	-	159.9	-	159.9
4'-OH	10.14 (1H, s)	-	10.18 (1H, s)	-
5'	6.86 (1H, d, *J*= 8.8 Hz)	115.7	6.88 (1H, d, *J*= 8.9 Hz)	115.7
6'	7.98 (1H, d, *J*= 8.8 Hz)	130.1	8.02 (1H, d, *J*= 8.9 Hz)	130.1
1''	5.45 (1H, d, *J*= 7.5 Hz)	100.9	5.48 (1H, d, *J*= 7.5 Hz)	100.9
2''	3.25 (1H, m)	74.2	3.20-3.35 (1H, m)	74.2
2''-OH	5.16 (1H, s)		5.23 (1H, s)	
3''	3.25 (1H, m)	76.2	3.20-3.35 (1H, m)	76.2
3''-OH	5.21 (1H, s)		5.27 (1H, s)	
4''	3.25 (1H, m)	69.9	3.20-3.35 (1H, m)	69.9
4''-OH	5.43 (1H, s)		5.50 (1H, s)	
5''	3.25 (1H, m)	74.1	3.20-3.35 (1H, m)	74.0
6''$_a$	4.31 (1H, dd, *J*= 2.2, 11.9 Hz)	62.9	4.30 (1H, dd, *J*= 2, 11.7 Hz)	62.9
6''$_b$	4.03 (1H, dd, *J*= 6.4, 11.8 Hz)		4.06 (1H, dd, *J*= 6.3, 11.7 Hz)	
1'''	-	124.9	-	124.8
2'''	7.37 (1H, d, *J*= 8.5 Hz)	130.7	7.39 (1H, d, *J*= 8.8 Hz)	130.7
3'''	6.81 (1H, d, *J*= 8.6 Hz)	115.0	6.81 (1H, d, *J*= 8.8 Hz)	115.0
4'''	-	159.7	-	159.7
4'''-OH	10.00 (1H, s)	-	10.06 (1H, s)	-
5'''	6.81 (1H, d, *J*= 8.6 Hz)	115.0	6.81 (1H, d, *J*= 8.8 Hz)	115.0
6'''	7.37 (1H, d, *J*= 8.5 Hz)	130.7	7.39 (1H, d, *J*= 8.8 Hz)	130.7
7'''	7.33 (1H, d, *J*= 15 Hz)	144.5	7.37 (1H, d, *J*= 16 Hz)	144.5
8'''	6.11 (1H, d, *J*= 15 Hz)	113.6	6.14 (1H, d, *J*= 16 Hz)	113.6
9'''	-	166.1	-	166.1

4.3. Bioactivity test results for compounds isolated from the plant *Thymelaea lythroides*

The isolated compounds were subjected to cytotoxicity and protein kinase bioassays. Some of the isolated pure compounds were also subjected to Hsp 90 inhibitor assays. The results are shown in Tables 4.13 and Table 4.14.

Table 4.13. Cytotoxicity test results for the compounds isolated from *Thymelaea lythroides*

Nr.	Compounds tested	L5178Y growth in %* (Conc. 10 µg/mL)
-	Control	0
3	(+)-Dapheolone	83.3
4	(-)-Wikstromol	7.6
5	δ-Sesamin	26.7
6	Daphnoretin	-10.7
7	Rutarensin	6
8	*trans*-Tiliroside	28.7

*Data provided by Prof. W. E. G. Müller, Mainz

The coumarin (+)-Daphneolone showed moderate activity against L5178Y cell line, while no activity was detected for the other compounds.

Table 4.14. Protein kinase assay results for the compounds isolated from *Thymelaea lythroides*

Compound tested (Conc. 1µg/mL)	Activity on various protein kinases based on IC50 [g/mL]*															
	AKT1	ALK	ARK 5	Aurora-B	AXL	FAK	IGF 1-R	MEK 1 wt	MET wt	NEK 2	NEK 6	PIM 1	PLK 1	PRK 1	SRC	VEGF-R2
Bassiatin	0	0	0	M	0	M	M	0	0	0	0	0	0	0	0	M
(+)-Dapheolone	0	0	0	M	0	0	0	0	0	0	0	0	0	0	0	0
(-)-Wikstromol	0	0	0	M	0	M	M	0	0	0	0	0	0	0	0	0
δ-Sesamin	0	0	0	M	0	M	M	0	0	0	0	0	0	0	0	0
Daphnoretin	0	0	0	M	0	M	M	0	0	0	0	0	0	0	0	M
Rutarensin	0	0	0	A	M	M	A	0	0	M	M	0	0	0	0	A
tran-Tiliroside	0	A	0	A	M	M	M	0	M	M	0	S	0	0	0	A

* Data provided by ProQinase, Freiburg.

S: strongly active, A: active, M: moderately active, 0: not active

The isolated compounds of *Thymelaea lythroides* were subjected to biochemical protein kinase activity assays using 16 different human protein kinases. Only compounds Tiliroside and Rutarensin inhibited several of the tested kinases.

The IC_{50} values observed for both compounds were in the low micromolar range against some protein kinases which inhibition is known to confer antitumoral effects.

The Hsp 90 chaperone showed no activity for all compounds isolated from *Thymelaea lythroides*.

4.4. Compounds isolated from the endophytic fungus *Chaetomium aureum*

Five endophytic fungi were isolated from the Moroccan medicinal plant *Thymelaea lythroides*, namely *Alternaria sp*., *Chaetomium aureus*, *Epicoccum nigrum*, *Cladosporium sp*. and *Pleospora sp*. Comparison of the HPLC chromatograms of the EtOAc extracts of all pure strain cultures and the preliminary biological screening assays, i.e. antibacterial, antifungal, and cytotoxicity showed that *Chaetomium aureum* is the most stable and active strain (Table 4.15).

The fungal strain of *Chaetomium aureum* (EMBL accession number HF546136) (Kabbaj *et al*., 2012) was identified based on analysis of the DNA sequences of internal transcribed spacer regions of its ribosomal RNA gene. A voucher strain (strain designation MM10S2-1) is kept in the Institute of Pharmaceutical Biology and Biotechnology, Düsseldorf, Germany.
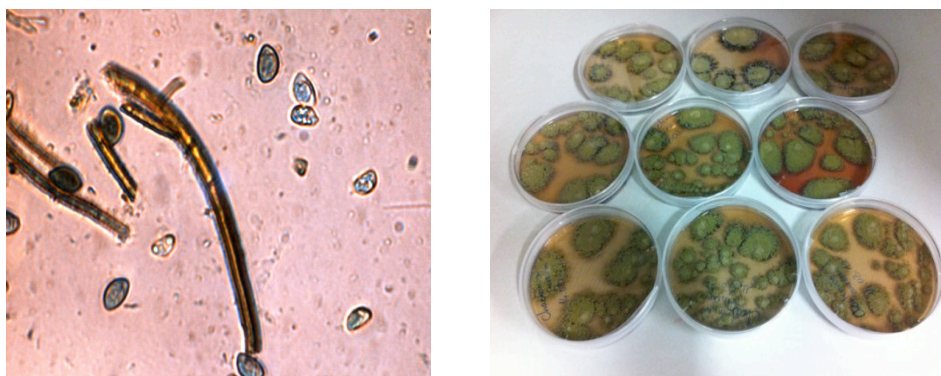
Figure 4.14. Photos of *Chaetomium aureum*

The pure fungal strain was cultivated on 11 media medium. Interestingly, chemical screening studies indicated a clear difference between *C. aureum* extracts obtained from liquid Wickerham medium, rice media culture, pulses media cultures and legumes media cultures. Comparison of the HPLC chromatograms of the EtOAc extracts of all cultures showed a different chemical pattern. While the rice culture extract showed (+)-sclerotiorin (9) as major substance, two unstable main components were detected in liquid cultures, with no traces of (+)-sclerotiorin (Figure 4.15). From the 10 others media, pulses media cultures showed the presence of the same two major compounds as in liquid cultures with the presence of (+)-sclerotiorin as minor compound, in legumes media steroids have been detected as major compounds.

In this part the investigation results on the natural products produced by *Chaetomium aureum* when grown on solid rice medium are presented.

Table 4.15. Preliminary biological screening assays of the five pure strains

Fungal strain	L5178Y	MRSA	*Strep. pneumonia*	*Entero. Faecalis*
Epicocom nigrum	100,3%	>62,5µg/ml	>31,25µg/ml	>62,5µg/ml
Alternaria sp.	95,5%	>31,25µg/ml	>62,5µg/ml	>31,25µg/ml
Cladosporium sp	25,2%	n.a	n.a	n.a
Chaetomium aureum	99,7%	>62,5µg/ml	> 62,5µg/ml	>62,5µg/ml
Pleospora sp.	101,3%	n.a	>125µg/ml	n.a
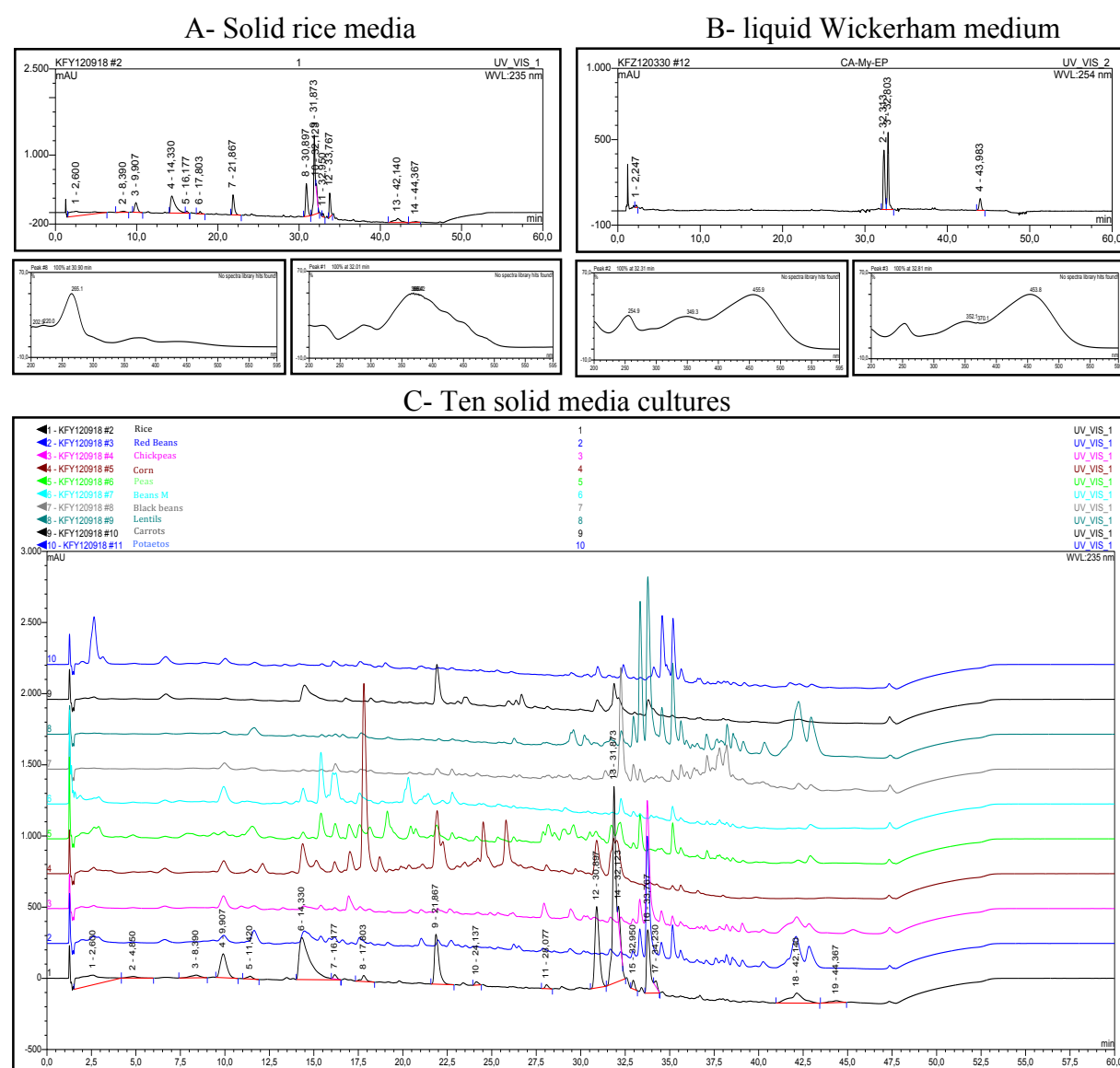
Figure 4.15. Comparison of the 11 media cultures of *Chaetomium aureum.* A- Solid rice media. B- liquid Wickerham medium. C- Ten solid media cultures

Compound isolated from the endophytic fungus *Chaetomium aureum*

(+)-Sclerotiorin (**9**, Known)

<table>
<tr><td colspan="2" align="center">**(+)-Sclerotiorin**</td></tr>
<tr><td>Synonym(s)</td><td>6H-2-Benzopyran-6,8(7H)-dione, 5-chloro-3-(3,5-dimethyl-1,3-heptadienyl)-7-hydroxy-7-methyl-, acetate (8CI)</td></tr>
<tr><td>CAS Registry Number</td><td>549-23-5</td></tr>
<tr><td>Biological source</td><td>*Chaetomium aureum*</td></tr>
<tr><td>Sample amount</td><td>105 mg</td></tr>
<tr><td>Physical Description</td><td>yellow crystals</td></tr>
<tr><td>Molecular Formula</td><td>$C_{21}H_{23}ClO_5$</td></tr>
<tr><td>Molecular Weight</td><td>390 g/mol</td></tr>
<tr><td>Retention time HPLC</td><td>32,01 min (standard gradient)</td></tr>
<tr><td>Optical rotation $[\alpha]_D^{26}$</td><td>+515 (c 1.0, CHCl$_3$)</td></tr>
<tr><td>UV spectrum λ_{max}</td><td>220, 288 and 370 nm</td></tr>
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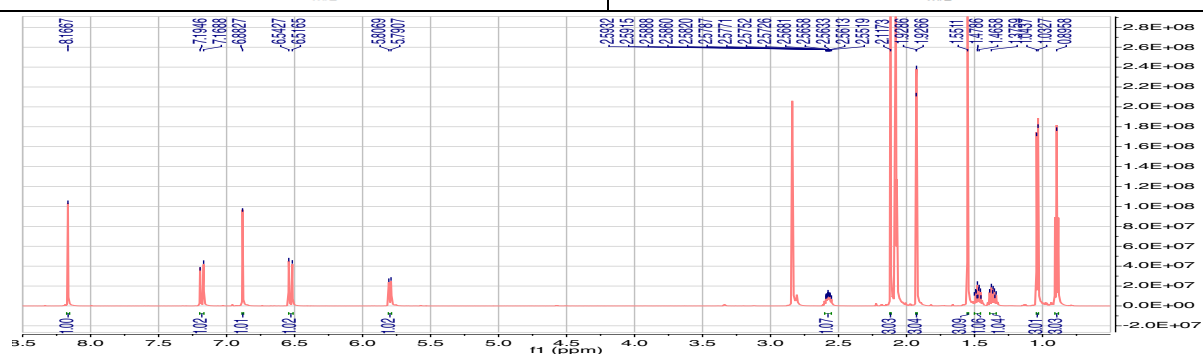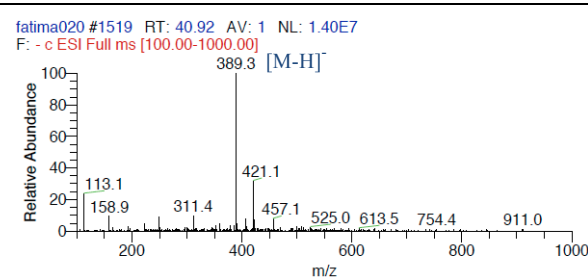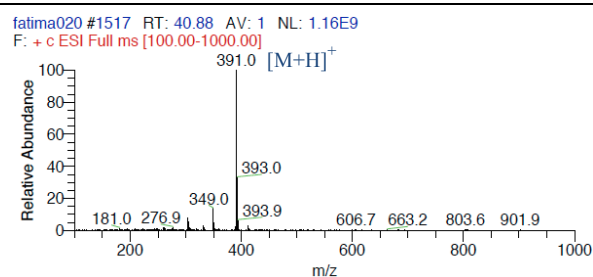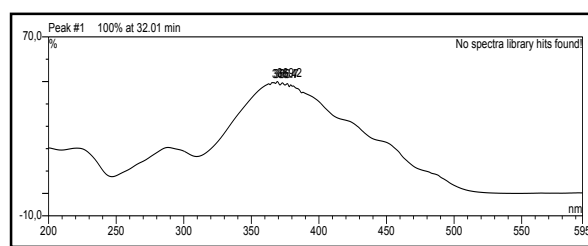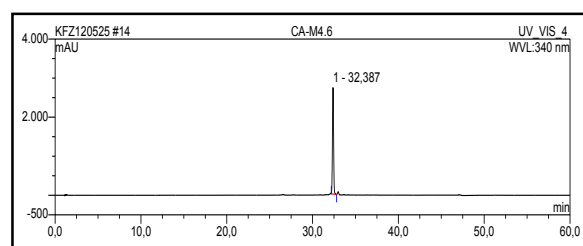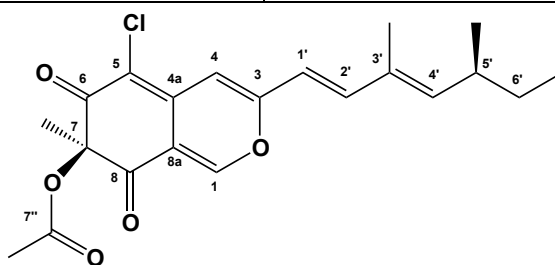
9 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive Sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated twice) then purified by silica gel F254 (Merck, Darmstadt, Germany) using gradient elution (*n*-hexane/EtOAc/CH_2Cl_2/MeOH), which afforded yellow needles (yield, 105 mg). It showed UV absorbance at λ_{max} (MeOH) 220, 288 and 370 nm. Positive and negative ESI-MS showed molecular ion peaks at m/z 391, $[M+H]^+$ (base peak) and m/z 389, $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 390 g/mol.

The ^{1}H NMR (Table 4.16) showed five methyl group, which appeared at (δ_H 0.89, 1.03, 1.55, 1.92 and 2.11 ppm), assigned to 6'-CH_3, 5'-CH_3, 7-CH_3, 3'-CH_3, and 7"-CH_3 respectively; one methylene group, which appeared as AB spin system at (δ_H 1.36 and 1.47 ppm), assigned to 6'- CH_2; and six methine proton at (δ_H 2.57, 5.80, 6.53, 6.88, 7.18 and 8.16 ppm), assigned to 5'-CH, 4'-CH, 1'-CH, 4-CH, 2'-CH and 1-CH.

The ^{13}C NMR showed the presence of 21 resolved signals, which were assigned to five methyl carbons, one methylene carbon, six sp^2 methine carbons, four sp^2 quaternary carbons, three oxygenated *sp^2* quaternary carbons and three carbonyl carbons.

Analyses of ^{1}H-^{1}H NMR COSY revealed the presence of two spin systems, as showed in Figure 4.16.A.

Furthermore, ^{13}C-^{1}H long range couplings of ^2J and ^3J observed in the ^{13}C-^{1}H heteronuclear multiple bond coherence (HMBC) spectrum gave the following linkages (Figure 4.16.B): from H-1 (δ_H 8.14 ppm) to C-3 (δ_H 159 ppm), C-4a (δ_H 139.8 ppm), C-8 (δ_H 192 ppm) and C-8a (δ_H 115.5 ppm); from H-4 to C-3, C-1' (δ_H 117 ppm), C-4a, and C-5 (δ_C 110 ppm); from H_3-7-CH_3 (δ_C 1.52 ppm) to C-7 (δ_C 85.4 ppm), C-6 (δ_C 186,2 ppm) and C-8; from H_3-7"-CH_3 to C-7" (δ_C 170,1 ppm); from H-1' (δ_C 6.50 ppm) to C-2' (δ_C 142.8 ppm), C-3' (δ_C 133.5 ppm), C-3 and C-4 (δ_C 106.7 ppm); from H-2' (δ_H 7.16 ppm) to C- 1', C-3', C-4' (δ_C 148.3 ppm), C-3'-CH_3 (δ_C 12.1 ppm) and C-3; from H_3-3'-CH_3 (δ_H 1.90 ppm) to C-2', C-4' and C-3'; from H-4' (δ_H 5.77 ppm) to C-2', C-3'-CH_3, C-5' (δ_C 35.4 ppm) and C-6' (δ_C 30.4 ppm); from H_3-5'-CH_3 (δ_H 1.01) to C-6', C-5' and C-4'; from H_2-6' (δ_H 1.33, 1.44) to C-4', C-5', C-5'-CH_3 (δ_C

20.1 ppm) and C-6'-CH$_3$ (δ_C 11.82 ppm) and from H$_3$-6'-CH$_3$ (δ_H 0.86 ppm) to C-5', C-5'-CH$_3$ and C-6'. The connection of the acetyl group was confirmed by the chemical shift of C-7" (δ_C 170,1 ppm) assigned to an ester bond and the long-rang coupling of J^4 observed from H$_3$-7"-CH$_3$ to C-7 in ^{13}C-^{1}H NMR spectrum (Figure 4.16.B). Furthermore, the chemical shift of C-1 (δ_C 154.1 ppm) and C-3 (δ_C 159.6 ppm) indicated the presence of an oxygenated carbon between both carbons and the correlation between the H-1 and the C-3 confirms ring closure. Thus, these data indicated that **9** was found to be 6H-2-Benzopyran-6,8(7H)-dione-5-chloro-3-(3,5-dimethyl-1,3-heptadienyl)-7-hydroxy-7-methyl-, acetate (8CI). On the basis of the coupling constants of H-1' with H-2' and the chemical shift of the neighbor carbon of C-5', the configurations of these two double bonds were both determined to be E.

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as [α]$_D$ value of **9** with those data previously published for (+)-sclerotiorin (Natsume *et al.*, 1988).

The compound was previously reported from *Penicillium sclerotiorum*, *P. multicolor*, *P. implicatum*, *P. hirayamae*, *P. frequentans*, *P.faveolata*, lichen mycobionts of *Pyrenula japonica*, ascommycete *T. luteus* and from unidentified fungus 98F134 (Gregory and Turner, 1963; Takenaka *et al.*, 1963; Takenaka *et al.*, 2000; Periyasamy *et al.*, 2012; Li *et al.*, 2010; Chidananda *et al.*, 2006). This is the first finding of this compound from *Chaetomium aureum.*
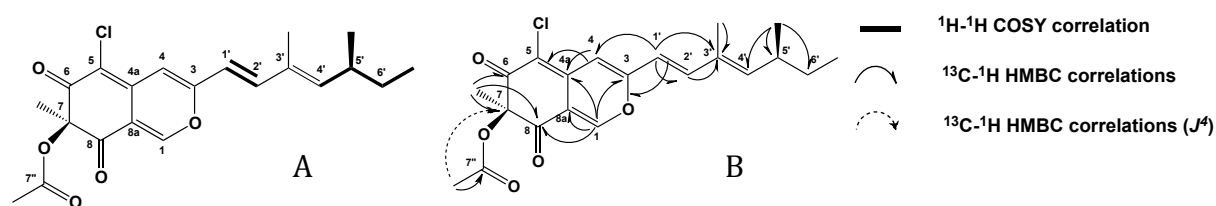
Figure 4.16. A) ^{1}H-^{1}H COSY correlations of **1 B)** selected ^{13}C-^{1}H HMBC correlations

Compound isolated from the endophytic fungus *Chaetomium aureum*

Isochromophilone VII (**10**, Known)

| **Isochromophilone VII**	
Synonym(s)	6,8(2H,7H)-Isoquinolinedione, 7-(acetyloxy)-5-chloro-3-(3,5-dimethyl-1,3-heptadienyl)-7-methyl- (9CI);
CAS Registry Number	175448-30-3
Biological source	*Chaetomium aureum*
Sample amount	1 mg
Physical Description	yellow needles
Molecular Formula	$C_{21}H_{25}ClO_6$
Molecular Weight	408 g/mol
Retention time HPLC	32.40 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	+563 (c 1.0, MeOH)
UV spectrum λ_{max}	370.7, 370.3 and 374.2 nm

10 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated twice), then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH, which afforded yellow needles (yield, 1 mg). It showed UV absorbance at λ_{max} (MeOH) 370.7, 370.3 and 374.2 nm. Positive and negative ESI-MS showed molecular ion peaks at m/z 409.1 $[M+H]^+$ (base peak) and m/z 407.2 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 408 g/mol. The molecular formula of **10** was determined to be $C_{21}H_{25}O_6C1$.

The ^{1}H NMR spectra (MeOD) (Table 4.16) showed 24 proton signals. To fulfill the molecular formula, the presence of one hydroxyl group was suggested.

From the ^{1}H-^{1}H COSY spectrum, three spin system, H-4'→H-6'-CH_3, between the two protons of H_α-1-H_β-1 and between H-1' and H-2' were determined (Figure 4.17).
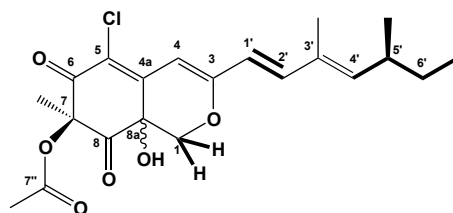
Figure 4.17. ^{1}H-^{1}H COSY correlations of **10**

^{13}C-^{1}H long range couplings of ^2J and ^3J observed in the HMBC spectrum showed cross peaks from H-3'-CH_3 (δ_H 1.82 ppm) to C-2' (δ_C 142.9 ppm), C-3' (δ_C 132.2 ppm) and C-4' (δ_C 148.1 ppm), forming the 3,5-dimethyl-1,3-heptadienyl moiety containing the above two proton sequences. The cross peaks from H-2' (δ_H 7.07 ppm), H-1' (δ_H 6.04 ppm) and H-4 (δ_H 6.18 ppm) to C-3 (δ_C 162.2 ppm), from H-4 to C-l' (δ_C 118.2 ppm) and from H-l' to C-4 revealed the presence of an extended conjugation system.

Furthermore, the cross peaks from H2-l (δ_H 4.02, 4.74 ppm) to C-3, C-4a (δ_C 142.8 ppm) and

C-8a (δ_C 67.9 ppm), and from H-4 to C-3 and C-8a, and ^{13}C shifts of C-l (δ_C 70.0 ppm) and C-3 revealed a pyran ring. The HMBC NMR showed correlations from H-7"-CH3 (δ_C 2.12 ppm) to C-7" (δ_C 169.7 ppm). The presence of another 6-membered ring was suggested because of the cross peaks from C-7-CH3 (δ_C 1.83 ppm) to C-6 (δ_C 185.4 ppm), C-7 (δ_C 83.4 ppm) and C-8. (δ_C 197.1 ppm), from H-l to C-8, and from H-4 to C-5 (δ_C 121.0 ppm) in the HMBC spectrum and the degree of unsaturation. The UV-spectrum also revealed that the structure contained a continous conjugated ketone. Finally the remaining hydroxyl group, acetyl group and Cl should be attached to C-8a, C-7 and C-5, respectively, due to the ^{13}C chemical shifts. Taken together, the structure was comparable with previously published data for isochromophilone VII isolated from *Penicillium sp.* FO-4164 (Yang *et al.*, 1995).

Isochromophilone VII was isolated only from *Penicillium sp.* FO-4164 (Yang *et al.*, 1995), this is the first report of Isochromophilone VII from *Chaetomiun aureum.*

Compound isolated from the endophytic fungus *Chaetomium aureum*

Sclerotioramin (**11**, Known)

| Sclerotioramin	
Synonym(s)	1H-2-Benzopyran-6,8(7H,8aH)-dione, 7-(acetyloxy)-5-chloro-3-(3,5-dimethyl-1,3-heptadienyl)-8a-hydroxy-7-methyl- (9CI)
CAS Registry Number	34695-81-3
Biological source	*Chaetomium aureum*
Sample amount	7 mg
Physical Description	red needles
Molecular Formula	$C_{21}H_{24}ClNO_4$
Molecular Weight	389 g/mol
Retention time HPLC	30.35 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	+505 (c 1.0, MeOH)
UV spectrum λ_{max}	203.2, 352.1 and 370.6 nm
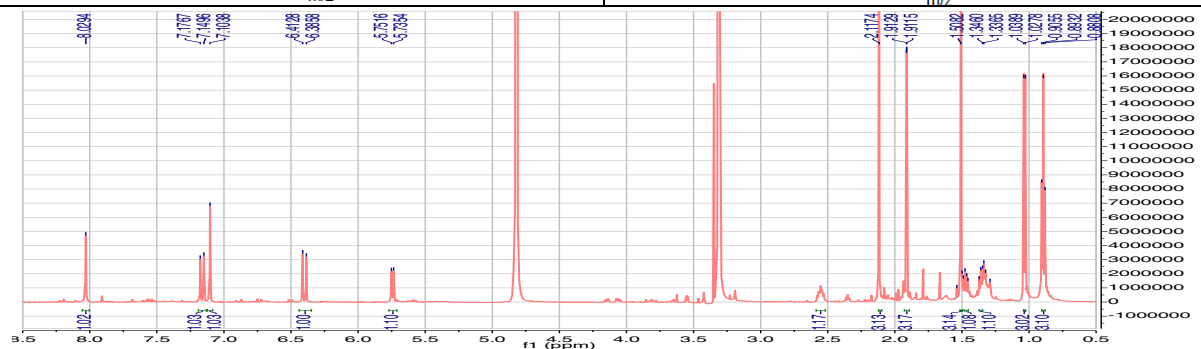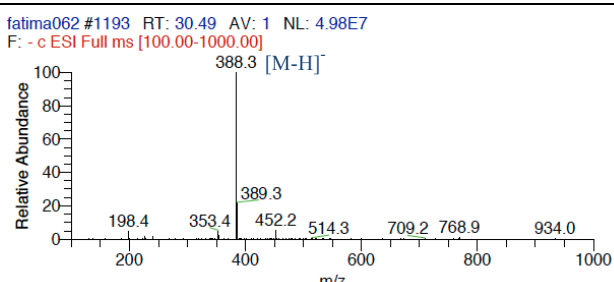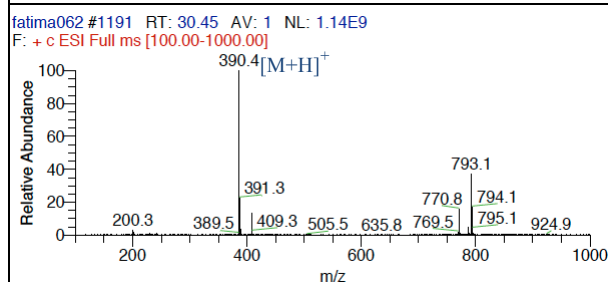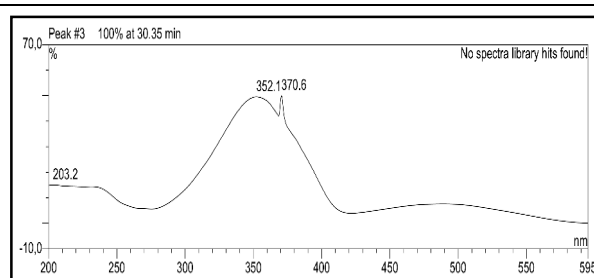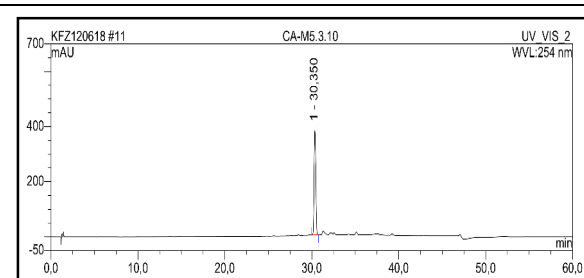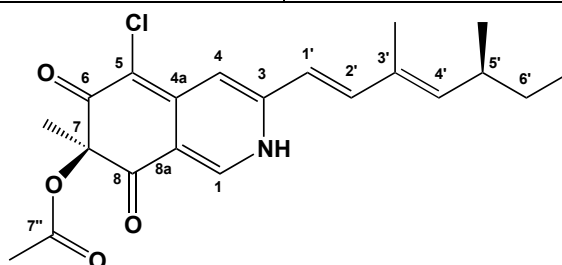
11 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive Sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated three times) then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH, which afforded red needles (yield, 7 mg). It showed UV absorbance at λ_{max} (MeOH) 203.2, 352.1 and 370.6 nm. Positive and negative ESI-MS showed molecular ion peaks at m/z 390.4 $[M+H]^+$ (base peak) and m/z 388.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 389 g/mol. The molecular formula of **11** was assigned as $C_{21}H_{24}ClNO_4$. To achieve the molecular formula, the presence of one secondary amine group was suggested.

The ^{1}H NMR spectra (MeOD) (Table 4.16) showed 23 protons signals, which were almost identical to sclerotiorin except the chemical shift of 2 signals at (δ_H 8.03 ppm) and (δ_H 7.10 ppm), assigned to H-1 and H-4. In the ^{13}C NMR showed the presence of 21 resolved signals, which were almost the same as sclerotiorin except the chemical shift of 3 signals at (δ_C 139.6 ppm), (δ_C 144.9 ppm) and (δ_C 112.5 ppm), assigned to C-1, C-3 and C-4.

The 2D NMR, ^{1}H-^{1}H NMR COSY and ^{13}C-^{1}H NMR HMBC showed the same correlation as sclerotiorin. The ^{15}N-^{1}H NMR HMBC showed the cross peaks from one nitrogen signal, N (δ_N -351.7 ppm) to H-1 (δ_H 8.03 ppm), H-4 (δ_H 7.10 ppm) and H-1' (δ_H 6.40 pmm). Thus, these data indicated the presence of nitrogen in the position **2**.

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as $[\alpha]_D$ value of **11** with the data previously published for (+)-sclerotioramin (Wang *et al.*, 2010).

Sclerotioramin is a natural product, which has been isolated, together with sclerotiorin, from the mycelium of *P. multicolour* and *P. sclerotiorum* grown under special conditions (Page *et al.*, 1957). The compound was previously also reported from *P. citreonigrum* (Wang *et al.*, 2010) from *Diaporthe sp* (Zang *et al.*, 2012), from *Penicillium implicatum* (Yuzuru *et al.*, 1959). But this is the first report of this compound from *Chaetomium aureum*.

Compound isolated from the endophytic fungus *Chaetomium aureum*

Sclerotioramin (**12**, Known)

Isochromphilone VI	
Synonym(s)	6,8(2H,7H)-Isoquinolinedione, 7-(acetyloxy)-5-chloro-3-[(1E,3E,5S)-3,5-dimethyl-1,3-heptadienyl]-2-(2-hydroxyethyl)-7-methyl-, (7R)- (9CI)
CAS Registry Number	167173-91-3
Biological source	*Chaetomium aureum*
Sample amount	5 mg
Physical Description	red needles
Molecular Formula	$C_{23}H_{28}ClNO_5$
Molecular Weight	433 g/mol
Retention time HPLC	28.2 min (standard gradient)
Optical rotation $[\alpha]_D^{26}$	+489 (c 1.0, MeOH)
UV spectrum λ_{max}	236.9, 306.9 and 371.0 nm
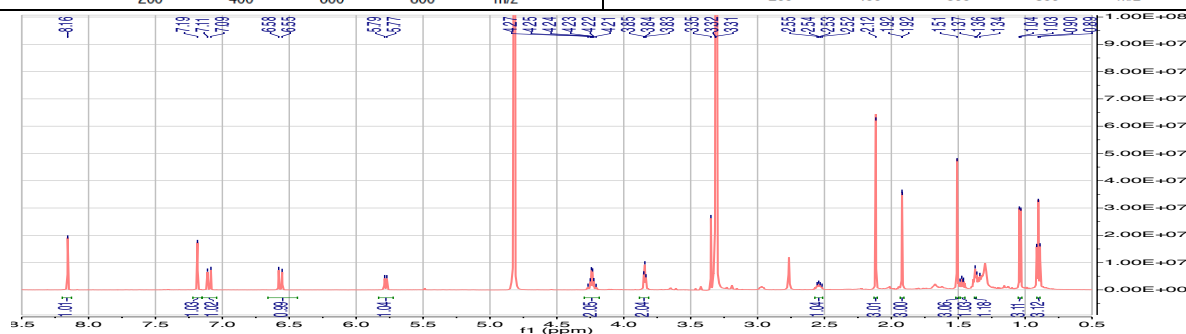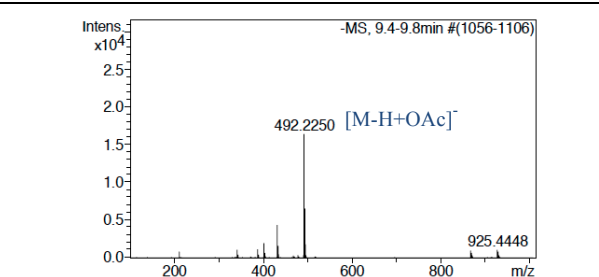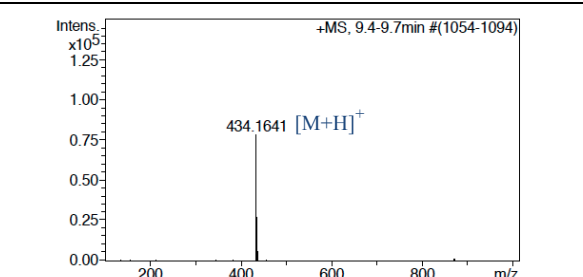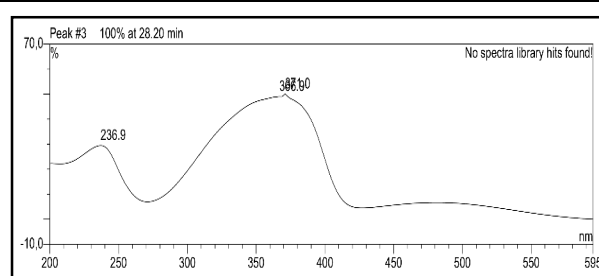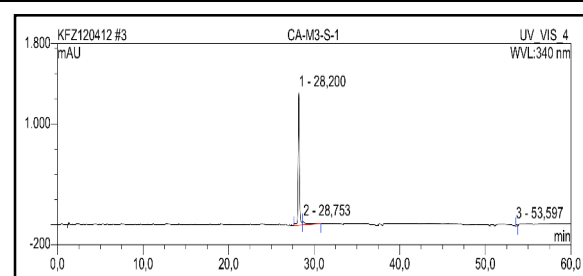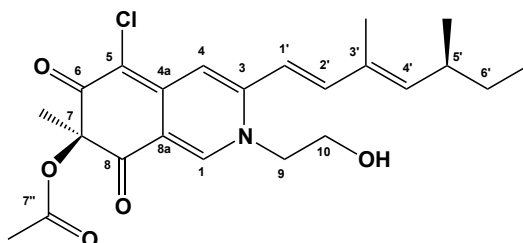
12 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated twice) then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH, which afforded red needles (yield, 5 mg). It showed UV absorbance at λ_{max} (MeOH) 236.9, 306.9 and 371.0 nm. Positive and negative ESI-MS showed molecular ion peaks at m/z 434.16 $[M+H]^+$ (base peak) and m/z 492.22 $[M-H+OAc]^-$ (base peak), respectively, indicating a molecular weight of 433 g/mol, with a 44 mass unit increase compared to sclerotioramin, which established a molecular formula of $C_{23}H_{28}ClNO_5$.

The ^1H NMR spectra (MeOD) (Table 4.16) showed 28 protons signals, which were almost identical to sclerotioramin except the presence of two more signals at (δ_H 3.84 ppm) and (δ_H 4.23 ppm), assigned to H-9 and H-10. In the ^{13}C NMR showed the presence of 23 resolved signals, which were almost the same as sclerotioramin except the presence of two more signals at (δ_C 61.1 ppm) and (δ_C 57.4 ppm), assigned to C-9 and C-10.

From the ^1H-^1H COSY spectrum, three spin system, H-4'→H-6'-CH_3, between the two protons H-9 and H-10 and between H-1' and H-2' were determined (Figure 3.18.A).
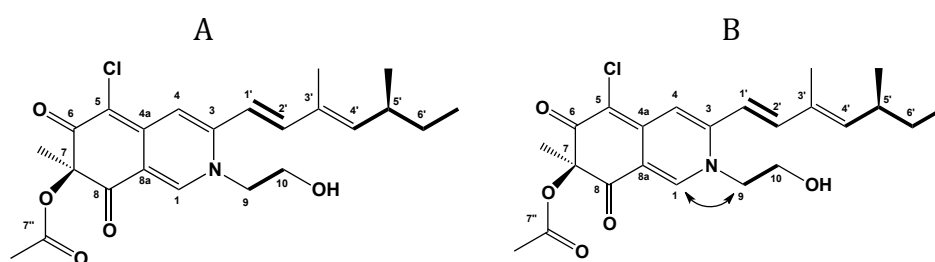
Figure 4.18. A) ^1H-^1H COSY correlations **B)** selected ^{13}C-^1H HMBC correlations of **12**

The more important correlations of ^{13}C-^1H long range couplings of ^2J and ^3J observed in the HMBC spectrum are the cross peaks from H-1 (δ_H 8.16 ppm) to C-9 (δ_C 57.4 ppm) and from H-9 (δ_H 3.84 ppm) to C-1 (δ_C 138.0 ppm), which confirm that the proton of NH in sclerotioramin has been replaced by a side chain of two carbons and a hydroxyl group (CH_2-CH_2-OH) (Figure 4.18.B).

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data as well as $[\alpha]_D$ value of **12** with the previously published data for (+)-isochromophilone VI (Arai *et al.*, 1995).

Isochromophilone VI was previously reported from *Penicillium multicolor* FO-3216 (Arai *et al.*, 1995), from *Diaporthe sp.* (Zang *et al.*, 2012), howerver, this is the first report of this compound from *Chaetomium aureum*.

Table 4.16. ^{1}H and ^{13}C NMR chemical shifts of sclerotion and its derivatives

No.	sclerotiorin δ_C	sclerotiorin δ_H	Isochromophilone VII δ_H	Sclerotioramin δ_C	Sclerotioramin δ_H	Isochromophilone VI δ_C	Isochromophilone VI δ_H
1	154.1	8.14 (s)	3.96 (d, *J*= 12.46 Hz) 4.74 (d, *J*= 12.47 Hz)	139.6	8.03 (s)	138.0	8.16 (s)
2	-	-	-	-	-	-	-
3	159.5	-	-	144.9	-	146.4	-
4	106.7	6.85 (s)	6.27 (s)	112.5	7.10 (s)	112.6	7.19 (s)
4a	139.8	-	-	148.3	-	148.3	-
5	110.2	-	-	101.1	-	101.2	-
6	186.3	-	-	185.9	-	185.5	-
7	85.5	-	-	85.9	-	86.2	-
7-CH₃	22.3	1.52 (s)	1.80 (s)	23.8	1.51 (s)	23.8	1.51 (s)
7''	170.1	-	-	171.5	-	171.5	-
7''-CH₃	19.6	2.09 (s)	2.07 (s)	20.1	2.12 (s)	20.1	2.12 (s)
8	192.7	-	-	195.0	-	195.1	-
8a	115.2	-	-	115.8	-	114.8	-
9	-	-	-	-	-	57.4	4.23 (dd, *J*= 4.86; 5.46 Hz)
10	-	-	-	-	-	61.1	3.84 (dd, *J*= 4.38; 5.16 Hz)
1'	117.3	6.50 (d, *J*= 15.7 Hz)	6.22 (d, *J*= 15.53 Hz)	118.3	6.40 (d, *J*= 16.22 Hz)	117.2	6.57 (d, *J*= 15.49 Hz)
2'	142.8	7.16 (d, *J*= 15.7 Hz)	7.02 (d, *J*=15.59 Hz)	143.4	7.16 (d, *J*= 16.22 Hz)	144.4	7.10 (d, *J*= 15.44 Hz)
3'	133.5	-	-	133.8	-	133.8	-
3'-CH₃	12.1	1.90 (s)	1.84 (d, *J*= 1.05 Hz)	12.5	1.91 (s)	12.6	1.92 (s)
4'	148.3	5.77 (d, *J*= 9.7)	5.65 (d, *J*= 9.72 Hz)	148.9	5.74 (d, *J*= 9.74 Hz)	148.7	5.78 (d, *J*= 9.75 Hz)
5'	35.4	2.54 (m)	2.54 (m)	36.2	2.55 (m)	36.2	2.53 (m)
5'-CH₃	20.1	1.01 (d, *J*= 6.61)	1.01 (d, *J*= 6.55 Hz)	20.5	1.03 (d, *J*= 6.68 Hz)	20.5	1.03 (d, *J*= 6.66 Hz)
6'	30.4	1.33-1.44 (m)	1.33-1.44 (m)	31.1	1.34-1.48 (m)	31.1	1.37-1.47 (m)
6'-CH₃	11.8	0.86 (t, *J*= 7.4, 7.4 Hz)	0.87 (t, *J*= 7.45; 7.45 Hz)	12.3	0.89 (t, *J*= 7.42; 7.42 Hz)	12.3	0.90 (t, *J*= 7.5; 7.3 Hz)

Compound isolated from the endophytic fungus *Chaetomium aureum*

Sclerotioramin (**13**, **New**)

SB 236050	
Synonym(s)	1H, 3H-Pyrano[4,3-b][1]benzopyran-9-carboxylic acid, 4,10-dihydro-7,8-dihydroxy-1-methoxy-3-methyl-10-oxo-
CAS Registry Number	335377-65-6
Biological source	*Chaetomium aureum*
Sample amount	40 mg
Physical Description	grau powder
Molecular Formula	$C_{15}H_{14}O_8$
Molecular Weight	322 g/mol
Retention time HPLC	22.04 min (standard gradient)
Optical rotation $[\alpha]_D^{20}$	+35.6 (c 1.0, CHCl$_3$)
UV spectrum λ_{max}	220.5, 316.0 and 370.0 nm
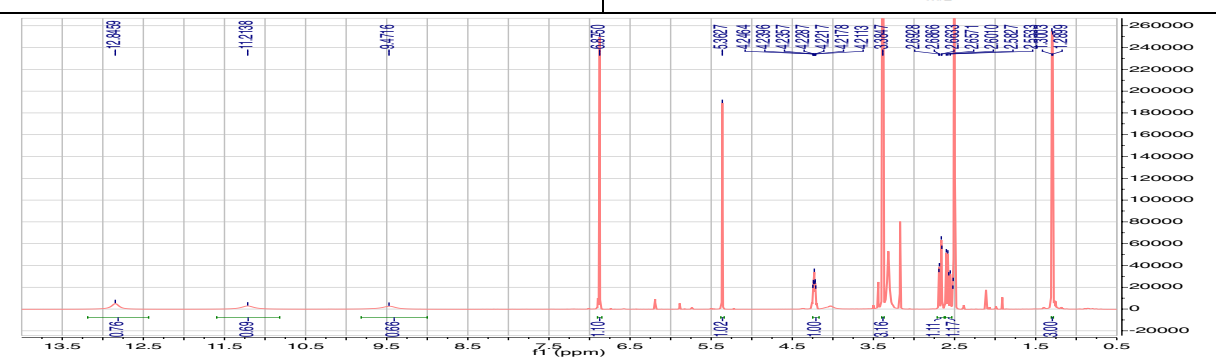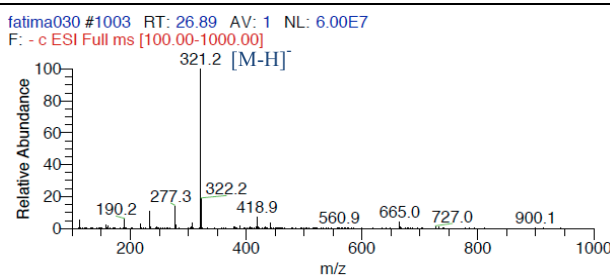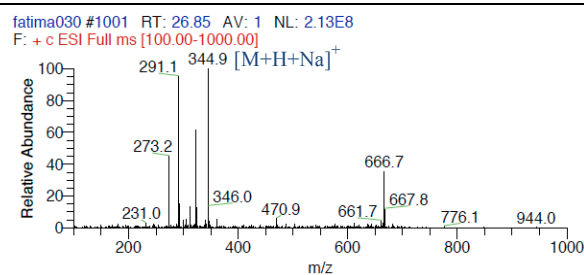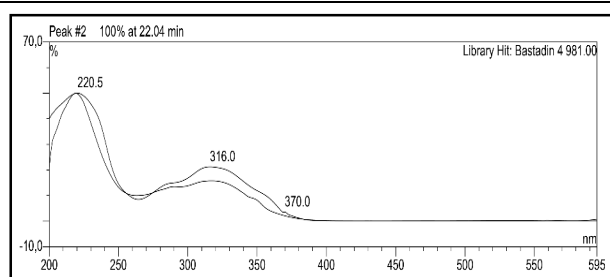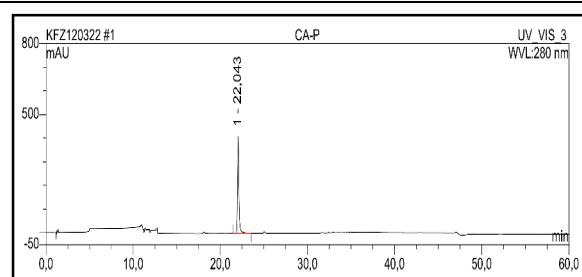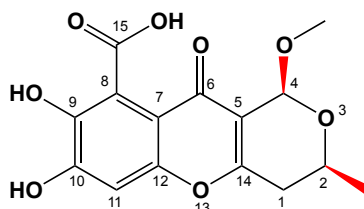
13 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction gave a precipitate, which was washed several times with methanol until obtaining grey powder (yield, 40 mg). It showed UV absorbance at λ_{max} (MeOH) 220.5, 316.0 and 370.0 nm. Positive and negative ESI-MS showed molecular ion peaks at m/z 344.9 [M+H+Na]$^+$ (base peak) and m/z 321.2 [M-H]$^-$ (base peak), respectively, indicating a molecular weight of 322 g/mol. The molecular formula of **13** was assigned as $C_{21}H_{24}ClNO_4$.

The ^{1}H NMR (DMSO-d_6) (Table 4.17) showed one methyl group at (δ_H 1.30 ppm) assigned to H-2-CH$_3$, one methoxy group at (δ_H 3.28 ppm) assigned to H-4-OCH$_3$, one methylene proton at (δ_H 2.67 ppm) and (δ_H 2.58 ppm) assigned to H-1, two aliphatic proton CH at (δ_H 4.22 ppm) and (δ_H 5.36 ppm) assigned to H-2 and H-4 respectively, one aromatic proton at (δ_H 6.87 ppm) assigned to H-11 and three hydroxyl group at (δ_H 9.47, 11.21 and 12.81 ppm) assigned to H-10-OH, H-9-OH and H-15-OH. The ^{13}C NMR and DEPT NMR showed the presence of 21 resolved signals, which were assigned to one methyl carbon (δ_C 20.3 ppm) assigned to 2-CH$_3$, one methylene carbon (δ_C 33.1 ppm) assigned to C-1, three methine carbons (δ_C 61.7, 93.8 and 102.1 ppm) assigned to C-2, C-4 and C-11 respectively, two carbonyl carbons (δ_C 172.1 and 167.5 ppm) assigned to C-6 and C-15 and seven sp^2 quaternary carbons.

The ^{1}H-^{1}H COSY spectra showed the presence of one spin system from H(1)→H(2)→H(2-CH$_3$) as shown in the Figure 4.19.A.
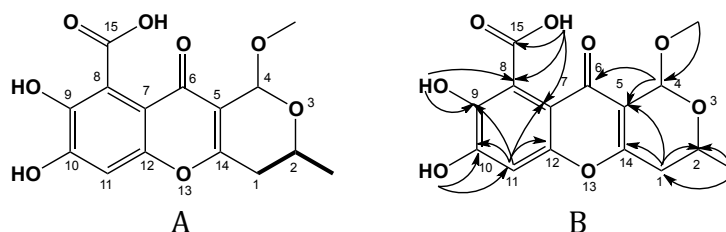
Figure 4.19. A) ^{1}H-^{1}H COSY correlations **B)** selected ^{1}H-^{13}C HMBC correlations of **13**

The ^{1}H-^{13}C HMBC spectra (Figure 4.19.B) showed the selected following correlations: The cross peak from H-2-CH$_3$ to C-2 and C-1, from H-1 to C-2, C-14 and C-5, from H-4-OCH$_3$ to C-4, from H-4 to C-5 and C-6, from H-11 to C-10, C-12, C-7 and C-9, from H-9-OH to C-8

and C-9, from H-10-OH to C-10 and C-11, from H-15-OH to C-7, C-8 and C-15.

The identification was further corroborated by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data of **13** and the previously published data for SB 236050 (Payne *et al.*, 2002).

Regarding the structure of SB 236050, which has two chiral carbon centers at C-2 and C-4, the relative stereochemistry was determined by REOSY NMR spectra as shown in Figure 4.20.
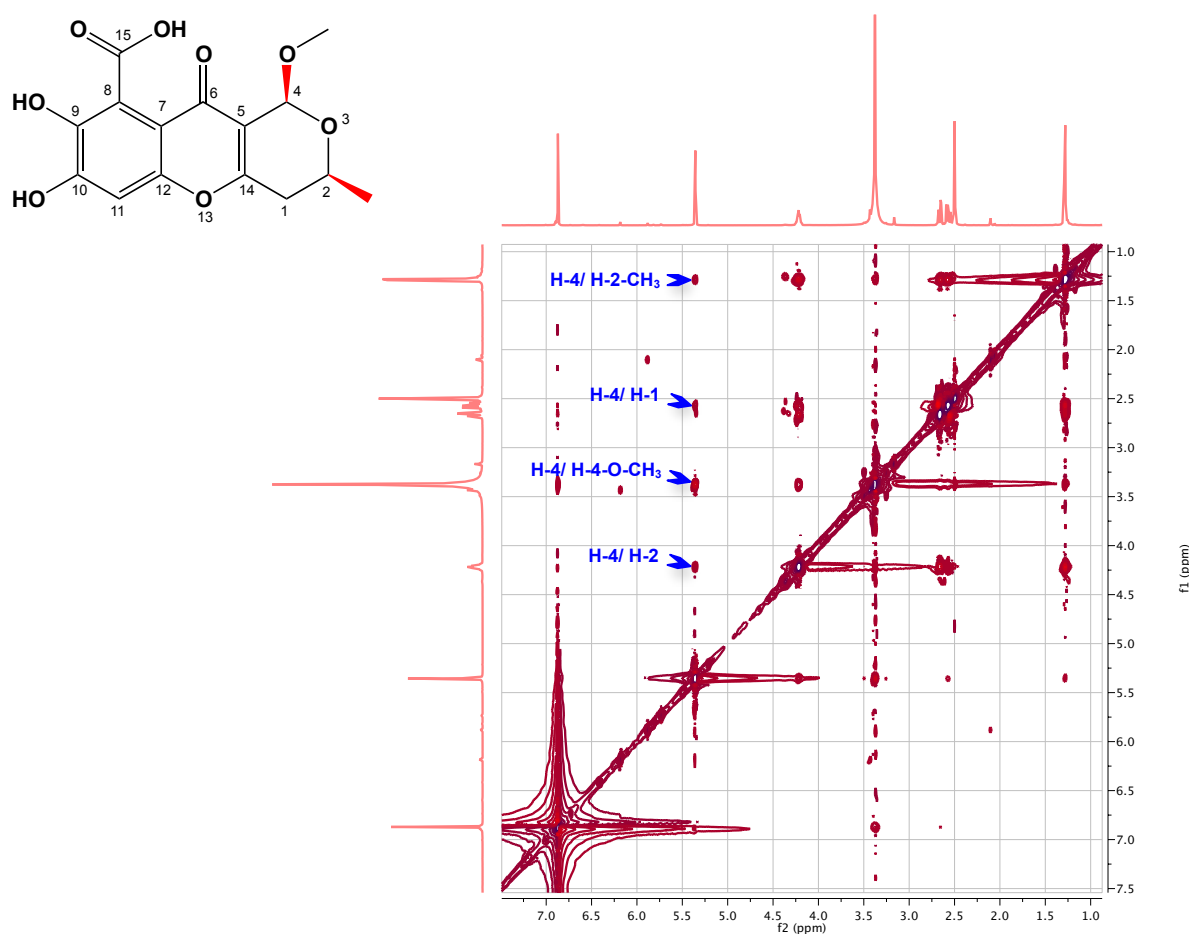
Figure 4.20. ^{1}H-^{1}H ROESY correlations

The H-4 correlated with H2-CH$_3$, H-1, H-4-OCH$_3$ and H-2 (see Figure 4.17). ^{1}H-^{1}H ROESY spectra permitted the assignment of the cis-stereochemistry of SB 236050 to be as shown in Figure 4.17, which is reported for the first time. The absolute stereochemistry could not be confirmed yet.

SB 236050 was previously reported from *Chaetomium funicola* (Payne *et al*., 2002). From the best of our knowledge, the relative stereochemistry of SB 236050 was not previously reported in the literature. This is the first report of the stereochemistry of SB 236050 isolated from *Chaetomium aureum*.

Table 4.17. Comparison of ^{1}H and ^{13}C NMR chemical shifts of **13** with SB 236050 (Payne *et al*., 2002)

N°	13 (DMSO)		SB 236050 (DMSO)	
	δ_H	δ_C	δ_H	δ_C
1	2.58 (1H, dd, *J*= 10.92, 17.71 Hz)	33.1	2.56 (1H, dd, *J*= 12, 15 Hz)	33.2
	2.67 (1H, dd, *J*= 3.76, 17.7 Hz)		2.67 (1H, dd, *J*= 4, 15 Hz)	
2	4.23 (1H, m)	61.7	4.21 (1H, m)	61.9
2-CH3	1.30 (3H, d, *J*= 6.23)	20.3	1.28 (1H, d, *J*= 6 Hz)	20.5
3	-	-	-	-
4	5.36 (1H, s)	93.8	5.35 (1H, s)	99.0
4-OCH3	3.40 (3H, s)	54.9	3.37 (1H, s)	55.0
5	-	112.3	-	112.1
6	-	172.1	-	172.2
7	-	114.9	-	115.4
8	6.87 (1H, s)	119.1	6.84 (1H, s)	119.0
9	-	141.3	-	141.8
9-OH	9.47	-	-	-
10	-	151.8	-	152.4
10-OH	11.21	-	-	-
11	-	102.1	-	102.2
12	-	149.9	-	150.2
13	-	-	-	-
14	-	162.3	-	162.3
15	-	167.5	-	167.8
15-OH	12.84	-	-	-

[a, b, c, d, f, x] could be opposite

Compound isolated from the endophytic fungus *Chaetomium aureum*

Xanthoradone D$_1$ (**14**, **New**)

Xanthoradone D$_1$	
Synonym(s)	1,4-Naphthalenedione, 6-[(3R)-3,4-dihydro-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-1H-naphtho[2,3-c]pyran-8-yl]-1,5-dihydroxy-2-methoxy
Biological source	*Chaetomium aureum*
Sample amount	3 mg
Physical Description	Orange-brown powder
Molecular Formula	C$_{26}$H$_{20}$O$_{10}$
Molecular Weight	492 g/mol
Retention time HPLC	30.89 min (standard gradient)
Optical rotation [α]$_D^{20}$	+305 (c 1.0, CHCl$_3$)
UV spectrum λ$_{max}$	202.9, 220.0 and 265.1 nm

14 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated three times) then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH, which afforded orange-brown powder (yield, 3 mg). It showed UV absorbance at λ_{max} (MeOH) 202.9, 220.0 and 265.1 nm. Positive and negative HR-ESI-MS exhibited a prominent peak at m/z 515.09 $[M+H+Na]^+$ (base peak) and m/z 491.17 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 492 g/mol, which established a molecular formula of $C_{26}H_{20}O_{10}$.

The ^1H NMR spectrum (Table 4.18) showed the presence of one olefinic methyl group, which appeared as one doublet at (δ_H 1.57 ppm), assigned to 2-CH_3; two methoxy group, which appeared at (δ_H 3.84 and 4.21 ppm), assigned to 8-OCH_3 and 3'-OCH_3 respectively; one olefinic methine group at (δ_H 4.79 ppm), assigned to 2-CH, one olefinic methylene group, which appeared as two doublet of doublet, assigned to 3-CH_2 and four aromatic methine group, which appeared at (δ_H 6.68, 6.96, 7.72 and 7.74 ppm), assigned to 7'-CH, 6-CH, 7'-CH and 6'-CH and three hydroxyl group at (δ_H 9.75, 11.53 and 13.88 ppm), assigned to 10-OH, 9'-OH and 12-OH respectively.

The ^{13}C NMR spectrum (in $CDCl_3$) showed 26 resolved signals, which were assigned to one methyl carbon, one methylene carbon, four sp^2 methine carbons, one oxygenated methine carbon, two oxygenated methyl carbons, eight sp^2 quaternary carbons, six oxygenated sp^2 quaternary carbons and three carbonyl carbons.

Analyses of ^1H-^1H NMR COSY revealed the presence of two spin systems, as shown in Figure 4.21, the aromatic spin system composed of H-6' and H-7' and the second spin system from 2-CH_3 to 5-CH. Furthermore, ^{13}C-^1H long range couplings of ^2J and ^3J observed in ^{13}C-^1H heteronuclear multiple bond coherence (HMBC) spectrum gave the following linkages (Figure 4.22): (A) Cross peaks from H_2-3 (δ_H 2.96 ppm) to C-14 (δ_C 20.4 ppm), C-2 (δ_C 76.2 ppm), C-13 (δ_C 99.4 ppm), C-5 (δ_C 115.7 ppm) and C-4 (δ_C 133.4); from H-5 (δ_H 6.95 ppm) to C-3 (δ_C 34.3 ppm), C-6 (δ_C 140.1 ppm), C-7 (δ_C 97.7 ppm), C-11 (δ_C 107.8 ppm), C-13 and

C-12 (δ_C 162.7 ppm); from H-7 (δ_H 6.66 ppm) to C-5, C-8 (δ_C 159.9 ppm), C-9 (δ_C 109.6 ppm), C-11; from H_3-14 (δ_H 1.56 ppm) to C-2 and C-3; from H_3-15 (δ_H 3.86 ppm) to C-8; from OH-10 (δ_H 9.74 ppm) to C-9, C-11 and C-10 (δ_C 155.0 ppm) and from OH-12 (δ_H 13.85 ppm) to C-11, C-12 and C-13. Furthermore, the chemical shift of C-2 (δ_C 76.2 ppm) indicated the presence of an oxygenated carbon and that the OH-12 (δ_H 13.85 ppm) is shifted to a lower field explains the presence of an ester bond between C-2 and C-13. This connection was confirmed by the chemical shift of C-1 (δ_C 171.3 ppm), which is typical for an ester group. Thus, these data indicated that **14** was found to have a dihydronaphthopyranone skeleton.
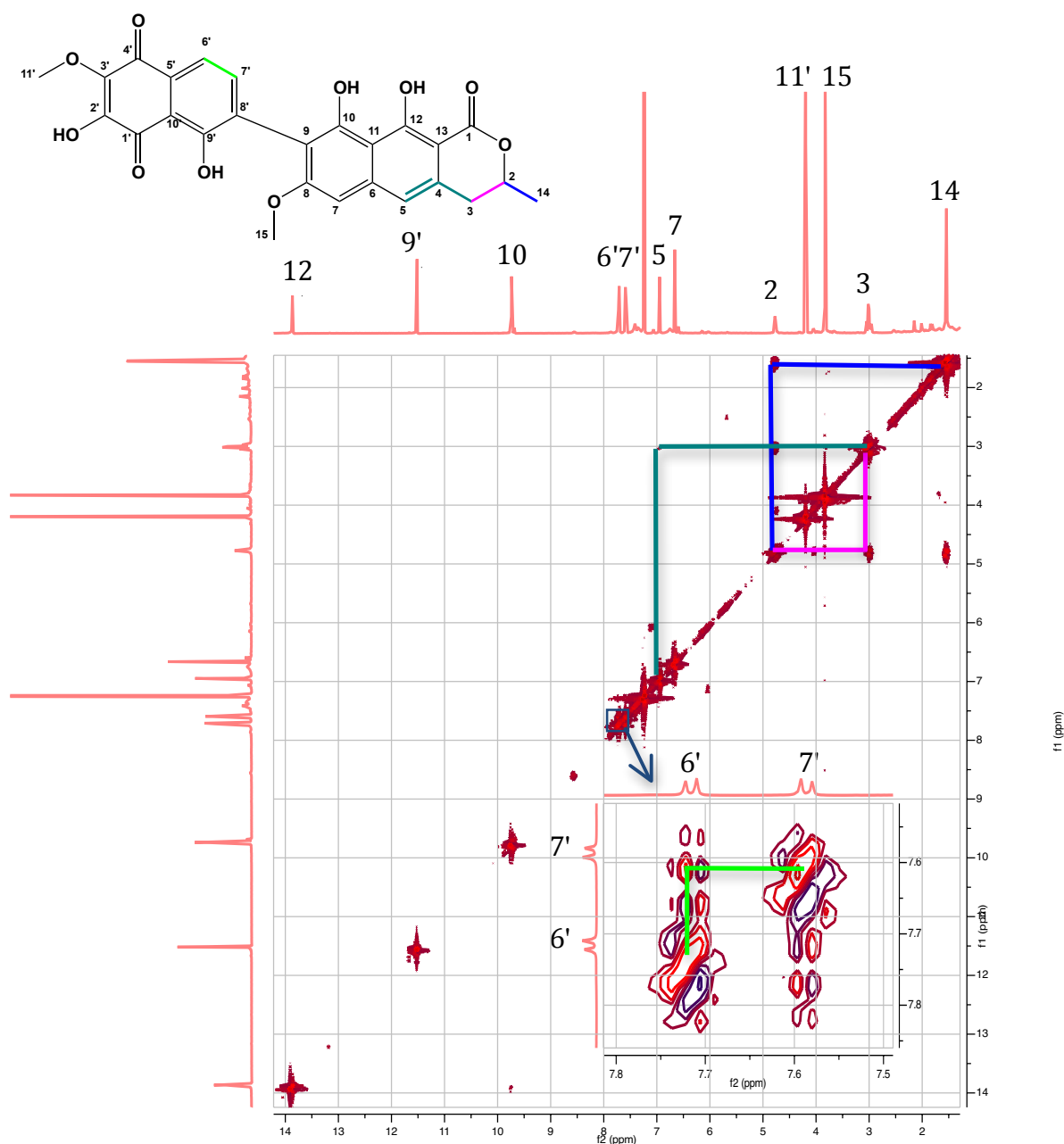
Figure 4.21. ^{1}H-^{1}H NMR COSY
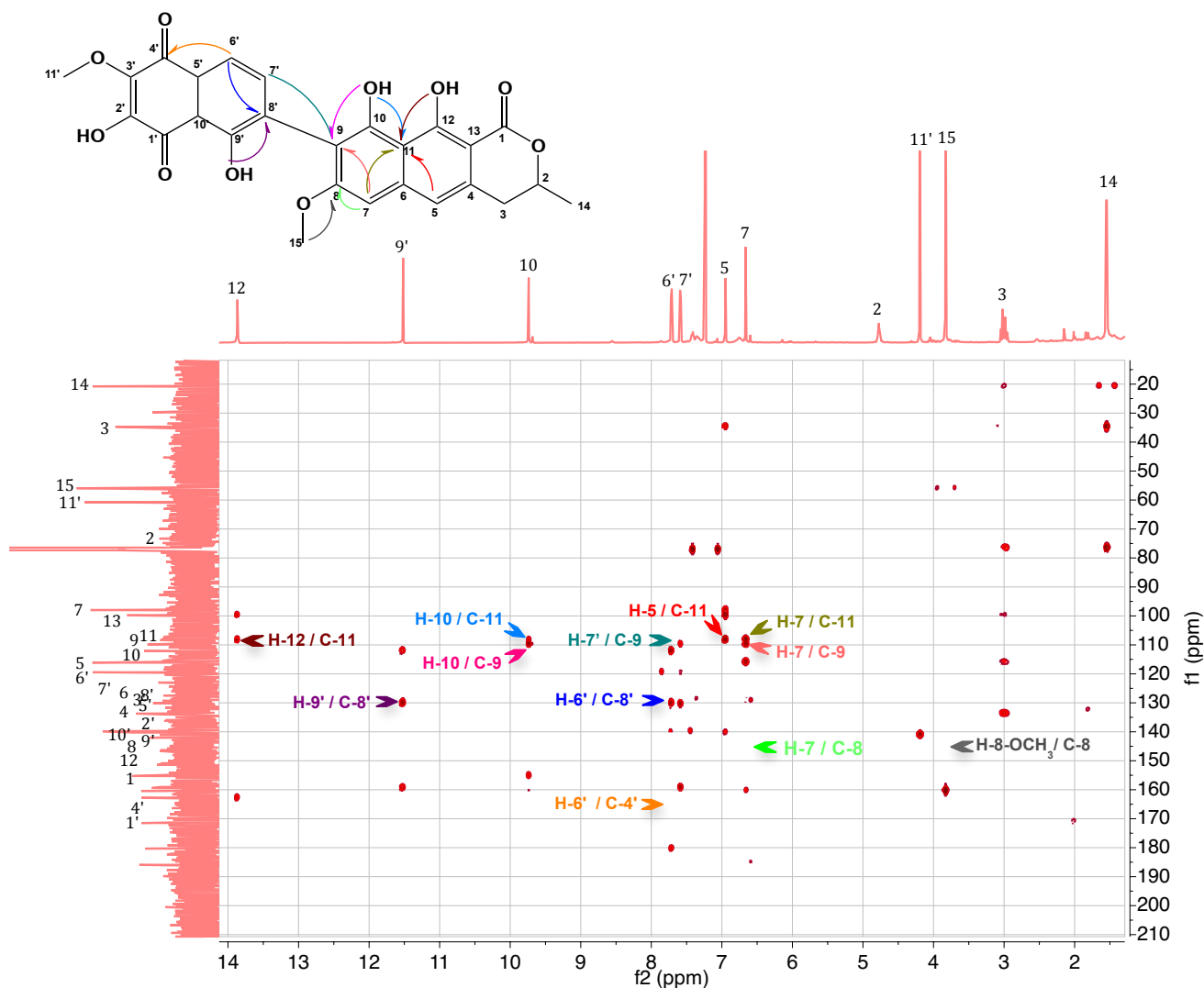
Figure 4.22. Selected ^{13}C-^{1}H HMBC correlations

(B) Cross peaks from H-6' (δ_H 7.71 ppm) to C-4' (δ_C 180.0 ppm), C-5' (δ_C 129.9 ppm), C- 7' (δ_C 139.8 ppm) and C-10' (δ_C 111.8 ppm); from H-7' (δ_H 7.58 ppm) to C-6' (δ_C 119.4 ppm), C-8' (δ_C 130.6 ppm), C-9' (δ_C 158.9 ppm) and C-9; from OH-9' (δ_H 11.51 ppm) to C-8', C-9' and C-10'; from H$_3$-11' (δ_H 4.19 ppm) to C-3' (δ_C 141.0 ppm). Therewith, the chemical shifts of C-1' (δ_C 185.0 ppm) and C-4' (δ_C 180.0 ppm) indicated the presence of two carbonyl carbons derived from quinone skeleton and the UV absorption at 165.1 nm confirmed the proposed structure. Thus, these data confirmed that **14** has another dihydronaphthoquinone skeleton.

The connection between the two dihydronaphthopyranone and dihydronaphthoquinone substructures was established by the presence of the correlation between H-7' and C-9,

between OH-10 and C-9 and between H-7' and C-8' confirming that they are connected at C-9-C-8'. The structure of **14** was elucidated as shown in Figure 3.20. The structure satisfied the degree of unsaturation and the molecular formula. Finally, **14** was identified as a new natural product for which the name xanthoradone D_1 was given.

Regarding the stereochemistry of xanthoradone D_1, which has one chiral carbon center at C-2, the structure was suggested as shown as in Figure 4.23 by TDDFT ECD calculations of their solution conformers.
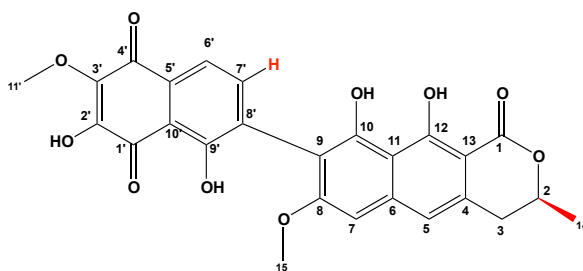
Figure 4.23. The structure of xanthorathode D_1

The comparison of the ^{1}H NMR spectra of **14** and **xanthoradone A** (Yamazaki *et al.*, 2009) (Table 4.15) showed a close relation-ship between both compounds except for the presence of an additional methyl group at (δ_H 2.21 ppm) and the absence of a hydroxyl group in **xanthoradone A**.

Compound isolated from the endophytic fungus *Chaetomium aureum*

Xanthoradone D$_2$ (**15**, **New**) (Mixture with its atropisomers xanthoradone D$_1$)

Xanthoradone D$_2$

Synonym(s)	1,4-Naphthalenedione, 6-[(3R)-3,4-dihydro-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-1H-naphtho[2,3-c]pyran-8-yl]-1,5-dihydroxy-2-methoxy
Biological source	*Chaetomium aureum*
Sample amount	5 mg
Physical Description	Orange-brown powder
Molecular Formula	C$_{26}$H$_{20}$O$_{10}$
Molecular Weight	492 g/mol
Retention time HPLC	30.89 min (standard gradient)
Optical rotation [α]$_D^{20}$	+3 (c 1.0, CHCl$_3$)
UV spectrum λ$_{max}$	202.9, 220.0 and 265.1 nm

15 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated three times) then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH, Compound **15** was obtained as a mixture with compound **14** forming an orange-brown powder (yield, 5 mg). It showed UV absorbance at λ_{max} (MeOH) 202.9, 220.0 and 265.1 nm. Positive and negative LC-ESI-MS of the mixture exhibited a prominent peak at m/z 493.2 $[M+H]^+$ (base peak) and m/z 491.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 492 g/mol, which established a molecular formula of $C_{26}H_{20}O_{10}$ identical to that of **14**.

In ^1H NMR spectrum (Table 4.18) showed the same protons signals as xanthoradone D_1, which are almost overlapped protons with proportion of (1:3). In Figure 4.24, the proton at (δ_H 7.57 ppm) assigned to H-6' of one of the isomers is very close to the proton at (δ_H 7.58 ppm) assigned to H-6' of the other one.
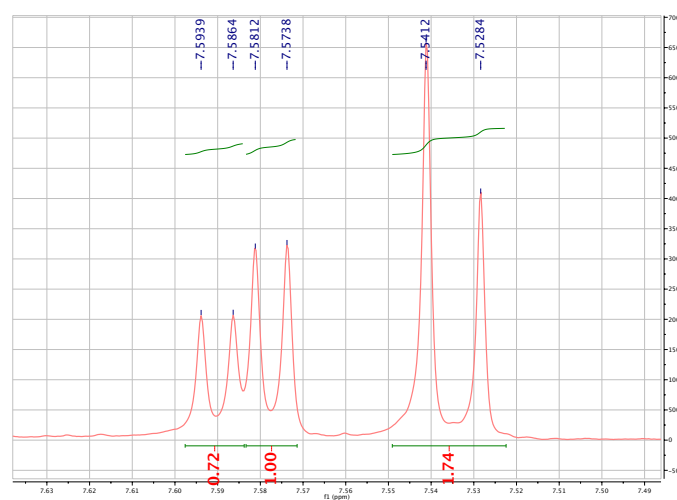
Figure 4.24. ^1H NMR of the overlapped proton H-6' and H-7'

Hence, **15** was identified as an stereoisomer of **14** for which the name xanthoradone D_2 is proposed. Even though a mixture was used, we were able to successfully determine the absolute configuration of both compounds with the help of the TDDFT ECD calculations.

Compound isolated from the endophytic fungus *Chaetomium aureum*

Xanthoradone E$_1$ and xanthoradone E$_2$ (**16/17**, **New**)

Xanthoradone E$_1$ / Xanthoradone E$_2$	
Synonym(s)	1,4-Naphthalenedione, 6-[(3R)-3,4-dihydro-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-1H-naphtho[2,3-c]pyran-8-yl]-5-hydroxy-2-methoxy
Biological source	*Chaetomium aureum*
Sample amount	6 mg
Physical Description	Orange-brown powder
Molecular Formula	C$_{26}$H$_{20}$O$_9$
Molecular Weight	476 g/mol
Retention time HPLC	32.92 min (standard gradient)
Optical rotation [α]$_D^{20}$	+1 (c 1.0, CHCl$_3$)
UV spectrum λ$_{max}$	220.1, 264.8 and 377.7 nm

No ionisation at the Positive mode

16 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive Sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated three times), then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH, Compound **16** was obtained as a mixture with compound **16** forming an orange-brown powder (yield, 6 mg). It showed UV absorbance at λ_{max} (MeOH) 220.1, 264.8 and 377.7 nm. Negative ESI-MS of **16/17** exhibited a prominent peak at m/z 475.3 [M-H]⁻ (base peak), indicating a molecular weight of 476 g/mol, which established a molecular formula of $C_{26}H_{20}O_9$ with a 16 mass unit decrease compared to **14/15**. The absence of one hydroxyl group was supposed.

In ^{1}H NMR spectrum (Table 4.18) showed the presence of 20 protons signals, which are almost overlapped with the proportional of (1:1). The proton signals are identical to those of **14/15** (Table 4.15), except the presence of one more aromatic proton at (δ_H 6.11 ppm) and the absence of one hydroxygroup at (δ_H 10.89 ppm).
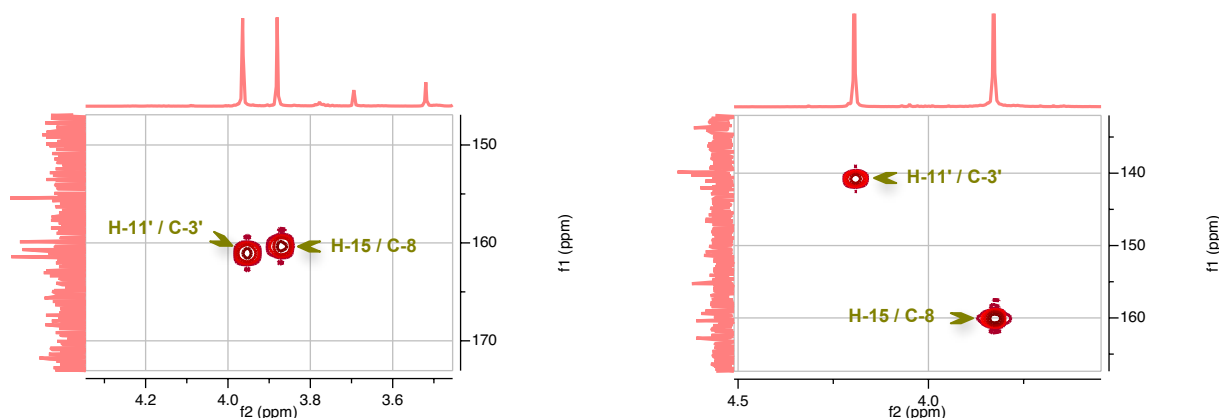
Figure 4.25. The comparison of H-C-3' of 14/15 and 16/17 in ^{13}C-^{1}H HMBC NMR

The ^{13}C NMR spectrum (in CDCl₃) showed 26 resolved signals, which are almost identical to those of **14/15**, except the chemical shift of 3 carbons, C-1' that in **14/15** was at (δ_C 186.0 ppm) and in **16/17** C-1' moved to (δ_C 190.7 ppm), C-2' that was upfield at (δ_C 159.0 ppm) in **14/15** compared to **16/17** (δ_C 110.1 ppm) and C-3' that was downfield (δ_C 141.0 ppm) in

14/15 compared to **16/17** (δ_C 161.3 ppm). These data confirm that the hydroxyl group in the position 2' of **14/15** is absent in the mixture of compounds **16/17**.

The ^{1}H-^{1}H NMR COSY of **16/17** spectra showed the same correlations as **14/15**. Furthermore, ^{13}C-^{1}H long range couplings of ^2J and ^3J observed in ^{13}C-^{1}H heteronuclear multiple bond coherence (HMBC) spectrum of **16/17** gave the same linkages as **14/15** except of the presence of one more cross peaks from H-2' (δ_H 6.11 ppm) to C-1' (δ_C 190.7 ppm), C-3' (δ_C 161.3 ppm), C-4' (δ_C 179.4 ppm) and C-10' (δ_C 114.2 ppm) as shown in Figure 3.23.
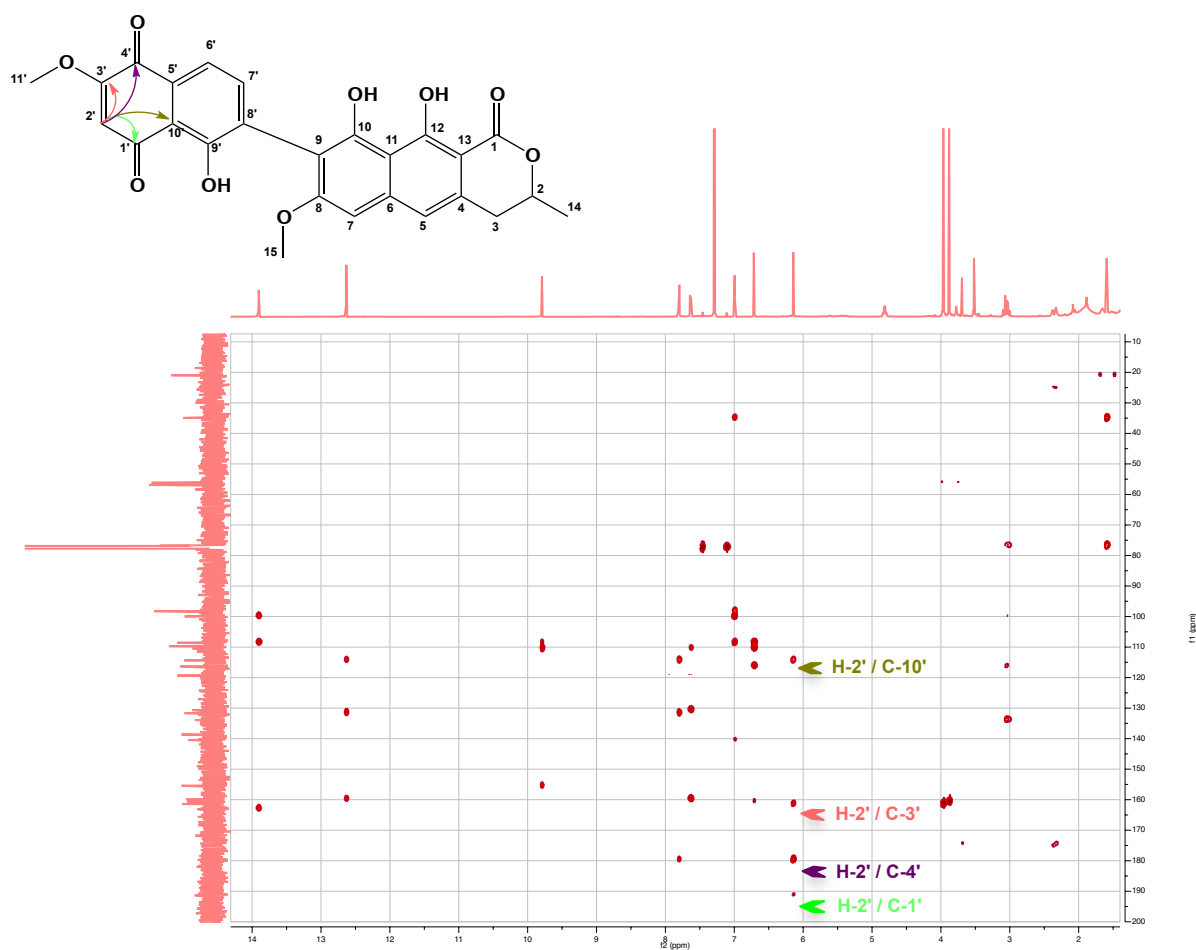
Figure 4.26. Selected ^{13}C-^{1}H HMBC NMR correlations of **16/17**

Hence, **16/17** was identified as mixture of two xanthoradones derivative for which the name xanthoradone E_1 and E_2 are proposed.

The stereochemistry of xanthoradones E_1 and E_2, which has one chiral carbon center at C-2 , was determined by TDDFT ECD calculations of their solution conformers.

Table 4.18. ^{1}H and ^{13}C NMR chemical shifts of xanthoradones A, D and E
(Yamazaki et al., 2009)

Position	Xanthoradone A (in CDCl$_3$)		Xanthoradone E (in CDCl$_3$)		Xanthoradone D (in CDCl$_3$)	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	171.5	-	171.5	-	171.3	-
2	76.8	4.79 m	76.5	4.79 m	76.7	4.77 m
3	34.7	ax: 3.00 dd (J= 17.0, 10.0 Hz) eq: 3.06 dd (J= 17.0, 4.0 Hz)	34.7	ax: 2.99 dd (J= 16.2, 10.4 Hz) eq: 3.05 dd (J= 16.1, 3.4 Hz)	34.8	ax: 2.99 dd (J= 15.8, 10.4) eq: 3.05 dd (J= 16.1, 3.4)
4	133.5	-	133.4	-	133.8	-
5	116.1	6.99 s	116.1	6.96 s	116.0	6.96 s
6	140.0	-	141.7	-	140.2	-
7	98.1	6.71 s	98.1	6.68 s	98.1	6.68 s
8	160.3	-	160.6	-	160.3	-
9	109.0	-	109.6	-	109.6	-
10	154.7	-	155.3	-	155.2	-
10-OH		9.73 s		9.76 s		9.75 s
11	108.4	-	108.1	-	108.0	-
12	162.7	-	162.4	-	162.7	-
12-OH		13.9 s		13.87 s		13.88 s
13	99.7	-	99.8	-	99.4	-
14	20.7	1.57 d (J= 7.0 Hz)	20.8	1.57 d (J= 3.1 Hz)	20.1	1.56 d (J=6.3)
15	56.0	3.84 s	56.0	3.85 s	55.4	3.84 s
1'	190.6	-	190.7	-	**186.0**	-
2'	109.6	6.08 s	110.1	6.11 s	**159.0**	-
2'-OH		-		-		-
3'	160.9	-	161.3	-	**141.0**	-
4'	179.8	-	179.4	-	180.2	-
5'	129.8	-	130.4	-	129.9	-
6'	121.3	7.67 s	**119.1**	**7.77 d (J= 7.7 Hz)**	119.4	7.73 d (J= 7.5 Hz)
7'	147.2	-	**138.3**	**7.60 d (J= 7.6 Hz)**	139.7	7.6 d (J= 7.6 Hz)
8'	130.6	-	131.5	-	130.4	-
9'	159.7	-	159.5	-	159.3	-
9'-OH		12.5 s		12.6 s		11.5 s
10'	112.0	-	114.2	-	111.9	-
11'	56.6	3.92 s	56.7	3.93 s	60.8	4.2 s
12'	20.5	2.21 s	-	-	-	-

Table 4.19. [1]H chemical shifts of the atropisomers of xanthoradone D and E

Position	Xanthoradone E (in CDl$_3$)		Xanthoradone D (in DMSO)	
	δ_H of D-1	δ_H of D-2	δ_H of E-1	δ_H of E-2
1	-	-	-	-
2	4.78 m	4.79 m	4.86 m	4.86 m
3	ax: 2.99 dd (J= 16.2, 10.4 Hz) eq: 3.04 dd (J= 16.0, 3.3 Hz)	ax: 2.99 dd (*J*= 16.2, 10.4 Hz) eq: 3.05 dd (*J*= 16.1, 3.4 Hz)	ax: 3.00 dd (*J*= 15.1, 10.9 Hz) eq: 3.14 dd (*J*= 15.7, 2.7 Hz)	ax: 2.99 dd (*J*= 15.8, 11.6) eq: 3.14 dd (*J*= 15.7, 2.7)
4	-	-	-	-
5	6.96 s	6.96 s	7.15 s	7.15 s
6	-	-	-	-
7	6.68 s	6.68 s	6.95 s	6.95 s
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
10-OH	9.76 s	9.76 s	9.63 br	9.63 br
11	-	-	-	-
12	-	-	-	-
12-OH	13.86 s	13.87 s	13.69 br	13.69 br
13	-	-	-	-
14	1.56 d (*J*=3.0 Hz)	1.57 d (*J*= 3.1 Hz)	1.45 d (*J*= 6.2)	1.45 d (*J*=6.2)
15	3.85 s	3.85 s	3.79 s	3.79 s
1'	-	-	-	-
2'	6.11 s	6.11 s	-	-
2'-OH	-	-	10.89 br	10. 89 br
3'	-	-	-	-
4'	-	-	-	-
5'	-	-	-	-
6'	7.77 d (J= 7.6 Hz)	7.77 d (*J*= 7.7 Hz)	7.58 d (*J*= 7.6 Hz)	7.57 d (*J*= 7.5 Hz)
7'	7.60 d (J= 7.6 Hz)	7.60 d (*J*= 7.6 Hz)	7.53 d (*J*= 7.6 Hz)	7.53 d (*J*= 7.6 Hz)
8'	-	-	-	-
9'	-	-	-	-
9'-OH	12.60 s	12.60 s	11.85 s	11.85 s
10'	-	-	-	-
11'	3.93 s	3.93 s	3.92 s	3.92 s

Compound isolated from the endophytic fungus *Chaetomium aureum*

Chaetoresorcinol (**18**, **New**)

Chaetoresorcinol	
Synonym(s)	Chaetoresorcinol
Biological source	*Chaetomium aureum*
Sample amount	6 mg
Physical Description	red oil
Molecular Formula	$C_{26}H_{20}O_9$
Molecular Weight	385 g/mol
Retention time HPLC	32.92 min (standard gradient)
UV spectrum λ_{max}	220.1, 264.8 and 377.7 nm
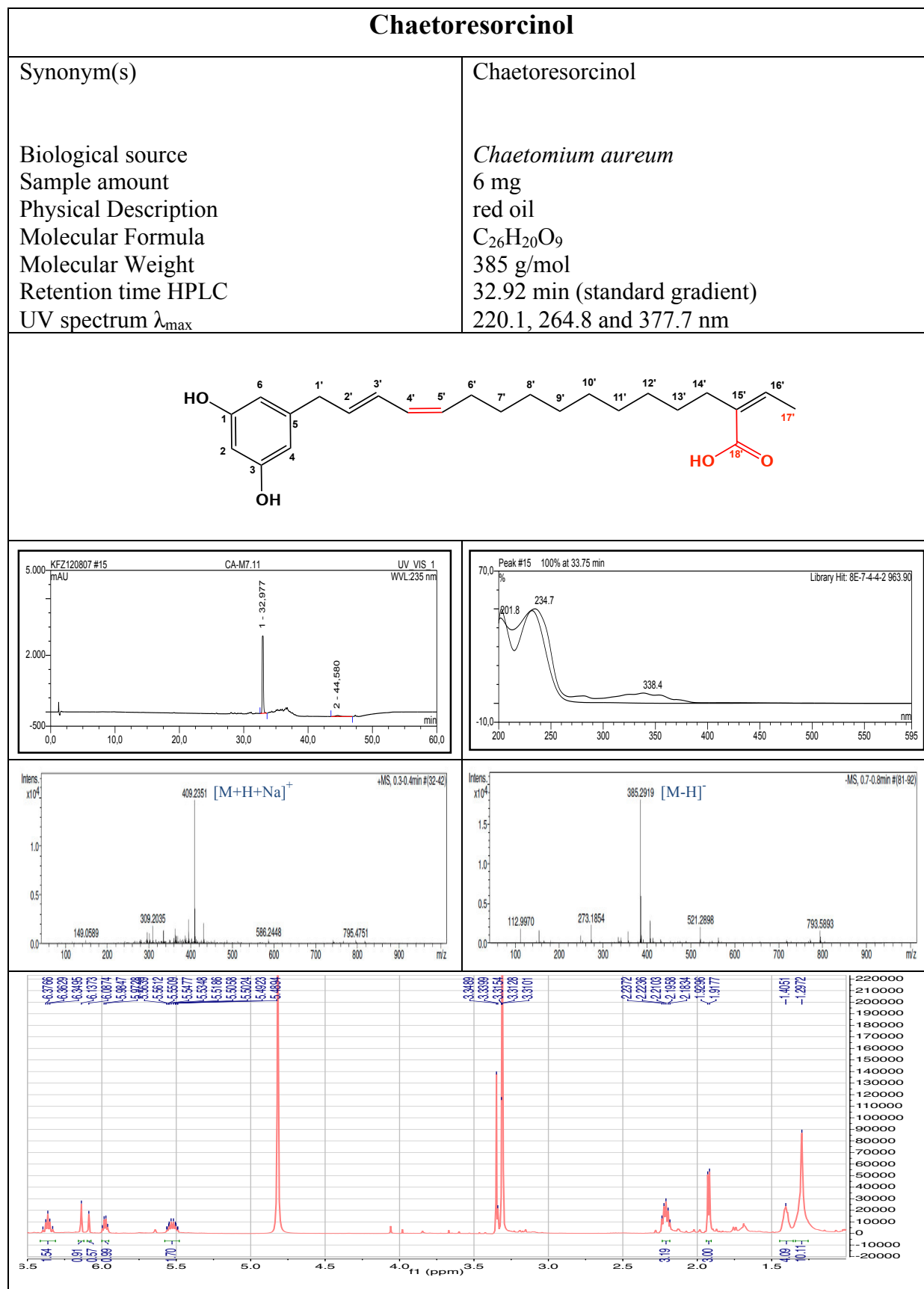
18 was isolated from *Chaetomium aureum* grown at room temperature for 30 days in Fernbach flasks on solid rice medium. The EtOAc extract was portioned between *n*-hexane and 90% MeOH. The MeOH fraction was chromatographed over a successive sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as eluent (repeated three times), then purified by semi-preparative HPLC (Merck, Hitachi L-7100) using a Eurosphere 100–10 C18 column (300 x 8mm, L x i.d.) with the following gradient (MeOH/H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100 % MeOH. Compound **18** was obtained as a red oil and showed three UV maxima at 201, 234 and 338 nm. ESIMS exhibited strong pseudomolecular base peak at *m/z* 385.3 [M-H]⁻ in the negative mode, indicating a molecular weight of 386 g/mol. The chemical formula of **18** was deduced as $C_{24}H_{33}O_4$ from the prominent peak appearing at *m/z* 409.2351 [M+Na]⁺ in the HRESIMS (calcd for $C_{24}H_{34}O_4Na$ 409.2355).

^{13}C and DEPT experiments (Table 4.20) exhibited 24 carbon atoms attributable to one olefinic methyl group, ten methylenes, eight olefinic methines, four quaternary carbons, and one carbonyl carbon.

Analysis of 2D NMR data indicated that **18** is a resorcinol derivative containing a side chain of 18 carbons.

^{1}H-^{1}H COSY confirmed the presence of three spin systems assigned for CH_3(17')H(16'), a continuous spin system CH_2(1')→CH_2(14') and the resorcinol ring system H(2)H(4)H(6), whereas the connection of these spin systems was established by inspection of ^2J and ^3J HMBC correlations (Figure 4.27).

CH_2-14' showed a ^2J correlation to the olefinic quaternary carbon C-15' (δ_C 135.2 ppm) and ^3J correlations to C-16' and C-18', appearring at δ_C 136.5 and 172.0 ppm, respectively (Figure 4.28). A strong ^3J correlation was also detected for the olefinic proton H-16' to C-14' (δ_C 35.9 ppm), the latter correlating also with the methyl group CH_3-17' *via* a ^4J correlation. On the other hand, the aliphatic methylene protons CH_2-1' correlated to two overlapping aromatic carbons, which were attributed to C-4 and C-6 (δ_C 108.0 ppm), as well as to an isolated aromatic carbon assigned to C-5 (δ_C 144.1 ppm), thus, establishing the connection between the identified spin systems.
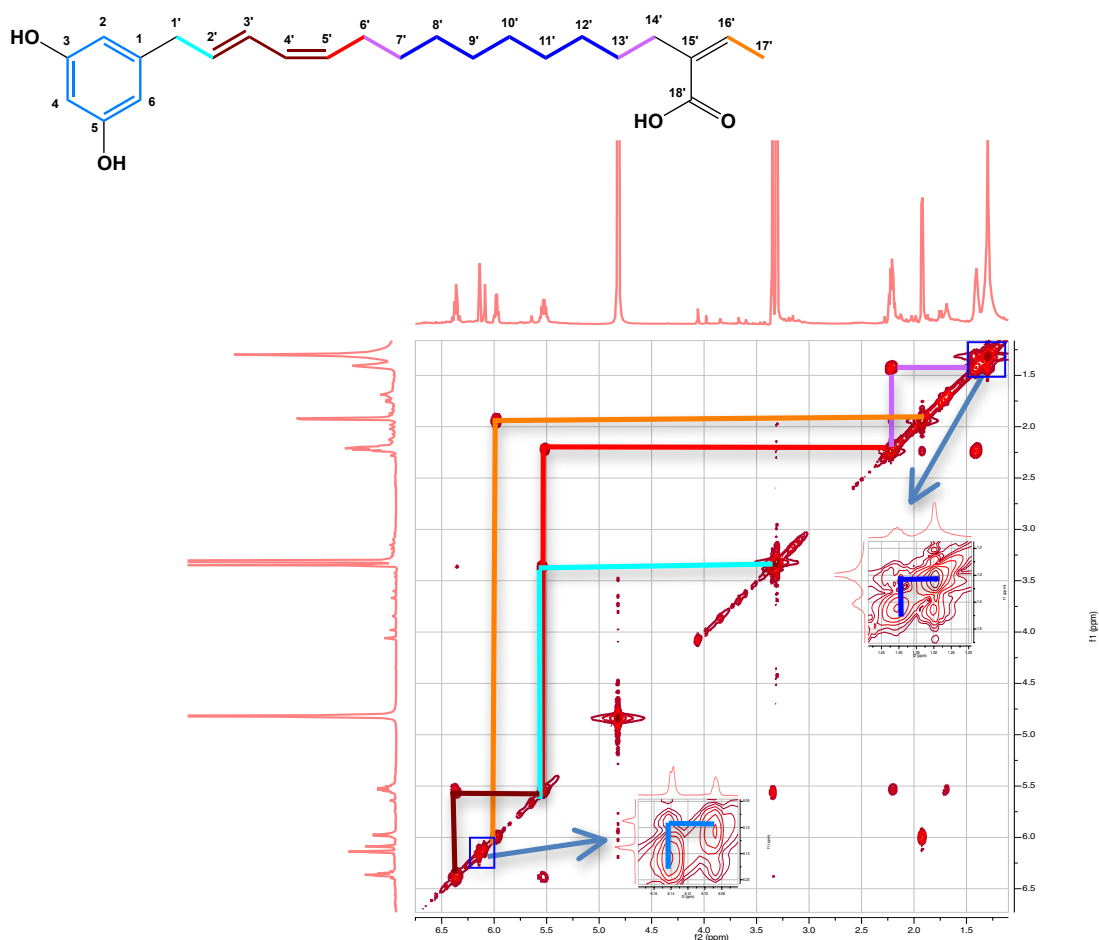
Figure 4.27. ^{1}H -^{1}H COSY NMR correlations of **18**

The configuration of the double bonds at C-2', C-4' and C-15' of the side chain was obtained by comparison of the observed chemical shifts of the respective carbons with ^{13}C NMR data of 5-heptadeca-8'Z,11'Z-dienylresorcinol (Barrow *et al.*, 1991; Jin and Zjawiony, 2006). 5-heptadeca-8'Z,11'Z,16-trienylresorcinol and 5-heptadeca-9'E,11'Z,16-trienylresorcinol (Jin and Zjawiony, 2006). For instance, $\Delta^{2',3'}$ and $\Delta^{15',16'}$ were assigned to have *E*-configuration since the neighboring carbons C-1' and C-14' resonated at about δ_C 35 ppm, whereas the allylic carbon of C-5' (C-6') appeared upfield (δ_C 28.6 ppm) in comparison to C-1' and C-14' and hence indicated a Z-configuration for the double bond $\Delta^{4',5'}$. This is in agreement with other known congeners (Suzuki *et al.*, 1996) where the allylic carbon of an *E*-configurated double bond resonates more downfield than that of a *Z*-configurated one. The configuration of the $\Delta^{15',16'}$ double bond was further confirmed by a ROESY experiment, where the olenific proton H-16' showed a strong correlation with CH_2-14' (Figure 4.29). Accordingly, **18** was identified as a new natural product for which the name chaetorcinol is proposed.
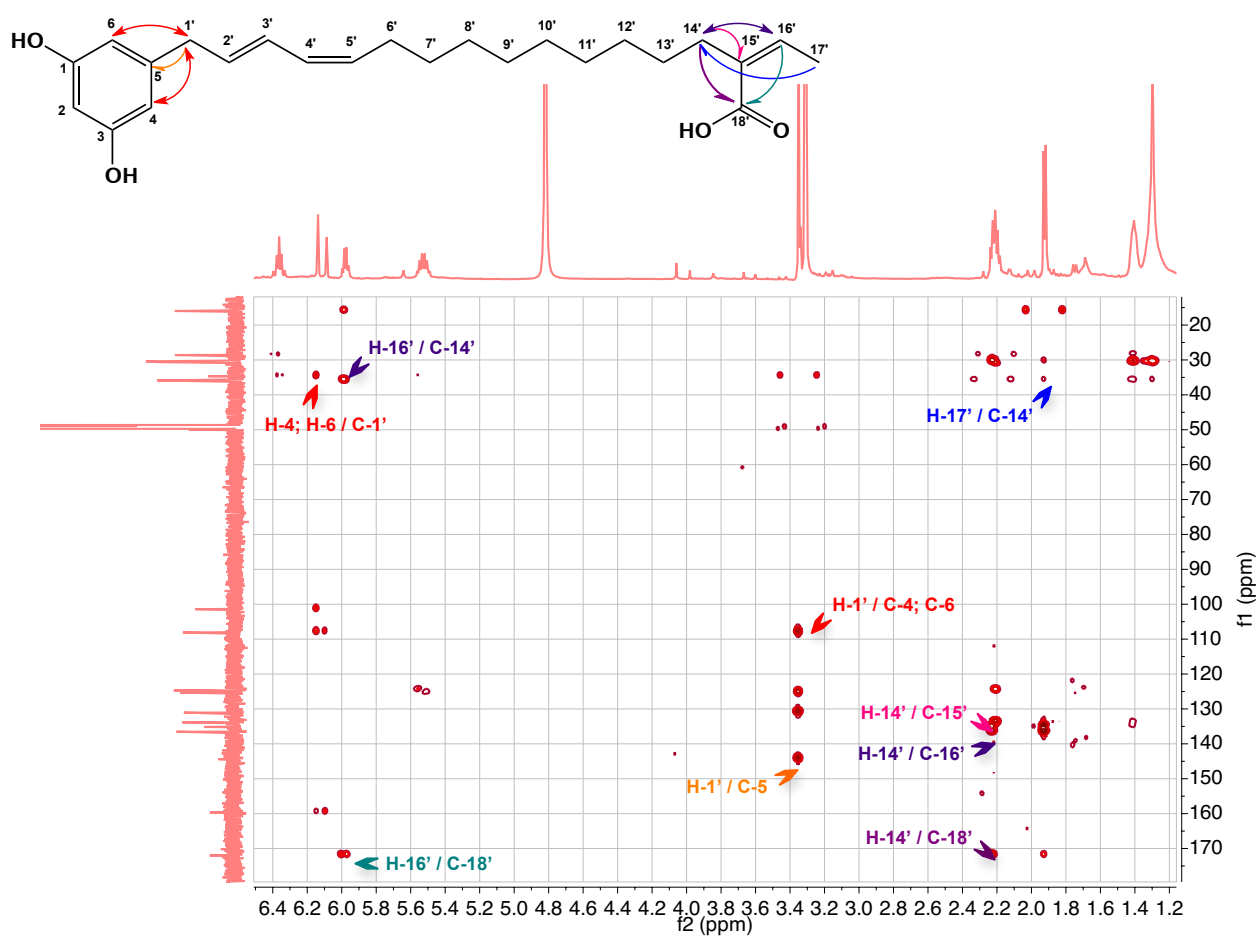
Figure 4.28. Selected ^{13}C-^{1}H HMBC NMR correlations of **18**
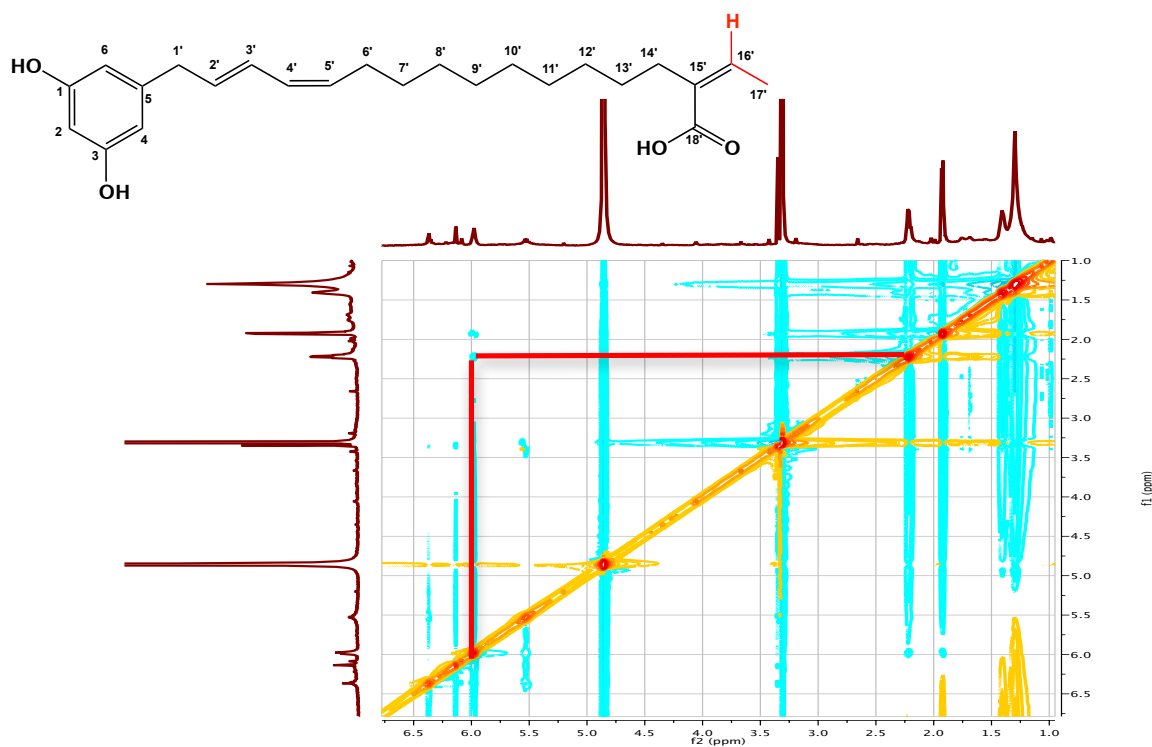
Figure 4.29. Selected ^{1}H -^{1}H ROESY NMR correlations of **18**

Table 4.20. ^{1}H and ^{13}C NMR data of 18 in MeOH at 600 MHz for ^{1}H and 150 for ^{13}C.

Position	18	
	δ_C	δ_H
1	159.7	-
2	101.1	6.08 (1H, s)
3	161.7	-
4	108.0	6.13[b] (1H, s)
5	144.1	-
6	108.0	6.14[b] (1H, s)
1'	34.7	3.35[c] (1H, br)
2'	131.05	5.54 (1H, m)
3'	125.3	6.35 (1H, m)
4'	124.7	6.38 (1H, m)
5'	133.8	5.52 (1H, m)
6'	28.6	2.20 (2H, m)
7'	30.34[a]	1.44 (2H, m)
8'	30.38[a]	1.33 (2H, m)
9'	30.44[a]	1.33 (2H, m)
10'	30.63[a]	1.33 (2H, m)
11'	30.69[a]	1.25 (2H, m)
12'	30.76[a]	1.25 (2H, m)
13'	30.86	1.37 (2H, m)
14'	35.9	2.22 (2H, m)
15'	135.2	-
16'	136.5	5.97 (q, J=7.1 Hz)
17'	15.9	1.92 (d, J=7.1 Hz)
18'	172.0	-

[a,b] may be exchangeable

[c] overlapped with solvent peak

4.5. Bioactivity test results for compounds isolated from the endophytic fungus *Chaetomium aureum*

The isolated compounds were subjected to cytotoxicity (Table 4.21), protein kinase bioassays (Table 4.22) and Hsp 90 chaperone inhibition assays (Figure 4.30). The new compounds were also subjected to antibacterial tests (Table 4.23).

Table 4.21. Cytotoxicity test results for the compounds isolated from endophytic fungus *Chaetomium aureum*

Nr.	Compounds tested	L5178Y growth in %* (Conc. 10 µg/mL)
-	Control	0
13	SB 236050	87
14	Xanthoradone D_1	-3.5
14/15	Xanthoradone D_1 + Xanthoradone D_2	- 3.5
16/17	Xanthoradone E_1 + Xanthoradone E_2	1.6

*Data provided by Prof. W. E. G. Müller, Mainz

Only SB 236050 showed moderate activity against L5178Y cell line, whereas no activity was detected for the other compounds.

Table 4.22. Protein kinase assay results for the compounds isolated from endophytic fungus *Chaetomium aueum*

Compound tested (Conc. 1µg/mL)	Activity on various protein kinases based on IC50 [g/mL]*															
	AKT1	ALK	ARK 5	Aurora-B	AXL	FAK	IGF 1-R	MEK 1 wt	MET wt	NEK 2	NEK 6	PIM 1	PLK 1	PRK 1	SRC	VEGF-R2
(+)-Sclerotiorin	A	A	M	S	M	A	A	0	S	A	A	A	M	M	A	A
(+)-Isochromophilone IV	0	0	0	M	0	M	M	0	0	0	0	M	0	0	M	0
(+)-Sclerotioramin	0	M	0	M	0	M	A	0	0	0	0	0	0	0	A	A
SB 236050	0	0	0	S	0	M	M	0	0	M	0	M	M	0	0	M
Chaetoresorcinol	0	A	0	A	M	A	S	0	M	A	M	0	0	0	A	A

* Data provided by ProQinase, Freiburg.

S: strongly active, A: active, M: moderately active, 0: not active

The results of the protein kinase assay showed a different activity profile than the cytotoxicity assay results. (+)-Sclerotiorin was active against most of protein kinases in spite of their low activity in the cytotoxicity assay, whereas its derivatives, isochromophilone VI and sclerotioramin inhibited only few of the tested enzymes. On the other hand, the new compounds, chaetoresorcinol and SB 236050, were also active against the tested protein kinases.

Table 4.23. Antibacterial test results for the new compounds isolated from endophytic fungus
Chaetomium aureum

Nr.	Name	The Minimum Inhibitory Concentration													
		MRSA [µg/ml]	*Strep. Pneumonia*	*Entero. Faecalis*	*Staph. Epidermidis*	*Entero. cloace*	*E. Coli*	*Klep. Pneumonia*	*Pseudo. aeruginosa*	*Acin. Baumanii*	*Entero-bacter*	*C. albicans*	*C. krusei*	*Asp. Faecalis*	*Asp. Fumigatus*
13	SB 236050	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
14	Xanthoradone D$_1$	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
14/15	Xanthoradone D$_1$ + Xanthoradone D$_2$	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
16/17	Xanthoradone E$_1$ + Xanthoradone E$_2$	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
18	Chaetoresorcinol	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	7.81

* Data provided by Mr. Pretsch, Vienna.

Chaetoresorcinol was moderately active against only *Asp. Fumigatus,* whereas the other compounds were not active.
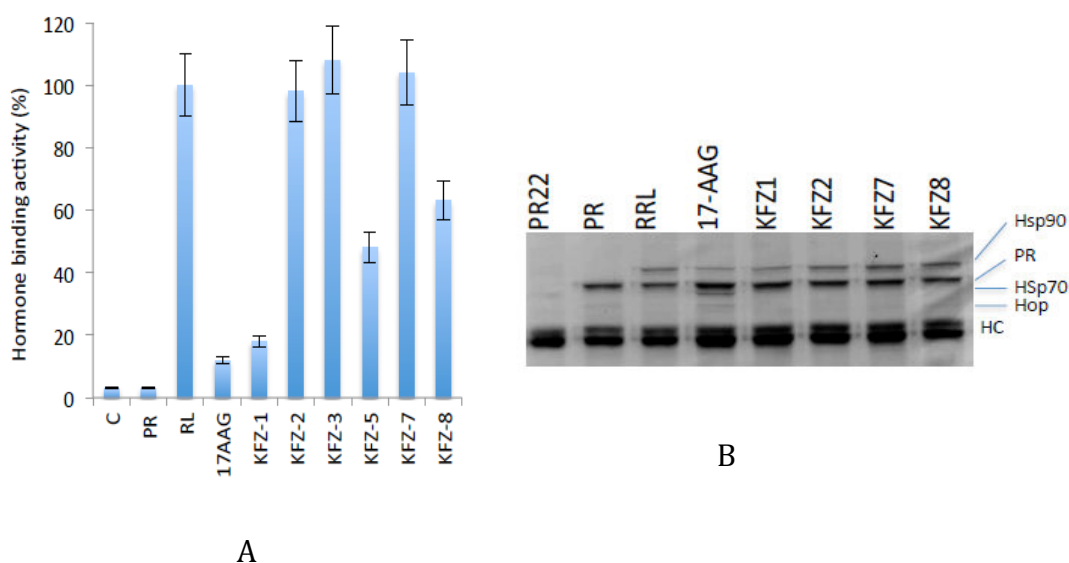
Figure 4.30. Effect of **1-6** on the Hsp90 chaperoning machine *in vitro*. A- PR hormone binding activity was reconstituted using rabbit reticulocyte lysate without (RL) or with metabolites KFZ-1: (+)-Sclerotiorin, KFZ-2: (+)-Isochromophilone VI, KFZ-3: SB 236050, KFZ-5: Chaetoresorcinol, KFZ-7: Sclerotioramin and KFZ-8: Isochromophilone VII. 17-AAG is used as a positive control. B- Samples from (A) were used for analysis of protein complexes by SDS-PAGE and Coomassie blue staining. PR22 indicates the PR antibody incubated with RRL. PR indicates the PR alone with no RRL.

As shown in (Figure 4.30), sclerotiorin efficiently inhibits the recovery of PR hormone binding activity using RRL. Sclerotiorin showed similar efficiency to that of 17-AAG, the classical inhibitor of Hsp90, whereas the other compounds showed much less activity than sclerotiorin.

Cytotoxicity test showed that sclerotiorin is not toxic to breast cancer cell line Hs578T, MCF7, MDA-MB-231, MDA-MB-453, Prostate cancer cell line LNCaP and cervical cancer cell line HeLa.

In an attempt to increase the cytotoxicity of sclerotiorin, a deacetylation was carried out.
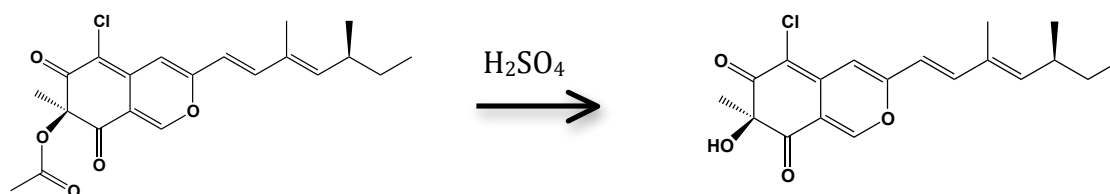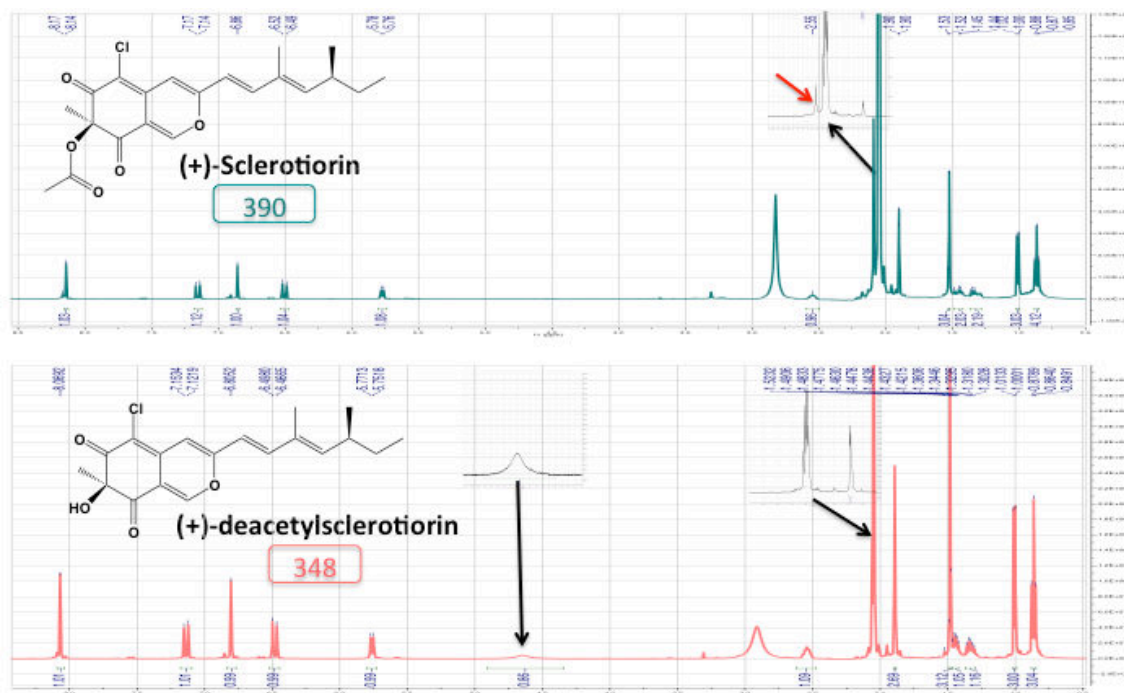
Figure 4.31. Comparison of ^{1}H NMR and the molecular weight of sclerotiorin and deacetylsclerotiorin

The ^{1}H NMR (DMSO) of deacetylsclerotiorin showed the abscence of the methyl group at (δ_H 2.11 ppm) and the presence of a broad proton signal at (δ_H 4.6 ppm) assigned to the hydroxyl group, which replaced the acetyl group.
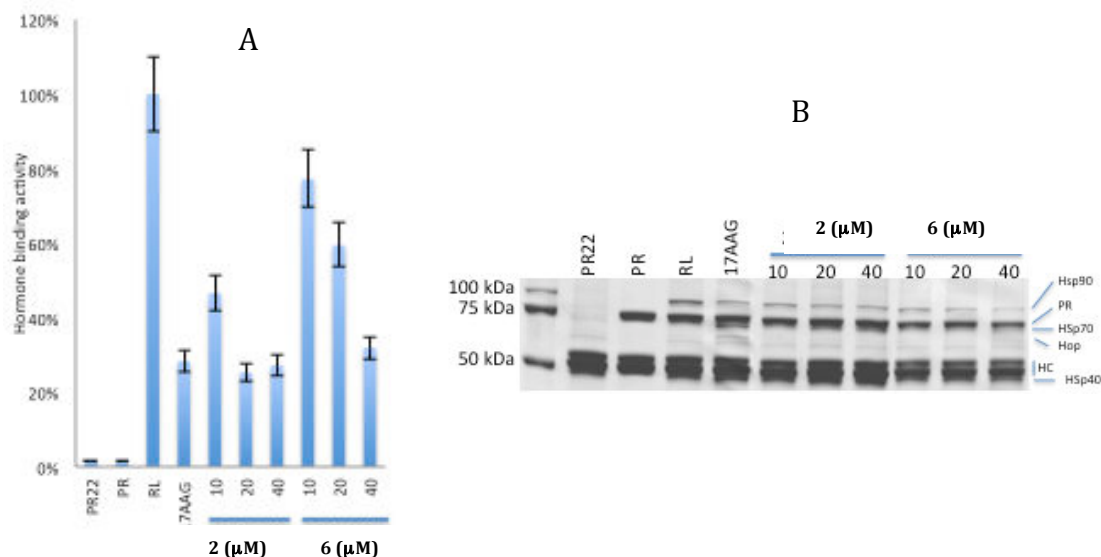
Figure 4.32. Comparison of sclerotiorin (**2**) and deacetylsclerotiorin (**6**). A- effect increasing concentration of sclerotiorin (**2**) and dacetylsclerotiorin (**6**) on PR hormone binding activity. B- Effect of sclerotiorin (**2**) and dacetylsclerotiorin (**6**) on PR protein complexes. Molecular chaperones are indicated on the right. HC represents the heavy chains of the PR antibody PR22.

The deacetylated sclerotiorin (**6**) is relatively less efficient in inhibiting the Hsp90 *in vitro*. The mechanism by which sclerotiorin and deacetylsclerotiorin inactivate the Hsp90 machine is not clear but it seems to be different from that of the 17-AAG.

As compared to 17-AAG, protein complexes analysis showed that sclerotiorin and deacetylsclerotiorin lower the level of Hsp90 without increase of Hop (Figure 4.32.B). Furthermore, deacetylsclerotiorin does not increase Hsp70 level in PR complexes (Figure 4.32.B).
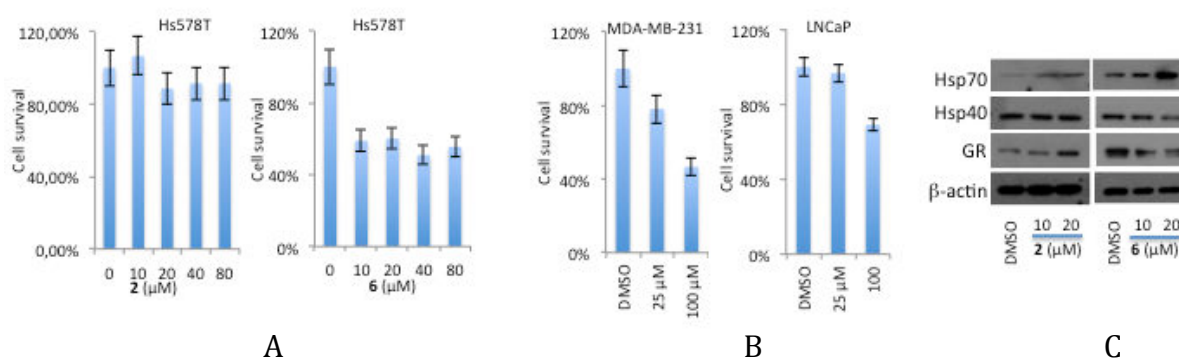
Figure 4.33. A- Comparison of the effect of sclerotiorin (**2**) and deacetylsclerotiorin (**6**) on cell survival of the breast cancer cell line Hs578T using MTS assay. B- Deacetylsclerotiorin (**6**) inhibits the growth of breast cancer cell line MDA-MB-231 and prostate cancer cell line LNCaP. C- Western blot analysis comparing the effect of sclerotiorin (**2**) and deacetylsclerotiorin (**6**) on the expression of Hs70, Hsp40 and GR. b-actin is used as a loading control.

Surprisingly, at inhibitory concentrations, deactylsclerotiorin becomes cytotoxic to Hs578T, MDA-MB-231 and LNCaP cell lines over a period of 48h (Figure 4.33.A-B). Western blot analysis (Figure 4.33.C) showed that sclerotiorin induces overexpression of Hsp70 and degradation of Hsp90 client proteins such as glucocorticoid receptor in Hs578T cells, indicating that the Hsp90 machine may be inhibited *in vivo*. It is worth noting however, that sclerotiorin also induces degradation of the heat shock protein 40 (Hsp40) (Figure 4.33.C) which may contribute to some of sclerotiorin's cytotoxicity.

5. Discussion

5.1. The use of medicinal plants in Morocco

The ethnopharmacological approach has been the platform for research programs with the objective to study plants used in traditional medicine in different cultures and to select one of the most used medicinal plant for its anticancer effect. For this, the choice of the National Institute of Oncology was taken based on the heterogeneous origin and the different socio-professional level of the patients, which are coming from different regions of Morocco.

In Morocco, the use of medicinal plants for therapeutic purposes is considered by many people as not being very efficient, but for being at least well tolerated because it is natural and is part of "soft" medicine. In all regions of Morocco, each home has a stock of medicinal plants that are used as home pharmacy for a number of diseases (Weniger, 1991). Moreover, people have free access to medicinal plants "without prescription" (Claisse, 1990).

Furthermore, the majority of patients who use medicinal plants have a low income and live far from the hospital, which explains their resorting to medicinal plants to be cured. The origin and their educational level were also a variable because in traditional medicine, the statements of an illiterate or of an expert are not the same. Many studies have shown that traditional medicine is still used allover Morocco. To approve the biological activity of each plant scientifically, plant extracts must be evaluated for their suspected biological effect.

5.2. Selection of interesting medicinal plants based on an ethnopharmacology survey

Fifty-five plants have been cited during this investigation. Among these plants 28 are proven to have anticancer activity. *Aristolochia longa*, *Trigonella foenum-graecum*, *Cassia absus* and *Nigella sativa* are the most used plants. However, there are plants that have been found to be toxic such as *Pinus halepinsis*, *Peganum harmala*, *Aristolochia longa* and *Euphorbia resinifera*. Regardless of their toxicity these plants are used by patients in the INO irrespective of the degree of toxicity or side effects.

Thymelaea lythroides was used by two patients, which have uterus cancer. The plant was taken as tea three times a day. Side effects by using this plant were not observed, in contrary to the effects of chemotherapy, but the effectiveness was similar.

From the best of our knowledge, *Thymelaea lythroides* has not been yet investigated for their anti-cancer activity, even though many herbalists have recommended the use of this plant for that purpose.

5.3. Chemical diversities of the genus *Thymelaea* secondary metabolites

Thymelaeaceae is a medium size family of dicotyledons comprising about 1200 species distributed in 67 genera and occurs in all continents in latitudes south of 40°N. It is found in both temperate and tropical regions, but is more diverse in the southern than in northern hemisphere (Ferreira *et al.*, 2009).

Thymelaea genera have a lot of uses in folk medicine. Over the years, this species has been used as a laxative, diuretic, antibacterial, antitussive, expectorant, antiparasitic and also as an antitumor agent (Ferreira *et al.*, 2009).

Thymelaea Mill. comprises 31 species of xerophyllous shrubs and herbs. *Thymelaea* is a Mediterranean genus belonging to a primarily tropical and subtropical family. The genus *Thymelaea* is poorly investigated. Only 4 species are studied: *T. hirsute*, *T. passerine*, *T. tartonraira* and *T. microphylla*. The Table 5.1 summarizes the bioactive metabolites reported from the genus *Thymelaea*.

Table 5.1. Bioactive metabolites from the genus *Thymelaea* (Borris *et al.*, 1988)

Compounds	Species	References
Coumarins		
Daphnetin	*Thymelaea hirsuta*	Rizk et al., 1975
Daphnetin-8-ß-D-glucoside	*Thymelaea hirsuta*	Rizk et al., 1975
Daphnin	*Thymelaea hirsuta*	Rizk et al., 1975
Daphnoretin	*Thymelaea hirsuta*	Rizk et al., 1975; Rizk et al., 1972
	Thymelaea tartonraira	Garcia-Grandos, 1980
Daphnorin	*Thymelaea hirsuta*	Rizk et al., 1975
Esculetin	*Thymelaea hirsuta*	Rizk et al., 1975
Scopoletin	*Thymelaea hirsuta*	Rizk et al., 1975
Sphondin	*Thymelaea passerina*	George and Rishi, 1982
Umbelliferone	*Thymelaea hirsuta*	Rizk et al., 1975
	Thymelaea passerina	George and Rishi, 1982
Flavonoids		
Genkwanin	*Thymelaea tartonraira*	Garcia-Grandos, 1980
Genkwanin-5-O-ß-D-	*Thymelaea tartonraira*	Garcia-Grandos, 1980

primeverosyl		
Kaempferol	*Thymelaea tartonraira*	Garcia-Grandos, 1980
Orientin	*Thymelaea tartonraira*	Garcia-Grandos, 1980
Vicenin-2	*Thymelaea hirsuta*	Nawwar et al., 1977
	Thymelaea tartonraira	Garcia-Grandos, 1980
Vitexin	*Thymelaea tartonraira*	Garcia-Grandos, 1980
Triterpenes		
ß-Amyrin	*Thymelaea hirsuta*	Garcia-Grandos, 1980
	Thymelaea passerina	George and Rishi, 1982
Betulin	*Thymelaea hirsuta*	Garcia-Grandos, 1980
Erythrodiol	*Thymelaea hirsuta*	Garcia-Grandos, 1980
Lanosterol	*Thymelaea hirsuta*	Garcia-Grandos, 1980
Lupeol	*Thymelaea hirsuta*	Garcia-Grandos, 1980
Sterols		
Campesterol	*Thymelaea hirsuta*	Rizk et al., 1974
Cholesterol	*Thymelaea hirsuta*	Garcia-Grandos, 1980
ß-sitosterol	*Thymelaea hirsuta*	Rizk et al., 1975
	Thymelaea passerina	George and Rishi, 1982
ß-sitosterol-ß-D-glucoside	*Thymelaea hirsuta*	Rizk et al., 1972
	Thymelaea passerina	George and Rishi, 1982
Stigmasterol	*Thymelaea hirsuta*	Gharbo et al., 1970
Lipids		
n-Docosanol	*Thymelaea hirsuta*	Rizk et al., 1974
n-Heptacosane	*Thymelaea hirsuta*	Rizk et al., 1974
n-Hexacosanol	*Thymelaea hirsuta*	Rizk et al., 1974
n-Nonacosane	*Thymelaea hirsuta*	Rizk et al., 1974
n-Octacosane	*Thymelaea hirsuta*	Rizk et al., 1974
n-Octacosanol	*Thymelaea hirsuta*	Rizk et al., 1974
	Thymelaea hirsuta	Rizk et al., 1974
n-Tetracosanol	*Thymelaea hirsuta*	Rizk et al., 1974
n-Triacontane	*Thymelaea hirsuta*	Rizk et al., 1974
Fatty acids		
Arachidic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Dihydromalvalic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Linoleic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Linolenic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Myristic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Oleic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Palmitic acid	*Thymelaea hirsuta*	Rizk et al., 1974
Stearic acid	*Thymelaea hirsuta*	Rizk et al., 1974

5.4. Methodologies for profiling of the metabolites

Isolation and structure elucidation processes enrich the chemistry of natural products (Butler, 2004). Hence, interesting fungal strains can be elected to be screened, which together with the use of spectroscopic methods in addition to chemoinformatics can be used as part of an effective dereplication protocol (Larsen *et al*., 2005). Secondary metabolites profiling is not an easy task to be applied since natural products display very diverse chemical structures. Thus, a single analytical method does not exist, which is capable of profiling all natural products in the investigated extract (Wolfender *et al*., 2005). However, advanced analytical and spectroscopic techniques, like the hyphenated techniques linked with HPLC, can provide a good idea about the substructures and/or functional groups in the chemical structure.

5.4.1. HPLC/UV

With the advancement of HPLC as well as much more stable and better columns for high resolution separation, combined with fast UV diode array detectors, it has become easy to acquire the UV spectrum of practically every single component from an extract, provided a suitable chromophore is existing. Consequently the UV spectrum has turned into one of the most readily accessible pieces of information related to structure of natural products that increased the interest in exploiting its usefulness (Cannall, 1998).

In the present study, a lot of chemical compounds that share similar chromophoric functions were examined by the hyphenated HPLC/UV-photodiode array detection (LC/UVDAD) technique, which showed very often similar UV spectra, even though there were significant differences in additional non-chromophoric functions.

5.4.2. HPLC/ESI-MS

Electrospray ionization mass spectrometry (ESI-MS) and the associated techniques provided the scientific community with a highly versatile method for studies of secondary metabolites. ESI-MS is a soft and sensitive ionization technique which can be adjusted to produce mainly protonated or sodiated ions (assuming positive ESI) from a wide range of natural products (Smedsgaard and Frisvad, 1996). Moreover, this method is also useful in establishing the related secondary metabolites. In the context of the present study, this proved extremely valuable in detecting azaphilone derivatives (9, 10, 11 and 12) and xanthoradone derivatives (14, 15, 16 and 17).

5.5. Secondary metabolites isolated from the plant *Thymelaea lythroides*

Thymelaea lythroides is growing wild in Mâamora forest, called by Moroccan people as "Methnan" and used as a folk remedy for otitis, diabetes, rheumatism and inflammation of the prostate (Gmira *et al*., 2007).

Eight compounds were isolated from areal parts of *Thymelaea lythroides* including one depsipeptide (**1**), two coumarins (**2**, **3**), one dicoumarin (**4**), two lignans (**5**, **6**), one flavonoid glucoside (**7**) and one dicoumarin glycoside (**8**).

4.5.1. Depsipeptides

Natural or synthetic compounds having sequences of amino and hydroxy carboxylic acid residues (usually α-amino and α-hydroxy acids), exhibit commonly but not necessarily regularly alternating units. In cyclodepsipeptides the residues are connected in a ring (IUPAC, 1997).

Romidepsin is an epsipeptide and is a member of the bicyclic peptide class of histone deacetylase inhibitors and was first isolated as a fermentation product from Chromobacterium violaceum by the Fujisawa Pharmaceutical Company (Yurek-George *et al*., 2007). It is being used in the treatment of some cancers where it is thought to reactivate silenced genes.

The depsipeptide etamycin, a newly isolated natural product from a marine actinomycete, shows potent activity in vitro and in mice against MRSA (Haste *et al*., 2010). It was first observed to have a positive role on gene expression in 1990. A clinical trial was conducted in 1996 for treating T cell lymphoma (Piekarz and Bates, 2004). Another natural depsipeptide HDAC inhibitor is spiruchostatin A (Yusuke *et al*., 2006).

5.5.2. Coumarins

Coumarin is a fragrant compound in the benzopyrone chemical class and is a colorless crystalline substance in its standard state. It is found naturally in many plants. Coumarins are used in the pharmaceutical industry as precursor molecules in the synthesis of a number of synthetic anticoagulant pharmaceuticals similar to dicoumarol. Coumarins have shown many biological activities, but are approved for only a few medical uses as pharmaceuticals. Reported coumarin activity includes anti-HIV, anti-tumor, anti-hypertension, anti-arrhythmia,

anti-inflammatory, anti-osteoporosis, antiseptic, and analgesic activity. It is also used in the treatment of asthma and lymphedema (Liu *et al*., 2011).

5.5.3. Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature. Over 4,000 flavonoids have been identified. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Preliminary research indicates that flavonoids may modify allergens, viruses, and carcinogens, and so may be biological "response modifiers". *In vitro* studies show that flavonoids also have anti-allergic, anti-inflammatory, anti-microbial, anti-cancer, anti-diarrheal and antiviral activities (Spencer, 2008; Yamamoto *et al*., 2001; Cushnie *et al*., 2005; Cushnie and Lamb, 2011; de Sousa *et al*., 2007; Schuier *et al*., 2005; González *et al*., 1990).

5.5.4. Lignans

Lignans are polyphenols found in plants. Their precursors are found in a wide variety of plant-based foods, including seeds, whole grains, legumes, fruits, and vegetables.

Lignan-rich foods are part of a healthful dietary pattern, but the role of lignans in the prevention of hormone-associated cancers, osteoporosis, and cardiovascular diseases is not yet clear.

5.6. Biosynthesis of the secondary metabolites

Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism, while primary metabolites play a key role in the survival of the species, playing an active function in the photosynthesis and respiration. Most of the secondary metabolites are classified based on their biosynthetic origin. Before looking at individual pathways in detail, it is helpful to consider the overall pathway of carbon from carbon dioxide and the photosynthetic formation of sugars. This is shown in Figure 5.1.

In this context a distinction may be drawn between primary and secondary metabolic pathways. The former are involved in the storage and release of energy and in the synthesis of essential cellular constituents such as the amino acids and the nucleic acids. The latter involve the biosynthesis of the metabolites, which are characteristic of each species. Different classes of these metabolites are often associated to a narrow set of species within a phylogenetic

group and constitute the bioactive compounds in several medicinal, aromatic, colorant, and spice plants and/or functional foods.
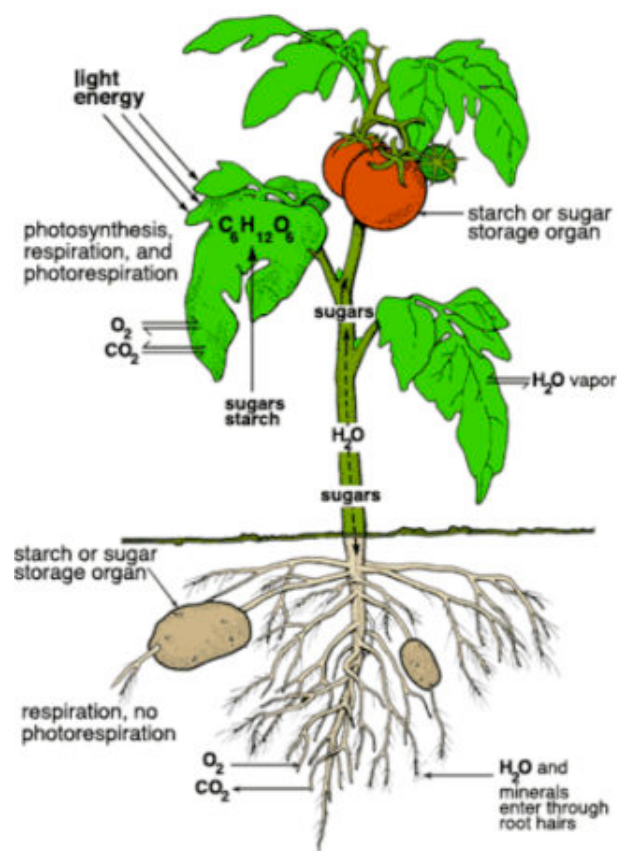
Figure 5.1. Photosynthesis, respiration, leaf and water exchange and translocation of sugar in plant (Silverstein *et al.*, 2008)

In this context a distinction may be drawn between primary and secondary metabolic pathways. The former are involved in the storage and release of energy and in the synthesis of essential cellular constituents such as the amino acids and the nucleic acids. The latter involve the biosynthesis of the metabolites, which are characteristic of each species. Different classes of these metabolites are often associated to a narrow set of species within a phylogenetic group and constitute the bioactive compounds in several medicinal, aromatic, colorant, and spice plants and/or functional foods.

In essence, secondary metabolism utilizes a limited number of metabolites available from primary metabolism in novel ways. Four major pathways have emerged and two of them in particular show a considerable degree of structural homogeneity (Scheme 5.1). In the first, the "**isoprene pathway**", the precursor is mevalonic acid derived from acetyl-CoA. In the

second, the "**polyketide pathway**", the primary metabolites are the CoA derivatives of lower fatty acids together with their carboxylated derivatives; typical examples are acetyl-CoA and malonyl-CoA, propionyl-CoA and methylmalonyl-CoA, and butyryl-CoA and eth- ylmalonyl-CoA. Multiple condensations lead to polyketomethylene structures; thus, 1 acetyl- CoA and n malonyl-CoA yield CH_3 -CO-(CH_2 - CO)$_n$ -CoA, and 1 propionyl-CoA and n methyl-malonyl-CoA give CH_3 -CH_2 -CO-(CH[CH_3]- CO)$_n$ -CoA.
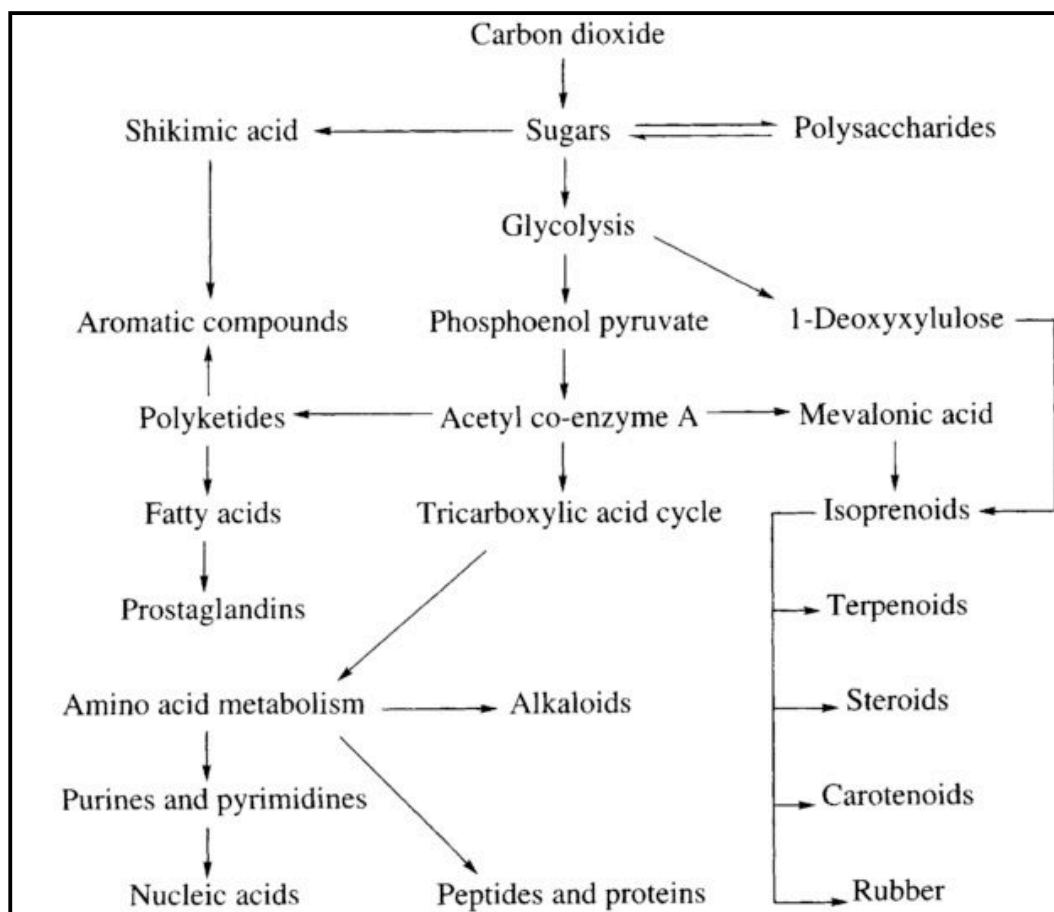
Scheme 5.1. The major biosynthesis pathways of secondary metabolites

The remaining two major pathways are less easily summarized because of the structural diversity of the secondary metabolites. The "**shikimate pathway**" uses shikimic acid itself as the primary metabolite or, alternatively, either an intermediate involved in shikimate formation or a further primary metabolite derived from shikimate (e.g., isochorismate). Many aromatic products originate in this way, as well as some nonaromatic, cyclohexane structures. Finally, the fourth major pathway involves secondary metabolites constructed from "**amino acids**" as precursors; many plant alkaloids are formed in this way. In some cases, secondary metabolite formation requires more than one amino acid. While these four pathways are the

major biosynthetic routes to secondary metabolites, there are still other possibilities such as the compounds that are derived directly from carbohydrates without cleavage of the carbon chain and those derived from intermediates of the tricarboxylic acid cycle. Moreover, many secondary metabolites are biosynthesized by a mixed process involving a combination of two (or more) of these major pathways. A classic example is the ergot alkaloid biosynthesis involving the amino acid, tryptophan, and addition of a single isoprene unit.

5.6.1. Biosynthesis pathway of the isolated compounds from *Thymelaea lythroides*

All isolated compounds were derived from the shikimate biosynthesis pathway. The shikimate pathway provides an alternative route to aromatic compounds, particularly the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan. This pathway is employed by microorganisms and plants, but not by animals; accordingly, the aromatic amino acids feature among the essential amino acids for man and have to be obtained in the diet. A central intermediate in the pathway is shikimic acid, a compound which had been isolated from plants of *Illicium* species (Japanese 'shikimi') many years before its role in metabolism had been discovered. Phenylalanine and tyrosine form the basis of phenylpropane units found in many natural products, e.g. coumarins, lignans, and flavonoids, and along with tryptophan are precursors of a wide range of alkaloid structures.

Nevertheless, distinct differences in the basic biosynthetic pathways exist. For example, plants and microorganisms can synthesize their amino acids from simple building blocks, whereas most animals lost this ability in the course of their adaptation to heterotrophic life. Thus, many biosynthetic pathways are regarded as specific for certain groups of organisms. The ability to form thousands of structurally diverse natural products due to secondary metabolism is considered a typical feature of plants and microbes.
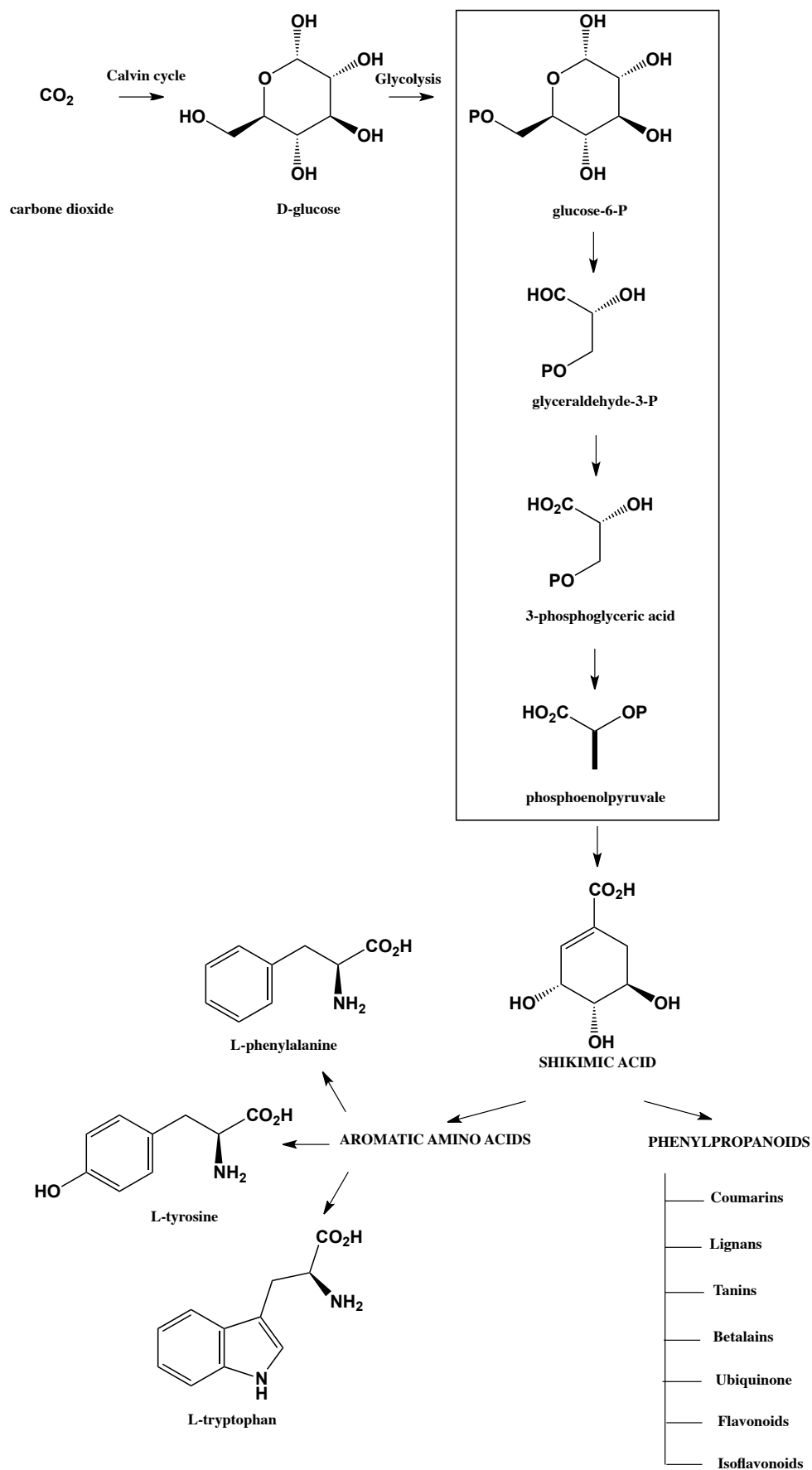
Scheme 5.2. The shikimate pathway: aromatic amino acids and phenylpropanoids

5.7. Bioactivity of the isolated compounds from *Thymelaea lythrodies*

5.7.1 Bioactivity of coumarins and dicoumarins isolated from *T. lythroides*

Coumarins and dicoumarins derivatives isolated from *T. lythroides* were subjected to cytotoxicity testing toward L5178Y (mouse lymphoma) cell line. (+)-Dapheolone (3) was the most active of the compounds, whereas daphnoretin (6) and rutarensin (7) were found to be inactive. However, dapheolone and daphnoretin were inactive in the protein kinase inhibition assay, nevertheless the dicoumarin glucosides rutarenside (7) was active against Aurora-B, IGF 1-R and VEGF-R2, which suggests that rutarensin can not pass through the cell membrane. Furthermore, all compounds were inactive in the Hsp 90 chaperone machinery inhibition assay.

Despite the activity of rutarensin against the mentioned protein kinases, it was inactive against the Hsp 90 chaperone machinery, indicating that the mechanism of the inhibition of the protein kinases could not have an interaction with protein kinases.

5.7.2. Bioactivity of lignans isolated from *T. lythroides*

(-)-Wiksromol (4) and *δ*-sesamin (5) were inactive against cytotoxicity tests toward the L5178Y (mouse lymphoma) cell line, protein kinase inhibition assay and Hsp 90 chaperone machinery inhibition assay, although in the literature, (-)-wikstromol (4) has cytotoxic potential against human cancer cell lines (Singh *et al*., 2007), and *δ*-sesamin (5) showed mild antiplasmodial activities and cytotoxicity (IC50 3.37 and 3.50 µg/mL resp.) without noticeable toxicity on mammalian normal cells (Risoleta *et al*., 2011).

5.7.3. Bioactivity of flavonoid glycosides isolated from *T. lythroides*

The flavonoid glycosides *trans*-tilliroside (8) was inactive against cytotoxicity tests toward the L5178Y (mouse lymphoma) cell line, which suggested that *trans*-tilliroside protein kinase activity against Aurora-B, ALK and VEGF-R2 is not occuring be *in vivo*, as the compound can not enter in the cell.

5.7.3. Bioactivity of depsipetides isolated from *T. lythroides*

Bassiatin (1) was inactive against cytotoxicity test toward the L5178Y (mouse lymphoma) cell line, protein kinase inhibition assay and Hsp 90 chaperone machinery inhibition assay, however, bassiatin was reported to have cytotoxicity activity against breast cancer cells (MCF-7) (Li *et al*., 2012).

5.8. Selection of the interesting endophytic fungus strains isolated from *Thymelaea lythroides*

Five endophytic fungus strains, *Chaetomium aureum*, *Pleospora sp.*, *Cladosporieum sp.*, *Alternaria sp.* and *Epicoccum nigrum,* were isolated from healthy stem tissues of *Thymelaea lythroides* (*Thymelaeaceae*). The pure fungus strains were cultivated on rice solid medium. Preliminary biological assay (antibacterial and cytotoxicity assay) with the analysis of HPLC chromatogram of the EtOAc extracts of the five pure fungus strains showed that extracts of *Chaetomium aureum* were much more active, displaying a variety of chemical classes based on analysis of UV spectra.

This fungus strain was cultivated more than five times under the same conditions to evaluate its stability. All extracts showed the same HPLC chromatogram, which reinforces the choice of this fungus as a subject of the study by the factor of stability.

5.9. Choice of culture media

Medium composition and culture conditions are one of the factors having great impact on growth and production of secondary metabolites from microorganisms (Bills, 1995). The physiology of secondary metabolism has often been ignored and still few of the regulatory features involved in the biosynthesis of natural products have been elucidated. Thus it is important to grow the organism in different media in order to have a broad range of secondary metabolites as possible candidates for a given strain to increase the chance to generate novel drug candidates (Larsen *et al*., 2005). Furthermore, some natural products are only produced under certain environmental conditions and only when all trace metals, phosphate and other medium factors are present in certain ranges of concentrations (Knight *et al*., 2003).

Thus, optimal media for good metabolite production can change for different genera being investigated (Larsen *et al*., 2005).

Different and relatively easy to control conditions to investigate in a discovery program include growing cultures at both solid and liquid conditions, incubation at two or more temperatures, incubation at two or more shaker speeds, incubation for at least two different time periods, media with at least two different pH levels, choosing carbon and nitrogen sources at different concentrations, high- or low phosphate content, adding trace minerals etc. (Knight *et al*., 2003).

Some authors strongly argue in favor of using solid substrate fermentations in studies of fungal metabolites since fungi, unlike other microorganisms, typically grow in nature on solid substrates such as wood, roots and leaves of plants (Nielsen *et al.*, 2004). On the other hand, some believe and argue that all metabolites can be expressed in liquid culture by varying carbohydrate composition, nitrogen source, oxygen tension, pH, redox potential, water activity, as the right conditions will produce intracellular conditions that will trigger production of a certain metabolite. It was found that metabolites associated to spore or sclerotia formation are often produced under solid conditions, whereas the production of other metabolites is enhanced under liquid conditions (Larsen *et al.*, 2005, Nielsen *et al.*, 2004).

In a screening effort aimed at the discovery of novel natural products, which includes a variety of different strains, one has to focus on a limited set of parameters, since otherwise the sheer number of extracts generated will easily be overwhelming. Thus, in this study selected fungal strain was cultured in liquid (Wickerham) medium as well as on eleven solid mediums. Bioactivity and chemical profiles of the obtained extracts from all cultures were compared and subjected to further investigation.

HPLC chromatograms of the EtOAc extracts of liquid and solid cultures showed different chemical patterns for the fungal strain investigated in this study. Moreover, when EtOAc extracts of liquid and solid cultures were compared, it was found that the yield of dry extract obtained from rice cultures was higher than that the others media. However, it cannot be excluded that this finding, at least to a certain degree, was due to the fact that more polar material, e.g. sugars or amino acids, were extracted from the culture medium in the case of the solid rice cultures compared to the liquid medium.

4.10. Chemical diversities of the genus *Chaetomium* secondary metabolites

Fungi have proved to be a rich source of a huge number of secondary metabolites with a large variety of chemical structures and diverse biological activities. Fungal metabolites are of considerable importance as new lead compounds for medicine as well as for plant protection (Zhang *et al.*, 2012).

Chaetomium is a large genus of the fungal family Chaetomiaceae (subdivision Ascomycota) which contains over one hundred of both marine- and terrestrial-derived species. Members of this filamentous fungal genus, *Chaetomium atrobrunneum, C. funicola, C. globosum* and *C.*

strumarium, are most commonly found in soil, air, and plant debris, and are encountered as causative agents of infections in humans. In the past decades, fungi of the Chaetomium genus have been revealed to be a rich source of fascinating and structurally complex natural products. To date, more than 200 compounds have been reported from this genus. A huge number of new and bioactive secondary metabolites associated with unique and diverse structural types, such as chaetoglobosins, epipolythiodioxopiperazines, azaphilones, depsidones, xanthones, anthraquinones, chromones, terpenoids, and steroids, have been isolated and identified. Many of the compounds have been reported to possess significant biological activities, such as antitumor, antimalarial, cytotoxic, enzyme inhibitory, antibiotic, phytotoxic, phytotoxic and other activities (Zhang *et al.*, 2012).

5.11. Secondary metabolites isolated from the endophytic fungi *Chaetomium aureum*

In this investigation, 18 compounds were isolated from the endophytic fungi *Chaetomium aureum* including four azaphilones derivatives (9-12), one pyranochromone (13), four xanthones derivatives (14-17) and one alkylresorcinol (resorcinolic lipid) (18).

One azaphilone derivative (19) was semi-synthetic from one of the isolated compounds.

5.11.1. Azaphilones

The azaphilones are a family of structurally diverse fungal polyketides possessing a pyranoquinone ring systems and containing a chiral quaternary center and a highly oxygenated bicyclic core, and sometimes bearing a five membered lactone ring. To date, 29 compounds of this class have been discovered in the genus *Chaetomium*, some of them displayed antifungal, antibacterial, antimalarial and selectively cytotoxic activities (Zhang *et al.*, 2012).

5.11.2. Pyranochromones

From the genus *Chaetomium* only three pyranochromones, SB236050, SB238569, and SB236049, were identified from a strain of *Chaetomium funicola*, which are metallo-ß-lactamase inhibitors in the *Bacillus cereus* II enzyme assay. Of these compounds SB238569 was the most active. Analysis of the crystal structure of SB236050 complexed in the active site of CfiA indicates that SB236050 exhibits key polar interactions with Lys184, Asn193, and His162 and a stacking interaction with the indole ring of Trp49 in the flap, which is in the closed conformation over the active site groove (Payne *et al.*, 2002).

5.11.3. Xanthones

Xanthones are a structurally diverse group of natural products, derived from plants, lichens, and fungi, with a broad range of biological activities. Two xanthones, O-methylsterigmatocystin and sterigmatocystin were first isolated in 1979 from cultures of *Chaetomium thielavioideum.*

5.11.4. Alkylresorcinols

Alkylresorcinols, also known as resorcinolic lipids, are phenolic lipids composed of long aliphatic chains and resorcinol-type phenolic rings. Alkylreosorcinol have been found to have multiple biological activities, including antimicrobial, molluscicidal, and antitumor properties.

5.12. Biosynthesis of isolated secondary metabolites from the endophytic fungi *Chaetomium aureum*

All isolated compounds are derived from the polyketide biosynthesis pathway. Polyketides constitute a large class of natural products grouped together on purely biosynthetic grounds. In addition to exhibiting a staggering range of functional and structural diversity, they boast a wealth of medicinally important activities, including antibiotic, anticancer, antifungal, antiparasitic and immunosuppressive properties. Even before the full extent of their utility was known, scientists became interested in how these complicated molecules are assembled. Their diverse structures can be explained as being derived from poly-β-keto chains.

The formation of the poly-β-keto chain could be envisaged as a series of Claisen reactions, the reverse of which are involved in the β-oxidation sequence for the metabolism of fatty acids. Thus, two molecules of acetyl-CoA could participate in a Claisen condensation giving acetoacetyl-CoA, and this reaction could be repeated to generate a poly-β-keto ester of appropriate chain length (Scheme 5.3).
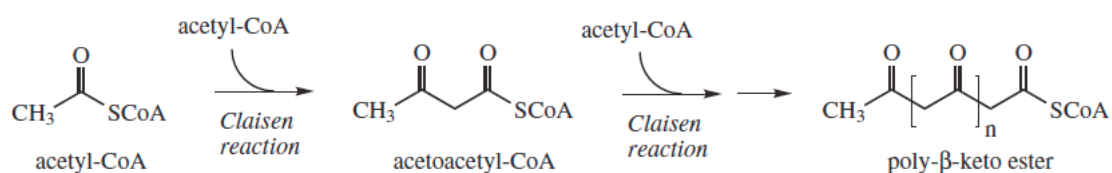
Scheme 5.3. The formation of the poly-β-keto chain

5.13. Bioactivity of isolated secondary metabolites from the endophytic fungi *Chaetomium aureum*

5.13.1. Bioactivity of azaphilone derivatives isolated from *Chaetomium aureum*

Sclerotiorin (9) inhibits efficiently the Hsp90 machine chaperoning activity. Sclerotiorin showed similar effectiveness to that of 17-AAG (Geldanalycin derivative), the classical inhibitor of Hsp90. However, sclerotiorin has no cytotoxic effect on breast cancer Hs578T, MDA-MB-231 and prostate cancer LNCaP cell lines. Interestingly, deacetylation of sclerotiorin increases its cytotoxicity toward the tested cell lines over a period of 48h. Sclerotiorin showed also strong activity against all of the tested protein kinases except MEK 1 wt. In the best of our Knowledge, the protein kinase MEK 1 wt is not Hsp 90 client protein (Jiao *et al.*, 2011; Citri *et al.*, 2006), which concord with the result of its resistance at (+)-sclerotiorin.
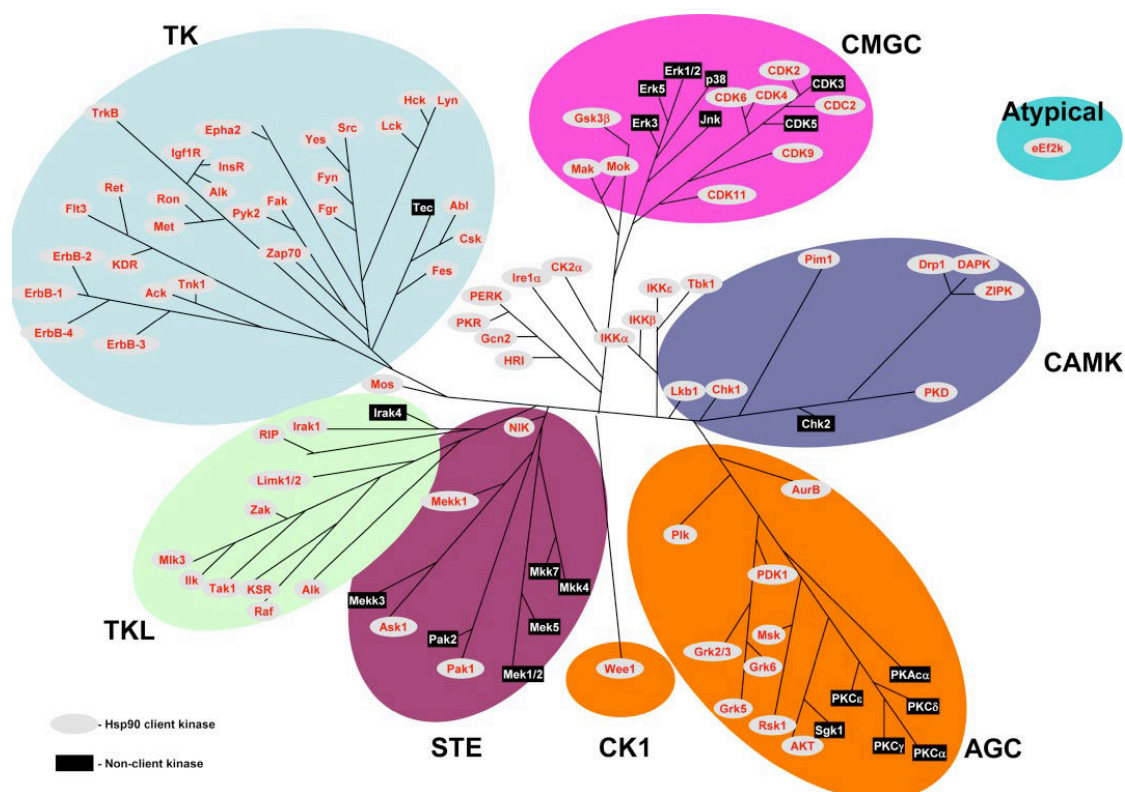
Figure 5.2. Diversity of established Hsp90 clients within the human kinome.

Hsp90 client kinases are represented by red letters on an elliptical gray background, whereas non-clients appear in white letters on a rectangular black background. The overall structure of the scheme was based on a previously published analysis of the human kinome.

Table 5.2. Distribution of Hsp90 clients within kinase families

Kinase Family	TK	TKL	CAMK	CMGC	AGC	CK1	STE	Other	Atypical	Total
Clients	28	12	7	9	8	1	4	10	1	80
Non-clients	1	2	1	8	6	0	7	0	0	25
Total	29	14	8	17	14	1	11	10	1	105
Total in group	90	43	74	61	63	12	47	83	45	518

The tabulation of the diversity of Hsp90 clientele according to kinase families. The data is based on data available in the literature.

- Sclerotiorin inhibits the Hsp90 chaperoning of progesterone receptor (PR) *in vitro*

Sclerotiorin efficiently inhibited the recovery of PR hormone binding activity using RRL (Figure 3.5.1). Sclerotiorin showed similar efficiency to that of 17-AAG, the classical inhibitor of Hsp90. Importantly, the oxygen atom of the heterocycle is essential for this inhibitory activity. Indeed, sclerotioramine and isochromophilone VI, where a nitrogen atom replaces the oxygen atom, are inactive. This is in line with previous reports showing that sclerotioramine and isochromophilone VI were unable to inhibit the activity of cholesterol ester transfer protein (CETP) (Tomoda *et al.*, 1999). Isochromophilone VII showed much less activity than sclerotiorin.

- Sclerotiorin is not cytotoxic to breast cancer Hs578T cells

The cytotoxicity assay showed that sclerotiorin is not toxic to breast cancer cell line Hs578T and MCF7, MDA-MB-231, MDA-MB-453, prostate cancer cell line LNCaP and cervical cancer cell line HeLa. This is in contrast to a report showing that sclerotiorin induces apoptosis in colon cancer (HCT-116) cells through the activation of BAX, and down-regulation of BCL-2 (Giridharan *et al.*, 2012).

- Deacetylsclerotiorin is less efficient at inhibiting the Hsp90 chaperoning of progesterone receptor (PR) *in vitro* than sclerotiorin.

The deacetylated sclerotiorin was relatively less efficient in inhibiting the Hsp90 *in vitro* than sclerotiorin. The mechanism by which sclerotiorin and deacetylsclerotiorin inactivate the Hsp90 machine is not clear but it seems to be different from that of the 17-AAG. As compared to 17-AAG, protein analysis of complex formation by SDS-PAGE showed that sclerotiorin and deacetylsclerotiorin lower the level of Hsp90 without increase of Hop. Furthermore, deacetylsclerotiorin did not increase Hsp70 level in PR complexes.

- Deacetylsclerotiorin is cytotoxic to cancer cells

At inhibitory concentrations, deacetylsclerotiorin becomes cytotoxic to Hs578T, MDA-MB-231 and LNCaP cell lines over a period of 48h. For Western blotting, cell lines were harvested at 48 h and cell lysates were made. 10 μg of each protein lysate was analyzed by Western blotting using home made antibodies (HMGR) for GR, for Hsp70 and for Hsp40 and sc-47778 (Santacruz) for β-actin. Western blot analysis showed that sclerotiorin induces overexpression of Hsp70 and degradation of Hsp90 client proteins such as glucocorticoid receptor in Hs578T cells, indicating that the Hsp90 machine may be inhibited in vivo. It is worth noting however, that sclerotiorin also induces degradation of the heat shock protein 40 (HSP40) which may contribute to some of sclerotiorin's cytotoxicity.

- Mechanism of action of Sclerotiorin

Sclerotiorin and several isochromophilone analogues were also shown to inhibit gp120-CD4 binding (Sun *et al.*, 1996) and the activity of cholesterol ester transfer protein (CETP) (Tomoda *et al.*, 1999). From these studies, it was concluded that the electrophilic sites at the C6 and C8 position are required for the sclerotiorin inhibition but the chlorine atom at the C-5 position and the acetyl residue at the C-7-OH are not. Furthermore, the oxygen atom of the heterocycle is also essential since sclerotioramine and isochromophilone VI where the oxygen atom is replaced by a nitrogen atom are less active. Long ago, sclerotiorin has been reported to react with primary amine (Eade *et al.* 1957) and this was the proposed mechanism for inactivation of CETP implying that sclerotiorin modifies primary amines such as lysine residues or the N-terminal amino acid in the CETP (Tomoda *et al.*, 1999). It is very possible that the inhibitory activity of sclerotiorin toward the Hsp90 machine could be through its reactivity with amine groups of components such as Hsp90, Hsp70, Hsp40, Hop and p23 leading to their inactivation. The loss of Hsp40 upon cell treatment with sclerotiorin could be the consequence of such covalent modifications.

5.13.2. Bioactivity of xanthone derivatives isolated from *Chaetomium aureum*

Xanthoradone D_1 and D_2 (14, 15) and xanthoradone E_1 and E_2 (16, 17) were subjected to cytotoxicity testing toward L5178Y (mouse lymphoma) cell line, protein kinases inhibition assays, antibacterial activity and Hsp 90 chaperone inhibition. All compounds were inactive. In contrast to literature, all xanthoradone derivatives were active against methicillin-resistant *Staphylococcus aureus*, produced by *Penicillium radicum* (Yamazaki *et al.*, 2009).

5.13.3. Bioactivity of alkylresorcinol derivative isolated from *Chaetomium aureum*

Chaetoresorcinol (18) was active against only *Asp. Fumigatus*, whereas it showed no antibacterial activities, which makes it selectively only against *Asp. Fumigatus.* Chaetoresorcinol was active against most of protein kinases, despite it inactivation against Hsp 90.

6. Conclusion

Medicinal plants and endophytic fungi produce natural products with a large diversity of chemical structures, which might prove to be suitable for specific medicinal applications. Most of these secondary metabolites show biological activities in pharmaceutically relevant bioassay systems and thus represent potential lead structures, which could be optimized to yield effective therapeutic and bioactive agents.

The Heat shock protein 90 plays a crucial role in maintaining oncogenic protein homoeostasis. Hsp90 inhibition offers great promise in the treatment of a wide variety of solid and haematological malignancies. Numerous natural Hsp90 inhibitors have been developed in recent years, some of which exhibit excellent antitumor activities and have entered clinical trials.

The aim of this work was the isolation of secondary metabolites from the selected plant *Thymelaea lythroides* and its endophytic fungus *Chaetomium aureum*, followed by structure elucidation and examination of the antitumor activity of isolated compounds by screening their inhibitory activity on the Hsp90 machinery.

The medicinal plant *Thymelaea lythroides* was selected from fifty five medicinal plants used as traditional medicine by the patients of the National Institute of Oncology in Morocco. Five endophytic fungal strains (*Alternaria sp., Chaetomium sp., Cladosporium sp.* and *Pleospora sp.*), were isolated from *Thymelaea lythroides*. The fungal strain *Chaetomium aureum* was chosen for further investigation. It was grown in liquid Wickerham medium as well as on eleven solid media for a period of three to four weeks. The extracts obtained were then subjected to different chromatographic separation techniques in order to isolate the secondary metabolites. Structure elucidation of the isolated secondary metabolites was performed using state of the art techniques, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy experiments.

Finally, the isolated compounds were subjected to various bioassays to examine their antitumor activity by testing their ability to inhibit the chaperone Hsp90 machinery. Besides, the isolated compounds were also tested for their antimicrobial, antifungal and cytotoxic activities as well as inhibitory profiles towards selected protein kinases.

6.1. Compounds isolated from the plant *Thymelaea lythroides*

Eight secondary meltabolites were isolated from the medicinal plant *Thymelaea lythroides* including one depsipeptide (**1**; bassiatin), two coumarins (**2**; daphneone and **3**; daphneolone), one dicoumarine (**4**; daphnoretin), two lignans (**5**; wikstromol and **6**; *δ*-sesamin), one flavonoid glucoside (**8**; trans tiliroside), and one dicoumarine glucoside (**7**; rutarensine).

Bassiatin (**1**) is an unusual plant secondary metabolite, which was previously reported exclusively from endophytic fungi. Thus, this is the first report of this compound from *Thymelaea lythroides*.

Daphneone (**2**) showed strong cytotoxic activity when tested against L5178Y mouse lymphoma cell line. Only trans tiliroside (**8**) and rutarensine (**7**) inhibited several of the tested protein kinases. All the compounds isolated from *Thymelaea lythroides* were inactive against the Hsp90 chaperone machinery.

6.2. Compounds isolated from the endophytic fungi *Chaetomium aureum*
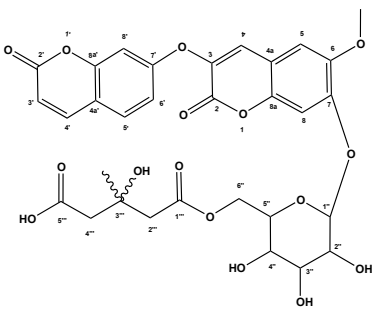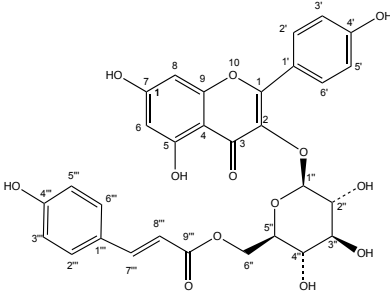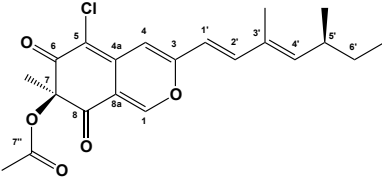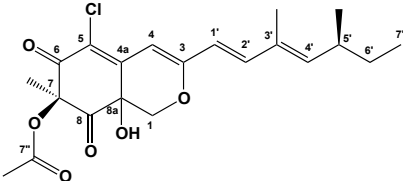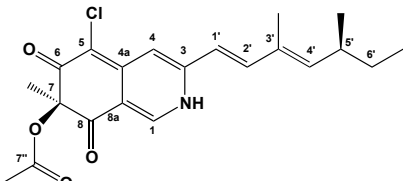
Six new compounds were obtained from this fungal strain, including a new alkylresorcinol derivative (**18**; chaetoresorcinol), four new xanthoradone derivatives (**14**; xanthoradone D1, **15**; xanthoradone D2, **16**; xanthoradone E1 and **17**; xanthoradone E2), and one pyrochromone derivative (13; SB 236050), together with four known azaphilone derivative (**9**; sclerotiorin, **10**; isochromophilone VII, **11**; sclerotioramin and **12**; isochromophilone VI).
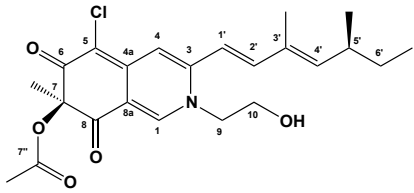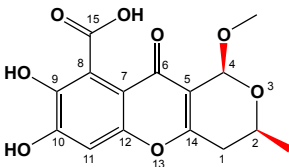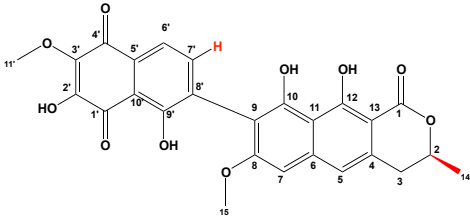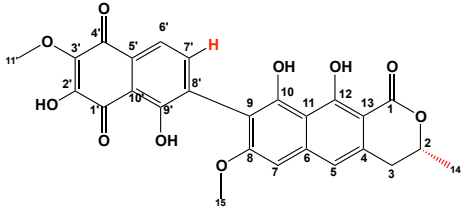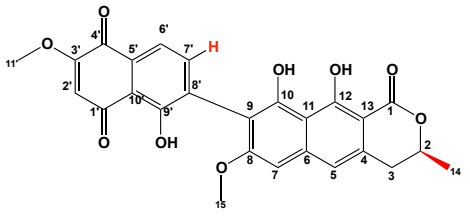
(+)-Sclerotiorin (**9**), (+)-isochromophilone VII (**10**), (+)-sclerotioramin (**11**) and isochromophilone VI (**12**) were not active upon testing their cytotoxic potential toward the L5178Y mouse lymphoma cell line. However, (+)-sclerotiorin (**9**) was active against all the tested protein kinases except MEK 1 wt, whereas its derivatives, isochromophilone VI (**12**) and sclerotioramin (**11**), inhibited only few of the tested enzymes. Furthermore, all compounds were tested in the Hsp 90 chaperone machinery inhibition assay. Only (+)-sclerotiorin exhibited prominent activity with similar efficiency as the famous Hsp-90 inhibitor 17-AAG (geldanamycin derivative).
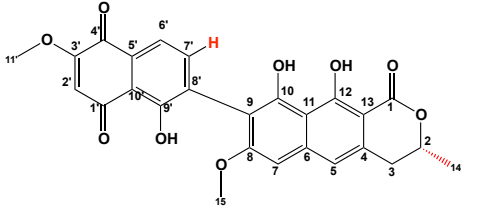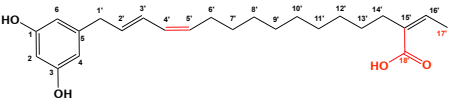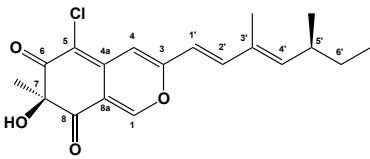
Chaetoresorcinol (**18**) showed selective moderated antifungal activity against *Aspergillus fumigatus*.

Table 6.1. Summary of the isolated compounds

Compound name	Structure	Source	Comment
Bassiatin		*Thymelaea lythroides*	Known
Daphneone		*Thymelaea lythroides*	Known
Daphneolone		*Thymelaea lythroides*	Known
Wikstromol		*Thymelaea lythroides*	Known
δ-Sesamin		*Thymelaea lythroides*	Known
Daphnoretin		*Thymelaea lythroides*	Known

Retarensin		*Thymelaea lythroides*	Known
trans-Tiliroside		*Thymelaea lythroides*	Known
(+)-Sclerotiorin		*Chaetomium aureum*	Known
(+)-Isochromophilone VII		*Chaetomium aureum*	Known
(+)-Sclerotioramin		*Chaetomium aureum*	Known

(+)-Isochromophilone		*Chaetomium aureum*	Known
SB 236050		*Chaetomium aureum*	New
Xanthoradone D₁		*Chaetomium aureum*	New
Xanthoradone D₂		*Chaetomium aureum*	New
Xanthoradone E₁		*Chaetomium aureum*	New

Xanthoradone E₂		*Chaetomium aureum*	New
Chaetoresorcinol		*Chaetomium aureum*	New
(+)-Deacetylsclerotiorin		Semi-synthetic	Known

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8. List of Abbreviations

$[\alpha]_D$	specific rotation at the sodium D-line
br	broad signal
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CI	chemical ionization
COSY	correlation spectroscopy
d	doublet
DCM	dichloromethane
dd	doublet of doublet
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ED	effective dose
EI	electron impact ionization
ESI	electrospray ionization
et al.	*et altera* (and others)
EtOAc	ethyl acetate
eV	electronvolt
FAB	fast atom bombardment
g	gram
HMBC	heteronuclear multiple bond connectivity
HMQC	heteronuclear multiple quantum coherence
H_2O	water
HPLC	high performance liquid chromatography
H_3PO_4	phosphoric acid
hr	hour
HR-MS	high resolution mass spectrometry
Hsp	Heat Shoc Protein
Hz	Herz
IZ	inhibition zone
L	liter

LC	liquid chromatography
LC/MS	liquid chromatography-mass spectrometery
m	multiplet
M	molar
MeOD	deuterated methanol
MeOH	methanol
mg	milligram
MHz	mega Herz
min	minute
mL	milliliter
mm	millimeter
MS	mass spectrometry
MTT	microculture tetrazolium assay
m/z	mass per charge
µg	microgram
µL	microliter
µM	micromol
NaCl	sodium chloride
ng	nanogram
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser and exchange spectroscopy
PCR	polymerase chain reaction
ppm	parts per million
PR	progesterone receptor
q	quartet
ROESY	rotating frame overhauser enhancement spectroscopy
RP 18	reversed phase C 18
RRL	rabbit reticulocyte lysate
s	singlet
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography

UV   ultra-violet

VLC   vacuum liquid chromatography

9. List of Figures

10. List of Tables

11. Attachements

Attachment 1. The ^{1}H NMR spectrum of bassiatin (**1**)

Attachment 2. The ^{1}H NMR spectrum of daphneone (**2**)
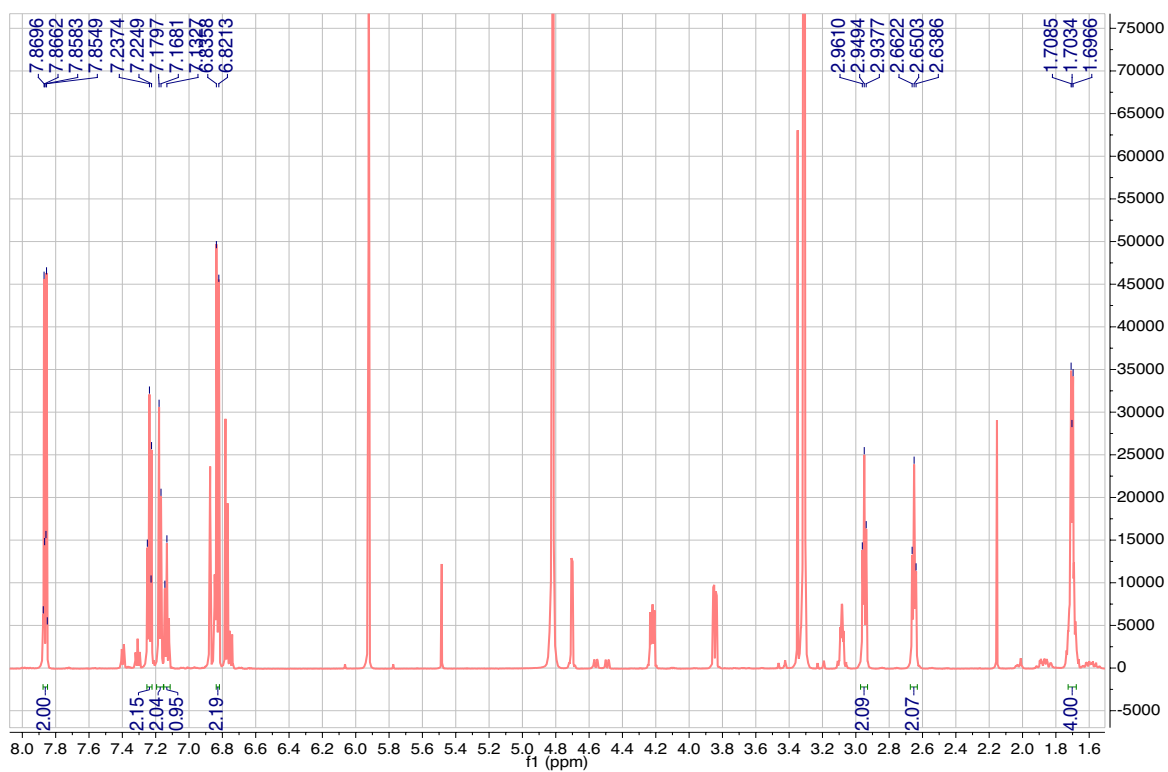
Attachment 3. The ^{1}H NMR spectrum of daphneolone (**3**)
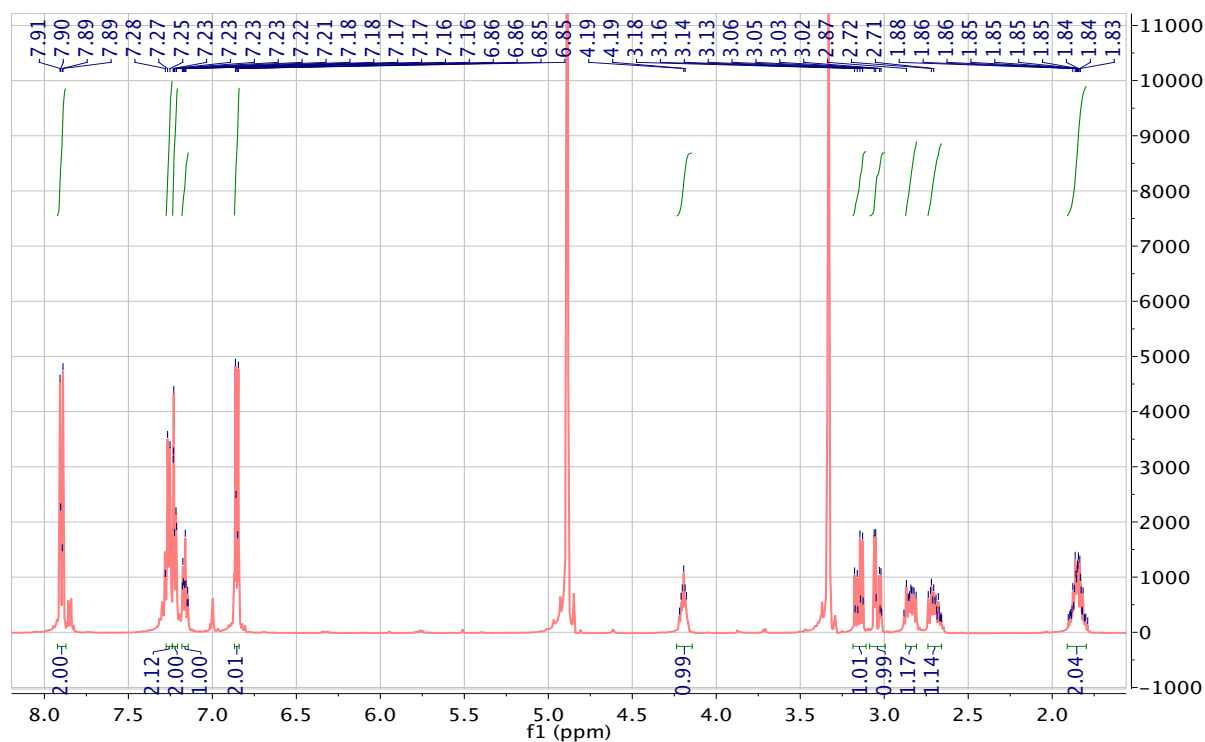
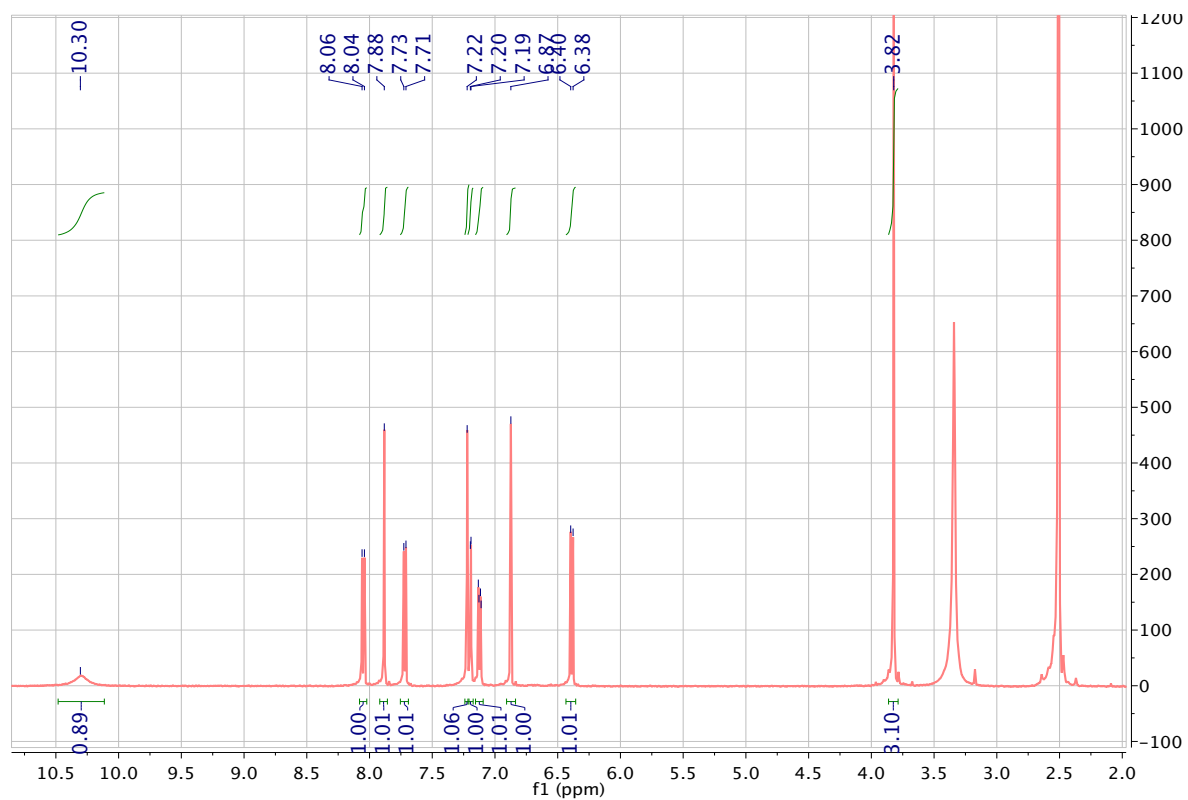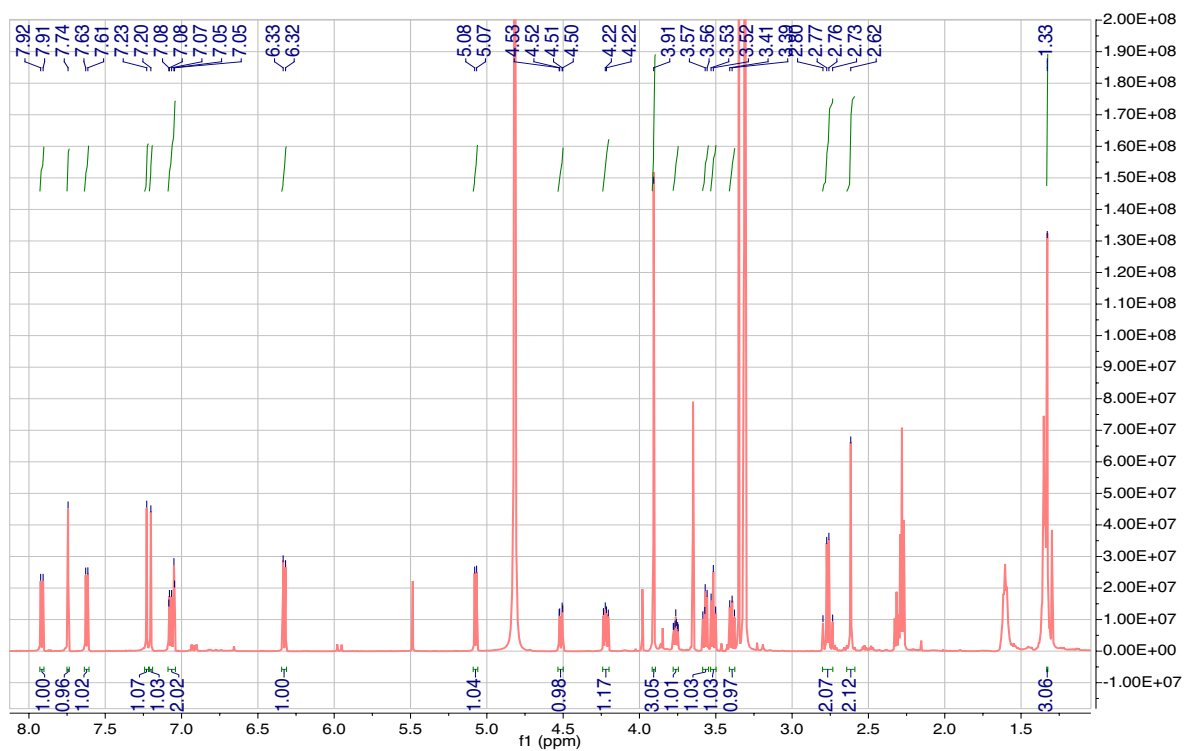**Attachment 4.** The ^{1}H NMR spectrum of (-)-wikstromol (**4**)
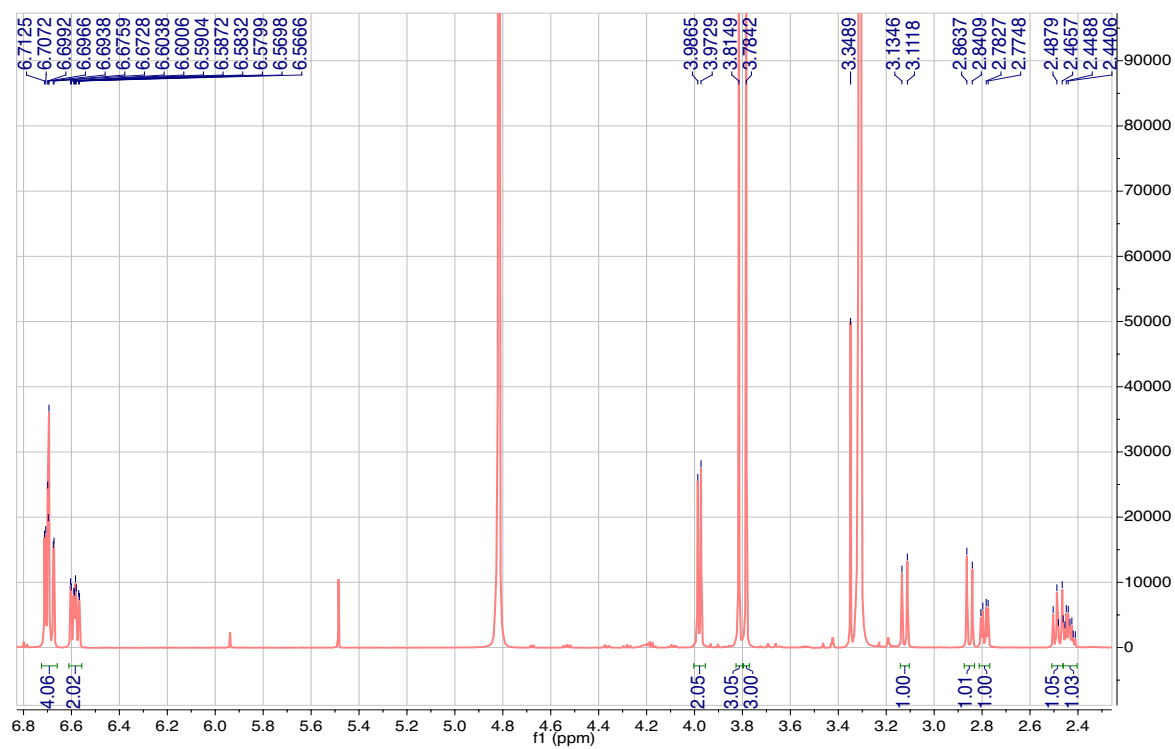
Attachment 5. The ^{1}H NMR spectrum of δ-sesamin (**5**)
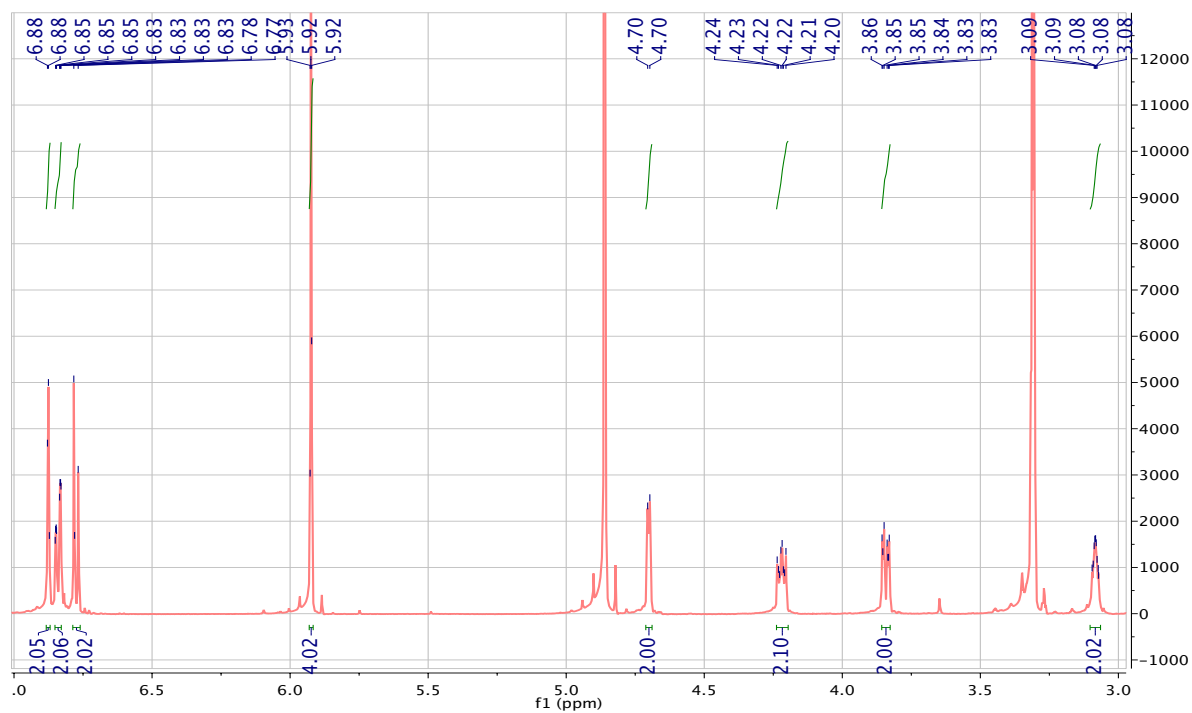
Attachment 6. The ^{1}H NMR spectrum of daphnoretin (**7**)

Attachment 7. The ^{1}H NMR spectrum of rutarensin (**7**)

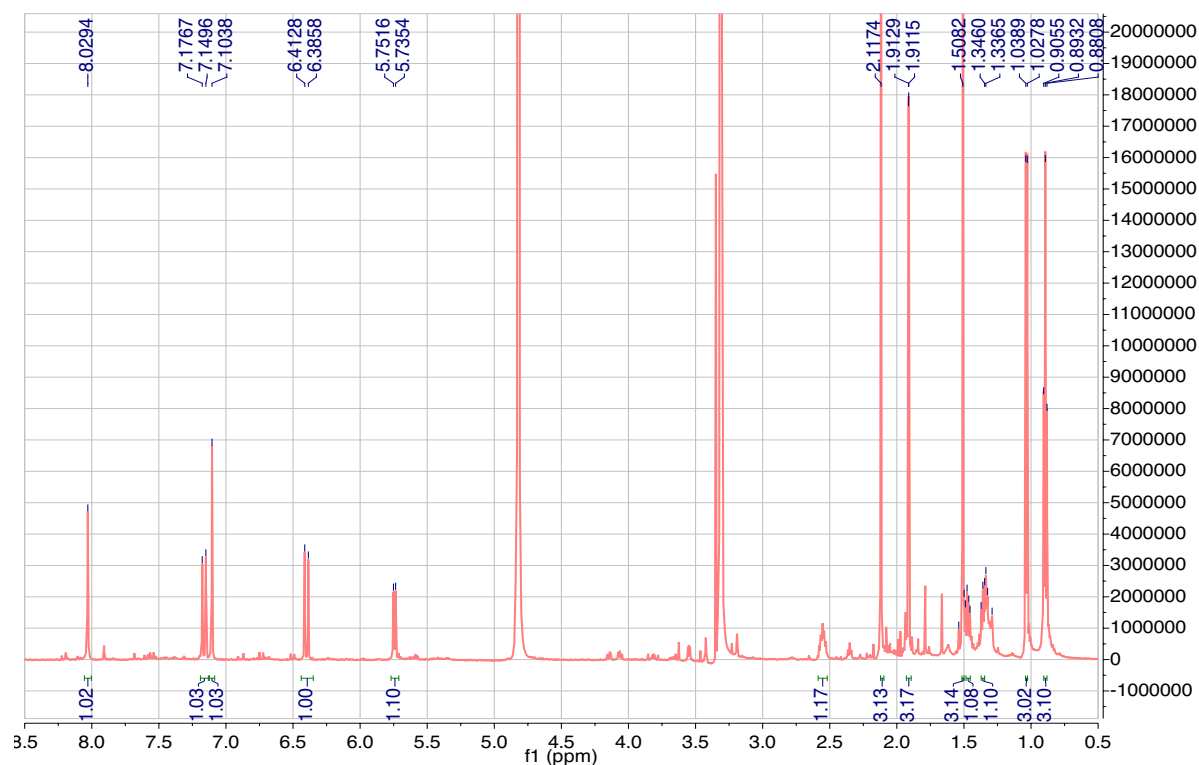**Attachment 8.** The ^{1}H NMR spectrum of *trans*-tiliroside (**8**)
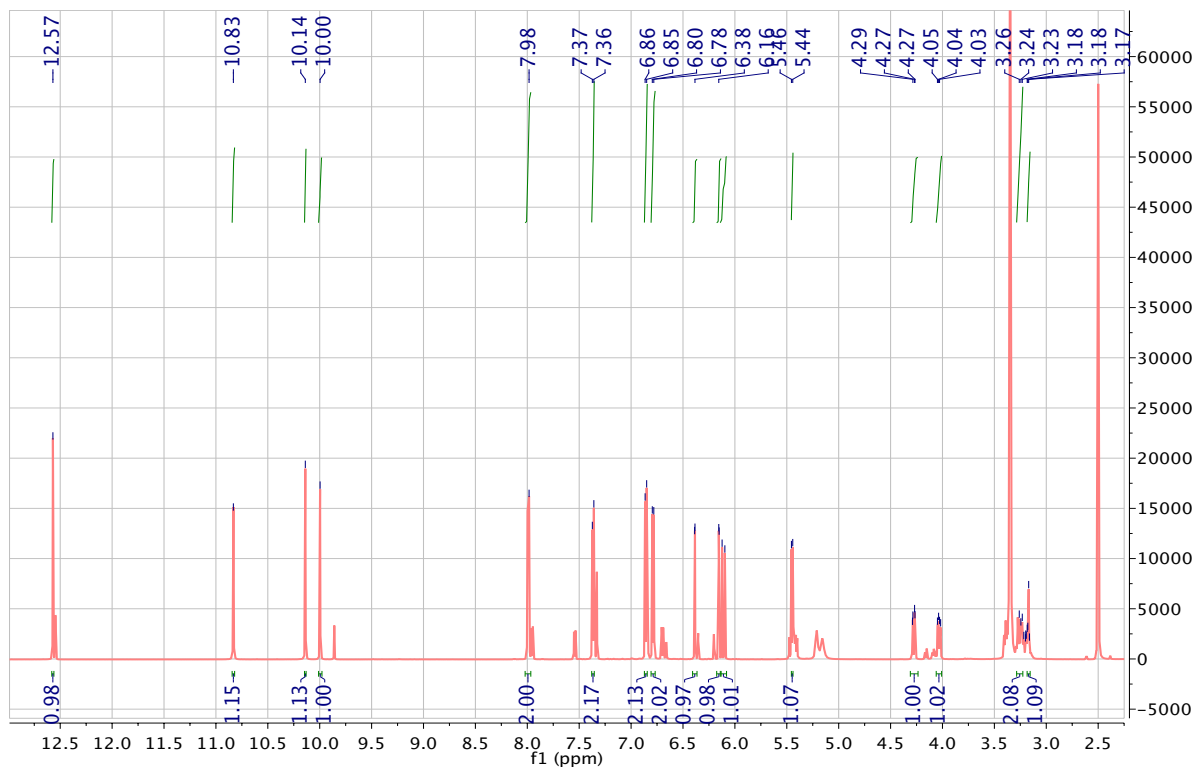
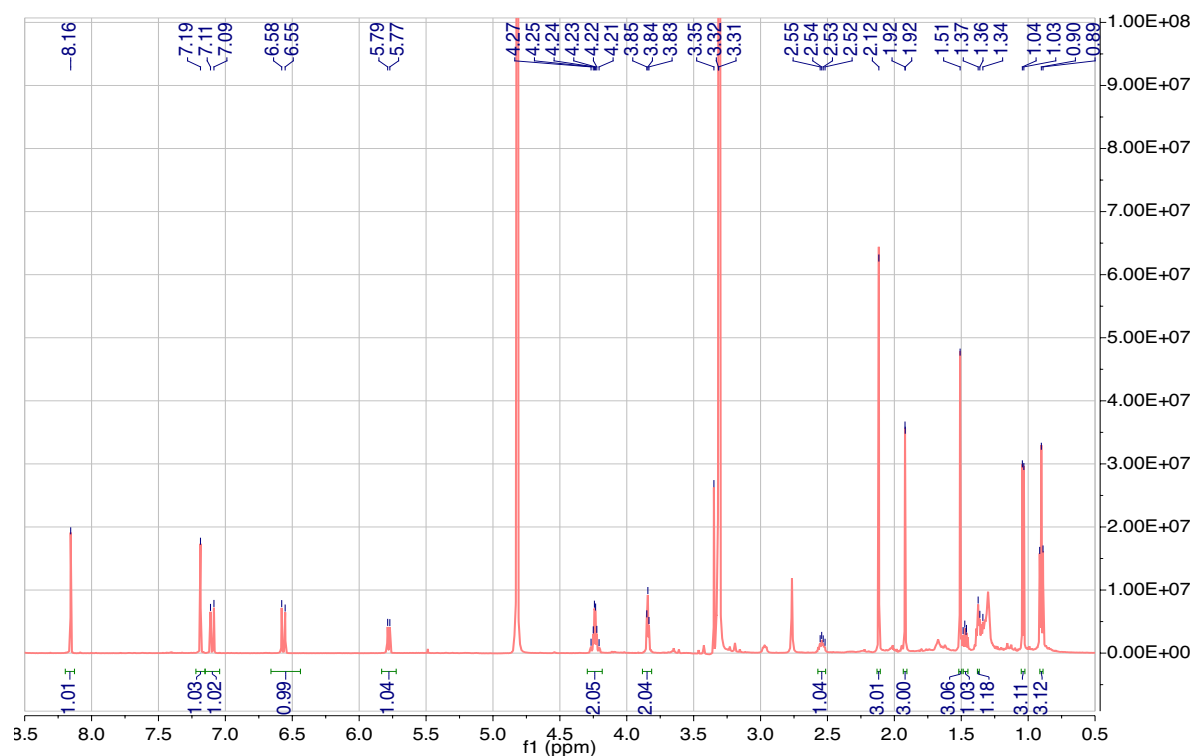**Attachment 9.** The ^{1}H NMR spectrum of (+)-sclerotiorin (**9**)
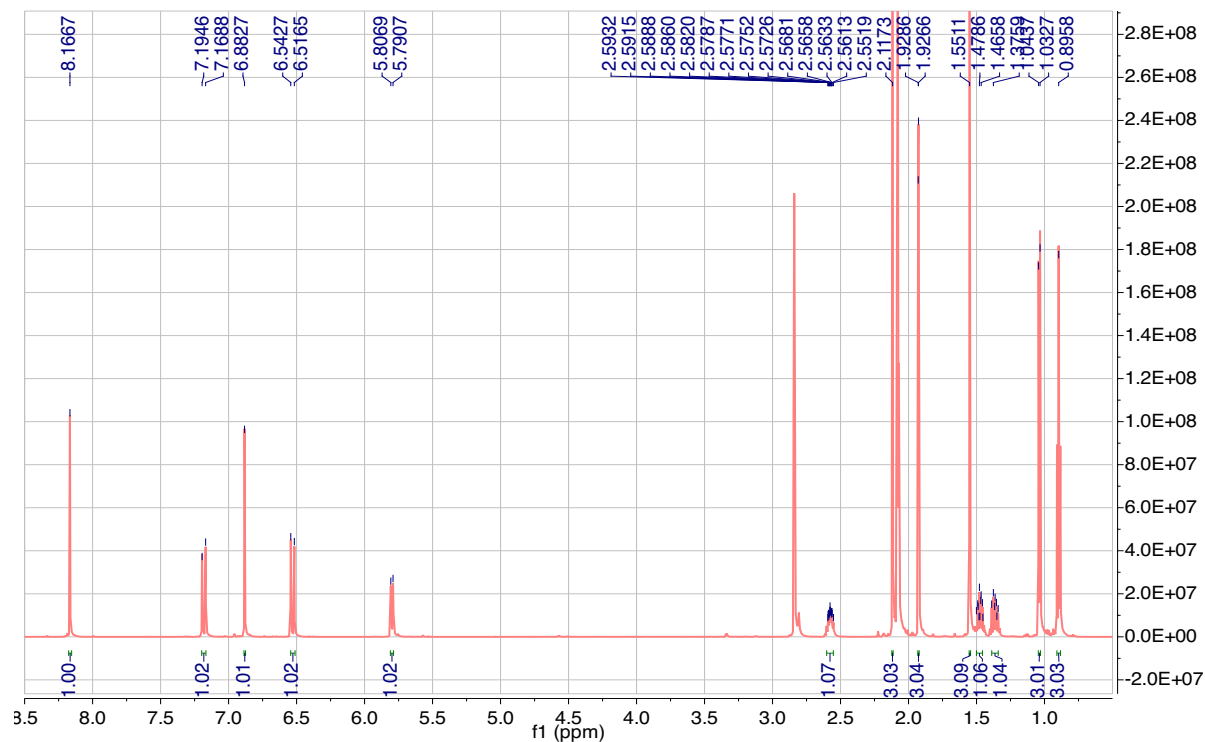
Attachment 10. The ^{1}H NMR spectrum of (+)-isochromophilone VII (**10**)
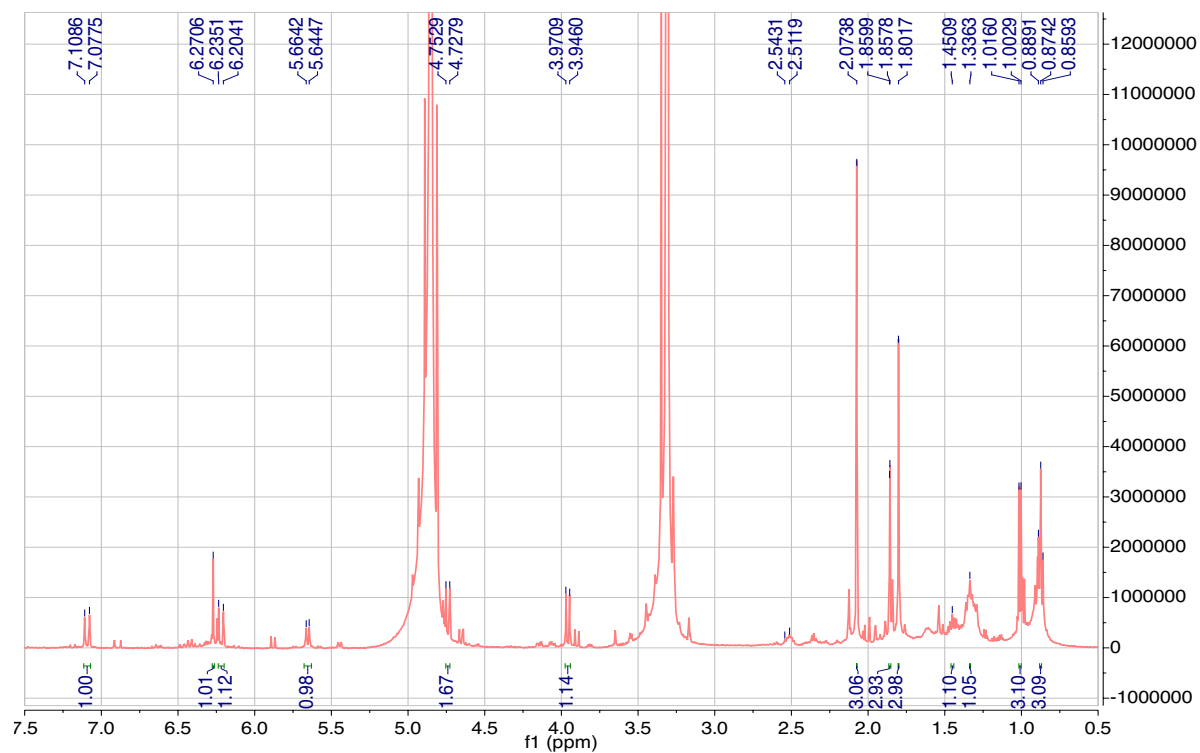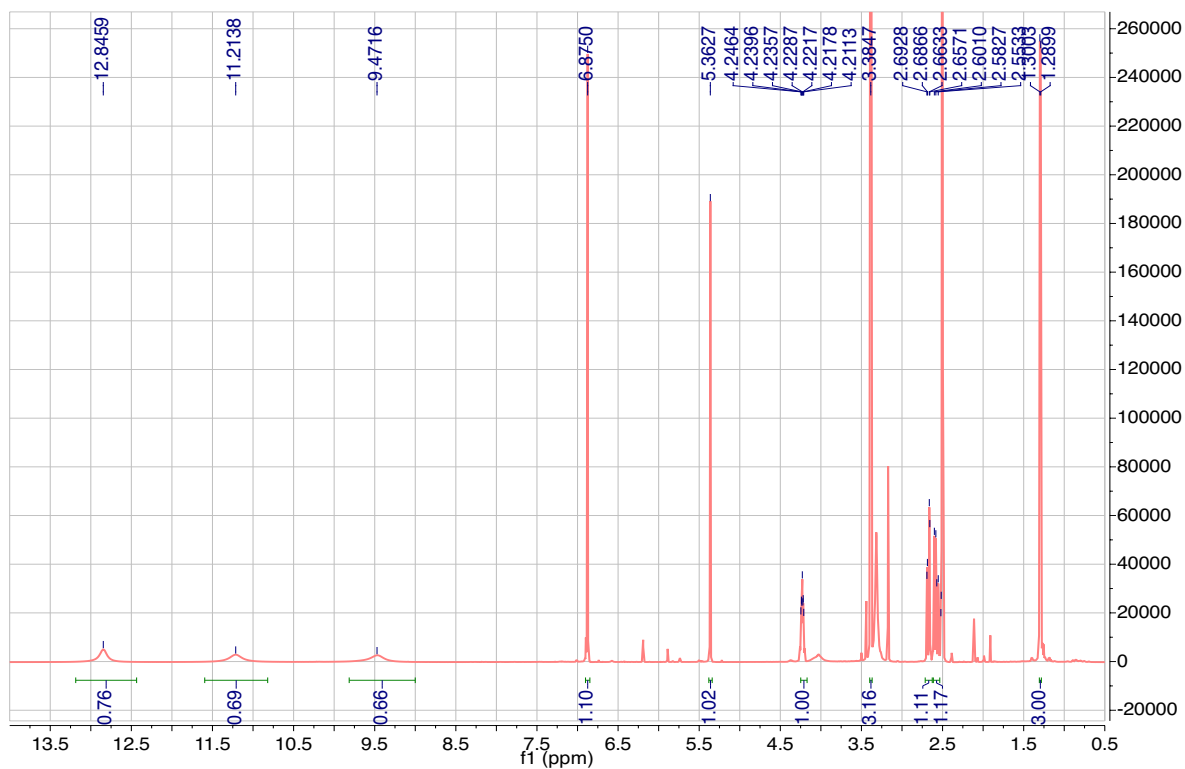
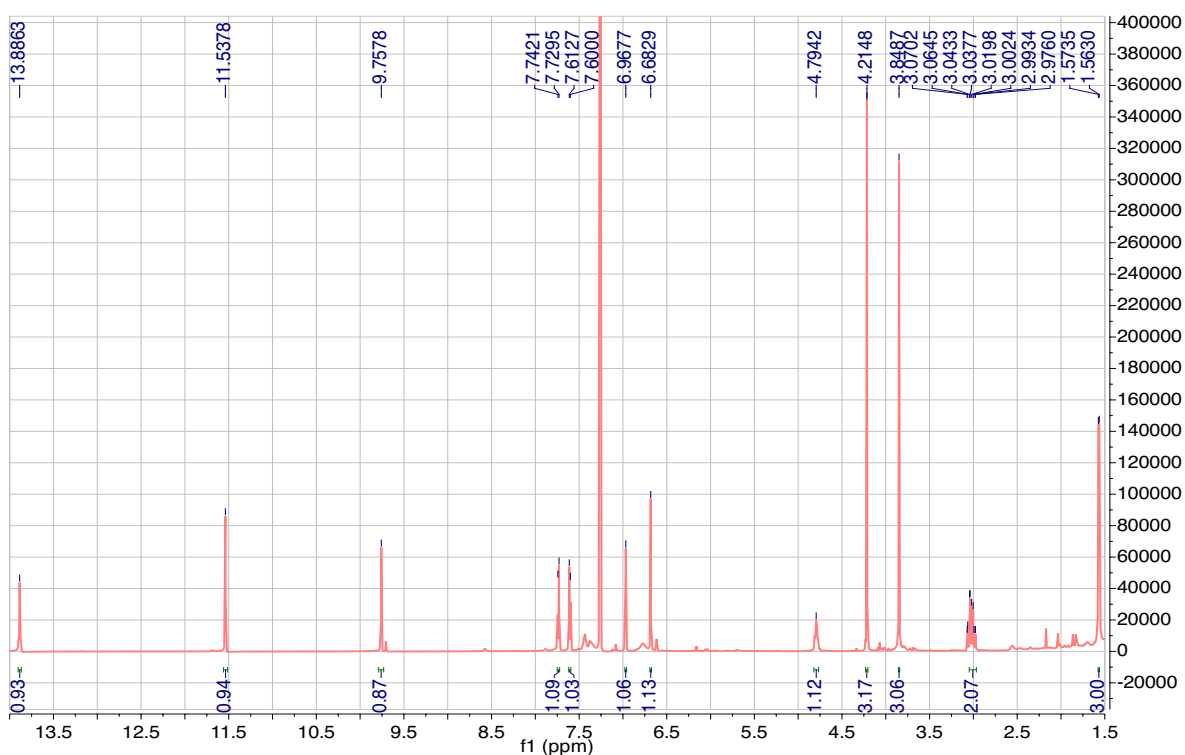**Attachment 11.** The ¹H NMR spectrum of (+)-sclerotioramin (**11**)

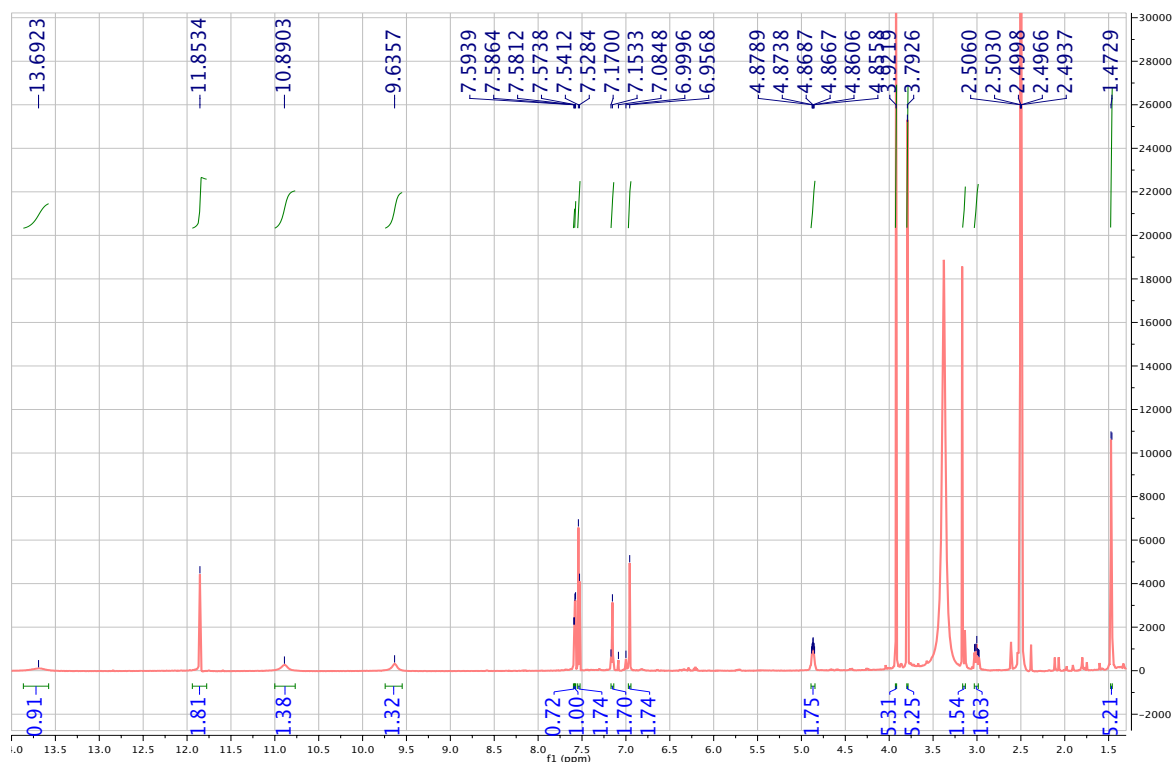**Attachment 12.** The ¹H NMR spectrum of (+)-isochromophilone VI (**12**)
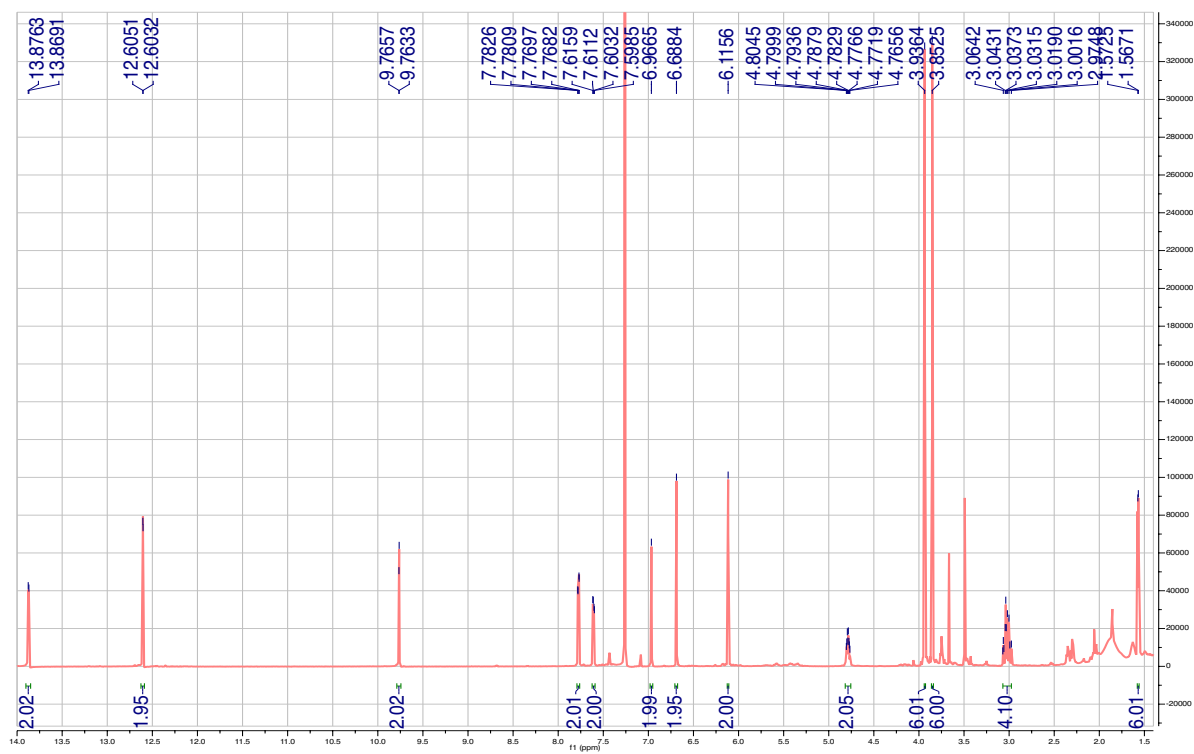
Attachment 13. The ¹H NMR spectrum of SB 236050 (**13**)

Attachment 14. The ¹H NMR spectrum of xanthoradone D₁ (**14**)
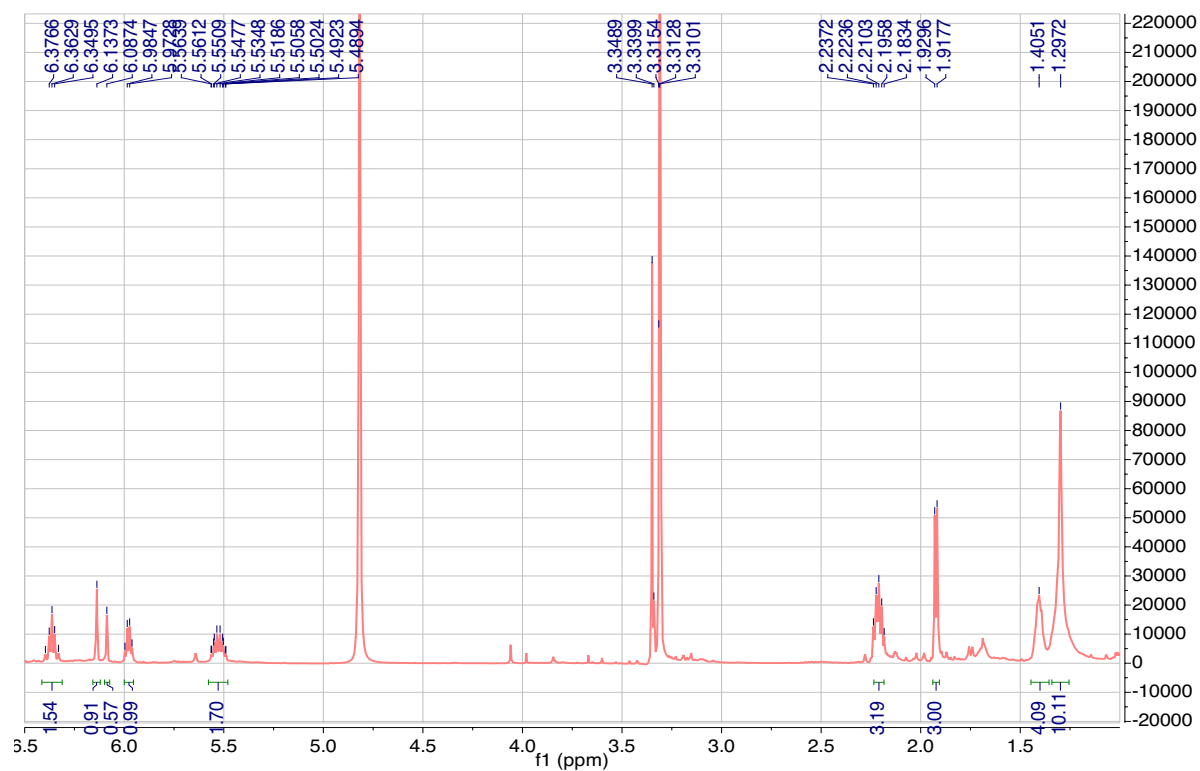
Attachment 14/15. The ^{1}H NMR spectrum of xanthoradone D$_1$ and D$_2$ (**14/15**)
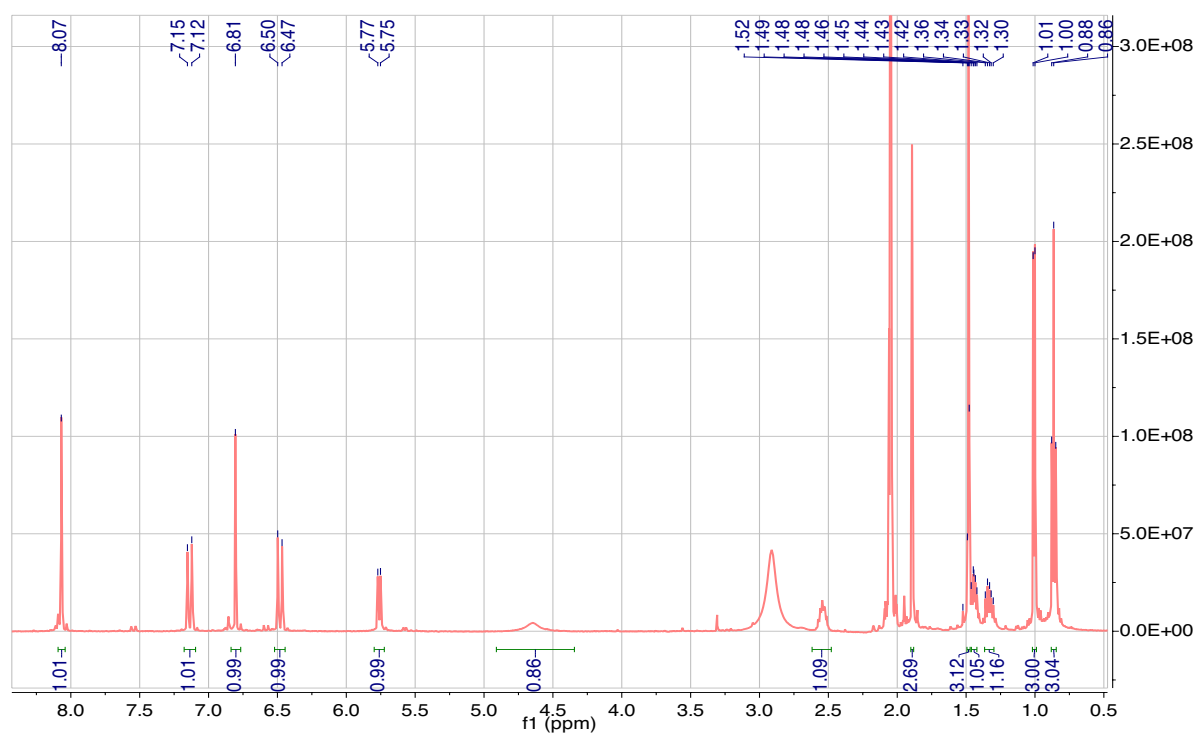

Attachment 16/17. The ^{1}H NMR spectrum of xanthoradone E$_1$ and E$_2$ (**16/17**)

Attachment 18. The ^{1}H NMR spectrum of chaetoresorcinol (**18**)

Attachment 19. The ^{1}H NMR spectrum of (+)-deacetylsclerotiorin (**19**)

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Curriculum vitae

Personal Data

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Study

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2008	Master Degree in Immunological and Biochemical Genius (IBG) at the university Mohammed V, Faculty of Science, Rabat. Mention: very well.
04/2008	Distance Learning General Course on Intellectual Property issued by the Worldwide Academy of the World Intellectual Property
2006	License in animal biology option Immunology at the university Mohammed V, Faculty of Science, Rabat
2004	University certification in Biology Geology at the university Mohammed V, Faculty of Science, Rabat. Mention: good.
2001	High school diploma experimental sciences, Lalla Nezha secondary school, Rabat

Professional experiences (training)

November 2011	Cooperation with the Institute of Pharmaceutical Biology and Biotechnology (Prof. Peter Proksch) at the University 'Heinrich Heine Düsseldorf' in Germany
April 2010	Ph D research at the University 'Bergische Universität Wuppertal' in Germany, Laboratory of Organic Chemistry under the supervision of Prof. Hans-Josef Altenbach.
09/2009-03/2010	Ethnopharmacological survey on cancer patients using traditional plants as herbal therapeutics in National Institute of Oncology in Rabat-Morocco
01/2008-07/2008	6-month training period end of study at the Faculty of Medicine and Pharmacy, Laboratory of Pharmacology and Toxicology under the supervision of Prof. A. Faouzi. Title of the topic "Quality control of antibiogram discs by high-performance liquid chromatography and research for new antibacterial herbal medicines."
03/2008-06/2008	3 months of Training in the laboratory of microbiology at the hospital IBN SINA in Rabat (realization and reading of antibiogramme, identification of the CMI of the resistant stumps).
02/2008	1 month training in the national laboratory of medicines control (Service of quality insurance, service physico-chimimie, service of the biological tries).
10/2007-11/2007	2 months of training at "Pharmaceutical Institute" in Ain Aouda (quality assurance Unit laboratory of quality control, production unit, stores raw materials and finished products, maintenance unit).
07/2005	1 month of training in the Laboratory of Biochemistry at the National Institute of Hygiene. (Analysis of: glycemia, Urea, creatinine, urates, Cholesterol, HDL cholesterol, LDL cholesterol, Triglycerides, direct and total ALAT, ASAT, PALE, GGT, Bilirubine, Sodium, Potassium, Calcium, Phosphates, magnesium).

Scientific Production

2013	**Kabbaj, F.Z.**; Lai, D.; Meddah, B.; Altenbach H.J.; Cherrah, Y.; Proksch, P.; Faouzi MA.; Debbab, A. Chemical constituents from aerial parts of *Thymelaea lythroides.* **2013**. *Biochem. Syst. Ecol*. *51*, 153–155
2013	**Kabbaj, F.Z.**; Lu, Su; Faouzi, My El Abbés; Meddah, Bouchra; Proksch, Peter; Cherrah, Yahya; Altenbach, Hans-Josef; Aly, Amal; Chadli, Ahmed; Debbab, Abdessamad. .Bioactive Metabolites from *Chaetomium aureum*: Structure elucidation and inhibition of the Hsp90 machine chaperoning activity. **2013**. J. Med. Chem. (*submitted*) Manuscript ID: jm-2014-00495q.
2013	**Kabbaj, F.Z.**; Aly, A.H.; Marmann, A.; Faouzi M.A.; Meddah, B.; Cherrah, Y.; Altenbach H.J; Proksch, P.; Debbab, A. New Xanthoradone Derivatives from *Chaetomium aureum* – Structures Elucidation and Biological Activity. **2013** (*In*

	progress)
2013	Marmouzi, I.; **Kabbaj, F.Z.**; Marmann, A.; Meddah, B.; Saidi, N.; Cherrah, Y.; Debbab A.; Faouzi, M.A.; Proksch, P. New secondary metabolites of Chaetomium globosum. 2013. GenBank. HG423571
2012	**Kabbaj, F.Z.**; Marmann, A.; El Amrani, M.; Proksch, P. and Debbab, A. Chaetomium aureum genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, isolate MM10S2-1. 2012. GenBank. HF546136.1

Languages

German	DSH 3
English	Upper intermediate (B2)
French	Mother tongue 2
Arabic	Mother tongue 1