Natural Products from Plant and Hypersaline Sediment Derived Fungi-Structure Elucidation and Biological Characterization

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Tag der mündlichen Prüfung:

Dedication

- To my mother, Basma, and my father, Saud, for always believing in me and endorsing me to further my education.

- To my husband, Raiyd, who has been a big part of my life, for his patience and encouragement.

- To my daughters, Tuleen and Tala who offered emotional support through laughing, and crying and for giving me reasons every day to push harder and try more.

- To my supervisor, for his guidance and unlimited support.

- To my teammates, colleagues, lab mates, mentors and everyone along the way who helped make this possible.

Erklärung

Ich versichere, dass ich die vorliegende Dissertation mit dem Titel " Neue Naturstoffe aus endophytischen Pilzen und Sedimenten hypersaliner Seen abgeleitet Pilze-Strukturaufklärung und biologische Charakterisierung" selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die eingereichte Arbeit habe ich in dieser oder ähnlicher Form noch keinem anderen Prüfungsausschuss vorgelegt.

Düsseldorf, den

Raha Orfali

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Zusammenfassung:

Pilze gehören zu den wichtigsten Gruppen eukaryotischer Organismen, die seit dem letzten Jahrhundert eine zentrale Rolle als Quelle für neue Leitstrukturen spielen. Verschiedene Umweltfaktoren wie unterschiedliche chemisch-physikalische Bedingungen und unterschiedliche biologische Faktoren haben Einfluss auf den Stoffwechsel von Pilzen und können die Produktion unterschiedlichster Sekundärmetabolite begünstigen. In der vorliegenden Arbeit wurden Pilze aus bisher nur wenig erforschten ökologischen Nischen auf ihre Naturstoffe untersucht.

Der erste Teil der Arbeit befasst sich mit dem endophytischen Pilz *Alternaria* sp., der aus den Samen der Heilpflanze *Ziziphus jujube* isoliert wurde. Der Pilz wurde sowohl in flüssigem Wickerham-Medium als auch auf festem Reismedium kultiviert.

1. *Alternaria* sp.

Aus dem Extrakt des *Alternaria* sp. der auf Reis kultiviert wurde, wurden insgesamt 21 Metabolite erhalten. Fünf dieser Verbindungen sind Isocumarin-Derivate, die zum ersten Mal aus dieser Gattung erhalten wurden. Von diesen Verbindungen weist das Citreoisocumarin eine antibiotische Aktivität gegenüber MRSA und anderen grampositiven Bakterien auf. Bei den restlichen 17 Verbindungen handelt es sich um Alternariol-Derivate. Die isolierten Alternariol-Derivate und einige strukturell verwandte Verbindungen zeigten sowohl eine hohe zytotoxische Aktivität gegenüber der Maus-Lymphom-Zelllinie L5178Y als auch ausgeprägte antibakterielle Aktivitäten. Die Substanz 7-Methoxyphthalid-3-essigsäure konnte zum ersten Mal aus der Natur isoliert werden.

Der zweite Teil der Arbeit beschäftigt sich mit halophilen Pilzen als Quelle für bioaktive Metabolite. Der Pilz *Penicillium* sp., der aus dem Sediment des Sees Wadi El-Natrun in Ägypten isoliert wurde, wurde hier als Beispiel für Pilze aus einem hypersalinen Habitat ausgewählt.

2. *Penicillium* sp.

Insgesamt wurden elf Verbindungen aus *Penicillium* sp. isoliert, von denen fünf neue Naturstoffe darstellten. Ferner wurden zwei neue Isochromane, ein neues Isocumarin, und zwei neue Epidithiodiketopiperazin-Analoga namens Pretichodermamid C und N-methylpretichodermamid B erhalten. Die letzte Verbindung erwies sich als wirksam gegenüber der L5178Y-Krebszelllinie.

Die hier präsentierten Ergebnisse unterstützen die These eines ökologisch basierten Ansatzes für die Auswahl von Pilzen für chemische Untersuchungen, da gezeigt werden konnte, dass dieser Ansatz in der Entdeckung neuer bioaktiver Pilzmetabolite resultiert.

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1. Introduction:

Natural products have provided a considerable value to the pharmaceutical industry over the past fifty years (Mishra and Tiwari, 2011). Historically, pharmaceutical companies have utilized plant extracts to produce therapeutic formulations since the discovery of antibiotics in the mid-twentieth century (Firn and Jones, 2003). Thus, enormous efforts have been initiated resulting in huge impact of these products on drug discovery (Brahmachari, 2013).

The studies of the information presented on sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products (Newman and Cragg, 2007). These natural products which are originating from microorganisms, plants- and animal-sources have been the most productive source of leads for the development of drugs to treat human diseases (Butler, 2005; Newman and Cragg, 2012). For instance, about 60% to 75% of new drugs in the areas of cancer and infectious diseases originated from natural sources between 1981 and 2002 (Newman *et al.,* 2003). Furthermore, 40 new drugs launched on the market between 2000 and 2010 originated from terrestrial plants, terrestrial microorganisms, marine organisms, terrestrial vertebrates and invertebrate sources (Newman and Cragg, 2012). In addition, this historical success in drug discovery and natural products is likely to continue. Thus, together with pharmacological screening, the chemical biodiversity of natural products has always provided highly useful leads for drug discovery (Brahmachari, 2013).

Recent technological advances in combination with the rational drug design including automated high-throughput screening (HTS) technology and combinatorial chemistry (Vincent *et al.,* 2006) have accelerated the screening of natural products and offered a unique opportunity to re-establish natural products as a major source of drug-leads (Cragg and Newman, 2013).

To conclude, natural products have been the traditional pathfinders which are exhibiting enormous structural diversity for new biologically active compounds. These natural products provide a compelling challenge for the global scientific community to undertake expanded exploration of “Nature” as a source of novel leads for the development of drugs and other valuable bioactive agents (Mishra and Tiwari, 2011). Since there are still a huge number of unstudied plants, mushrooms, marine organisms, insects, and microorganisms, there is a wealth of molecular diversity which is waiting to be discovered and utilized (Dias *et al.,* 2012).

1.1. The potential of fungal natural products in drug discovery

The kingdom fungi is an important source of many novel metabolites which are directly used as drugs or function as lead structures for synthetic modification of potential drug-leads (Aly *et al.,* 2011). It continues to provide a unique structural diversity which has been a driving tool in the development of new useful products. Fungal natural products could now be utilized as pharmaceuticals against a large number of diseases and they provide unique and inspirational chemicals for innovative drugs (Zhou *et al.,* 2014; Niu *et al.,* 2014; Ebrahim *et al.,* 2013; Akone *et al.,* 2013; Pham *et al.,* 2013).

Macro- and micro-fungi have been a part of human life since thousands of years. They have been domesticated to be used in baking, industrial fermentation and biotechnological industries. In addition, other species are cultivated or collected to be used as food (Dias *et al.,* 2012). Macro-fungi have been described throughout the history in the form of traditional medicine and remedy and the most of this bioactive compounds still remain unidentified (Sullivan *et al.,* 2006). In Asia, a variety of mushrooms have been used as a popular medicine to prevent or treat different diseases (Lindequist *et al.,* 2010). For instance, the medicinal higher fungus *Ganoderma lucidum* has been used in Traditional Chinese Medicine for over 4000 years for treating disorders of many systems including reproductive, excretory, digestive, circulatory, immune, cardiovascular, nervous, and respiratory systems (Shiao, 2003). In contrast to the Traditional Chinese Medicine`s holistic approach, researchers from different countries have investigated *Ganoderma lucidum* for over 30 years in an attempt to isolate its main active chemical constituents (Chen, J and Chen, T, 2003). This study revealed that triterpenes were the main active chemical components and thus have been classified as potent anti-cancer agents and have also demonstrated to exert cytotoxic effects on various cancer cells (Silva, 2003; Tang *et al.,* 2003; Liu *et al.,* 2012; Li *et al.,* 2013). Thus, mushrooms have been valued as a traditional source of natural bioactive compounds since many centuries and have been targeted as promising therapeutic agents (Spiteller, 2008). In addition, the investigation of microfungi for bioactive metabolites was initiated by the discovery of **penicillin G** from *Penicillium notatum* by Alexander Fleming in 1928 and the observation of the broad therapeutic use of this agent in 1940 started a new era in medicine and promoted intensive research of fungi and microorganisms as a source of novel bioactive agents especially for antibiotics (Larsen *et al.,* 2005). Since then, several further clinical studies took place by many pharmaceutical companies resulting in the

discovery of novel antimicrobial structural classes such as **griseofulvin** (Grove *et al.,* 1952), **cephalosporin C** (Newton and Abraham, 1995), and **fusidic acid** (Godtfredsen *et al.,* 1962). Nowadays, many of these compounds can be produced in large quantities and at a reasonable cost by fermentation employing wild type or genetically altered fungi (Strobel and Daisy, 2003). Moreover, intensive studies which are aiming at the improvement of potency, efficacy, spectrum of activity and activity against resistant pathogens are still ongoing. For instance, there are three semisynthetic ketolide derivatives of erythromycin (Mishra and Tiwari, 2011) and nine *β*-lactams (two cephalosporins, six carbapenems and one penem) in clinical trials or undergoing drug registration (Fabbretti *et al.,* 2011).

Apart from the use of micro-fungi as a source of antimicrobial agents to combat microbial infectious diseases, their use in heterogeneous fields such as the use of fungal natural products as immunosuppressants during organ transplantations is important. The discovery of **cyclosporine** was a new era in the field of immunopharmacology (Shaw, 1989) and since then, natural products have played a pivotal role in the development of such drugs (Butler, 2004; Gabardi and Cerio, 2004). The antihyperlipidemic drugs, statins, represent another group of important fungal- derived drugs. In fact, statins are the most potent cholesterol-lowering agents available. The first natural isolated fungal statin compound was **lovastatin** (Mevacor®) which was isolated from *Monascus ruber* (Negishi *et al.,* 1986). This was followed by synthetic analogues derived from the natural fungal metabolites. Atorvastatin (Lipitor®) and simvastatin (Zocor®) feature as the top seller drugs reflecting the widespread implementation of clinical guidelines and recommendations relating to coronary heart disease (Sweetman, 2009).

Since several years, microfungi especially those which are isolated from soil samples started to show a reduced hit-rate of novel lead compounds. Natural product chemists started to turn their interest to less investigated drug sources such as endophytic fungi associated with plants (Gunatilaka, 2006; Zhang *et al.,* 2006; Verma *et al.,* 2009; Aly *et al.,* 2010; Kharwar *et al.,* 2011; Aly *et al.,* 2013) and marine fungi (Paz *et al.,* 2010; Rateb and Ebel, 2011; Debbab *et al.,* 2012; Ebada and Proksch, 2013) which turned out to be a vast untapped reservoir of metabolic diversity.

The kingdom fungi is considered as a mega-diverse group which inhabits almost every niche on earth (Strobel, 2002a; Hyde, 2005). However, only about 7 % of the estimated 1.5 million species of fungi have been described (Hawksworth, 2004) and only a small number has

been explored for the production of pharmacologically active metabolites (Suryanarayanan *et al.,* 2009). Accordingly, many undescribed species of fungi still exist in unexplored habitats for chemical investigation. These observations argue strongly to continue studying fungal chemistry in order to meet the increasing demand for new medicinally and agriculturally beneficial agents.

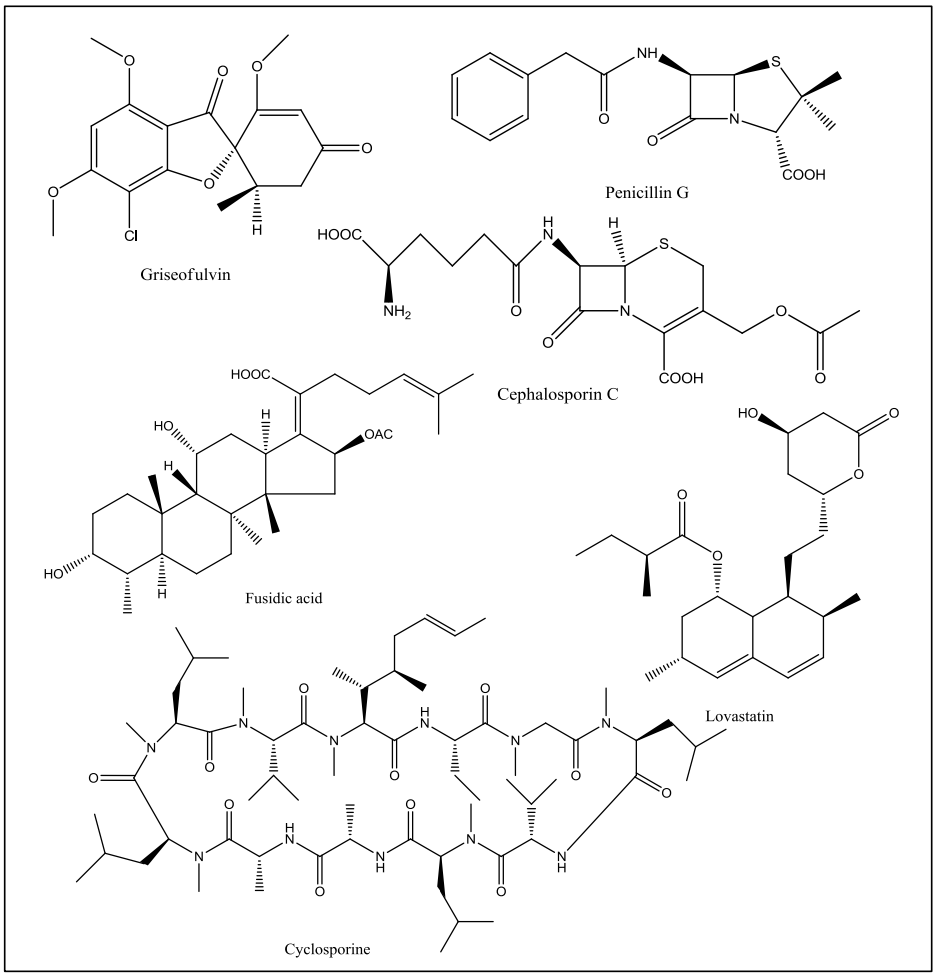


Figure 1.1: Fungal natural products as drugs or drug-lead compounds.

1.2. Endophytic fungi

Endophytic fungi are a highly diverse polyphyletic group including primarily ascomycetous fungi that occur ubiquitously in plants (Hyde and Soytong, 2008). They reside in

the intercellular spaces of stems, petioles, roots, leaves, and even seeds of plants without causing any obvious negative impacts (Bacon and White, 2000). Most plant species that have been previously studied host at least one endophytic organism (Strobel, 2003). There are over 300 000 plant species inhabiting our planet and it could be assumed that each individual plant hosts from few to hundreds of endophytic fungi (Strobel, 2003; Huang *et al.,* 2007a). These subtle inhabitants of the tissues of higher plants generally could contain some types of novel endophytic microorganisms (Gamboa *et al.,* 2002; Strobel and Daisy, 2003).

Bioactive natural compounds produced by endophytic fungi have a promising potential which is may be used for human health. Functional metabolites of these fungi have already demonstrated a considerable potential to impact the pharmaceutical arena (Tan and Zou, 2001; Strobel and Daisy, 2003; Strobel *et al.,* 2004; Gunatilaka, 2006; Zhang *et al.,* 2006; Verma *et al.,* 2009; Aly *et al.,* 2010; Kharwar *et al.,* 2011; Aly *et al.,* 2013). For instance, endophytes are believed to carry out a resistance mechanism to overcome pathogenic invasion by producing secondary metabolites bearing antimicrobial activity. An example is the novel diterpenoid **guanacastepene** produced by an endophytic fungus isolated from a branch of *Daphnopsis americana* growing in Costa Rica which proves to be a representative of a potentially new class of antibacterial agents showing activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Singh *et al.,* 2000).

On the other hand, the tetramic acid **cryptocin** which was obtained from the endophytic fungus *Cryptosporiopsis quercina* that inhabits the inner bark of the stems of *Tripterygium wilfordii* demonstrated an excellent antifungal activity against a wide range of plant pathogens including *Pyricularia oryzae* which is one of the most devastating crop diseases worldwide (Li *et al.,* 2000).

As antiviral agents, the discovery of compounds having such activity is in its infancy stage (Singh *et al.,* 2004). However, some promising endophytic fungi proved to be the source of hinnuliquinone, a potent inhibitor of the HIV-1 protease which was isolated from a fungal specimen associated with the leaves of *Quercus coccifera* (Singh *et al.,* 2004).

Searching for new immunosuppressants from endophytes resulted in the isolation of **subglutinol A** and **B**. These diterpene pyrones which were isolated from *Fusarium subglutinans* (harbored by the perennial twining vine *Tripterygium wilfordii*) showed substantial

immunosuppressive activity and were devoid of cytotoxic effects which are characteristic of cyclosporine A (Lee *et al.,* 1995; Strobel and Pliam, 1997).

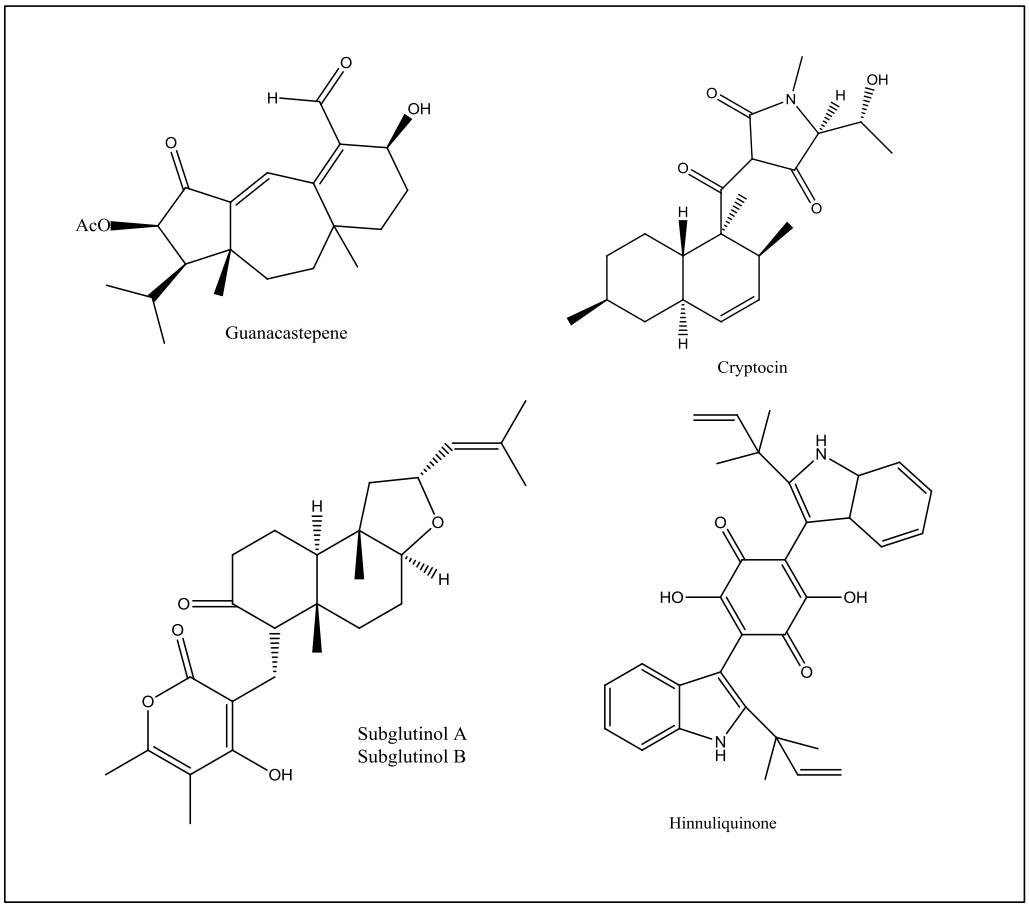


Figure 1.2: Bioactive metabolites from endophytic fungi.

As cytotoxic agents, many of the endophytic fungal metabolites possess strong cytotoxicity against different cancer cell lines which could be useful for the discovery of lead anticancer drugs. The diterpenoid **paclitaxel** represented the first member of the major group of anticancer agents that are produced by endophytes (Stierle *et al.,* 1993; Stierle *et al.,* 1995). This compound was the world's first billion-dollar anticancer drug and it is being unique in its mode of action by halting the proliferation of cancer cells. It was found to preclude tubulin molecules from depolymerizing during the processes of cell division yielding activity against a broad band

of tumor types including breast, ovarian, lung, head and neck cancers as well as advanced forms of Kaposi's sarcoma (Yuan *et al.,* 2001; Flores-Bustamante *et al.,* 2010). Paclitaxel® was found in *Taxus brevifolia* (Suffness, 1995). However, these trees are rare, slow growing and produce only small amounts of paclitaxel which makes its cost unsuitable to most people of the world (Yuan *et al.,* 2001). In the ongoing search for alternative and cheaper sources of paclitaxel, the research group of Gary Strobel discovered a novel paclitaxel-producing endophytic fungus, *Taxomyces andreanae*, which is associated with *Taxus brevifolia* (Stierle *et al.,* 1993; Stierle *et al.,* 1995).

After the huge clinical success of paclitaxel, a number of studies for screening the high-yielding paclitaxel species were conducted at geographically and ecologically diverse sites all over the world. Paclitaxel was found in endophytic fungi harbored in other *Taxus* species as well as in non-*Taxus* plants (Bashyal *et al.,* 1999; Wang *et al.,* 2000; Liu *et al.,* 2009).

Recently, around 200 endophytic fungi belonging to more than 40 fungal genera from several different orders have been reported to produce paclitaxel (Flores-Bustamante *et al.,* 2010; Hao *et al.,* 2013) indicating the widespread biogenetic capacity for the production of this important drug from fungi rather in plants. Moreover, it was also demonstrated that the production of known herbal compounds in endophytes is not restricted to paclitaxel but extends also to further pharmacologically important natural products such as the antineoplastic **camptothecin** (CPT) and its structural analogues (Puri *et al.,* 2005; Kusari *et al.,* 2009a; Kusari *et al.,* 2011; Shweta *et al.,* 2010), anticancer drugs **vincristine**, the pro-drugs **podophyllotoxin** (Eyberger *et al.,* 2006; Puri *et al.,* 2006) and **deoxypodophyllotoxin** (Kusari *et al.,* 2009b), anti-depressants **hypericin** and **emodin** (Kusari *et al.,* 2008; Kusari *et al.,* 2009c) and others. However, it is quite disappointing that none of these endophytes have been successfully translated into industrial bioprocesses so far and this is mainly due to the inability of the hosting fungus to reproduce high-titer yields in axenic cultures. Meanwhile, introduction of innovative chemical techniques and biotechnological approaches are now emerging to tackle this issue (Kusari and Spiteller, 2011; Kusari *et al.,* 2014).

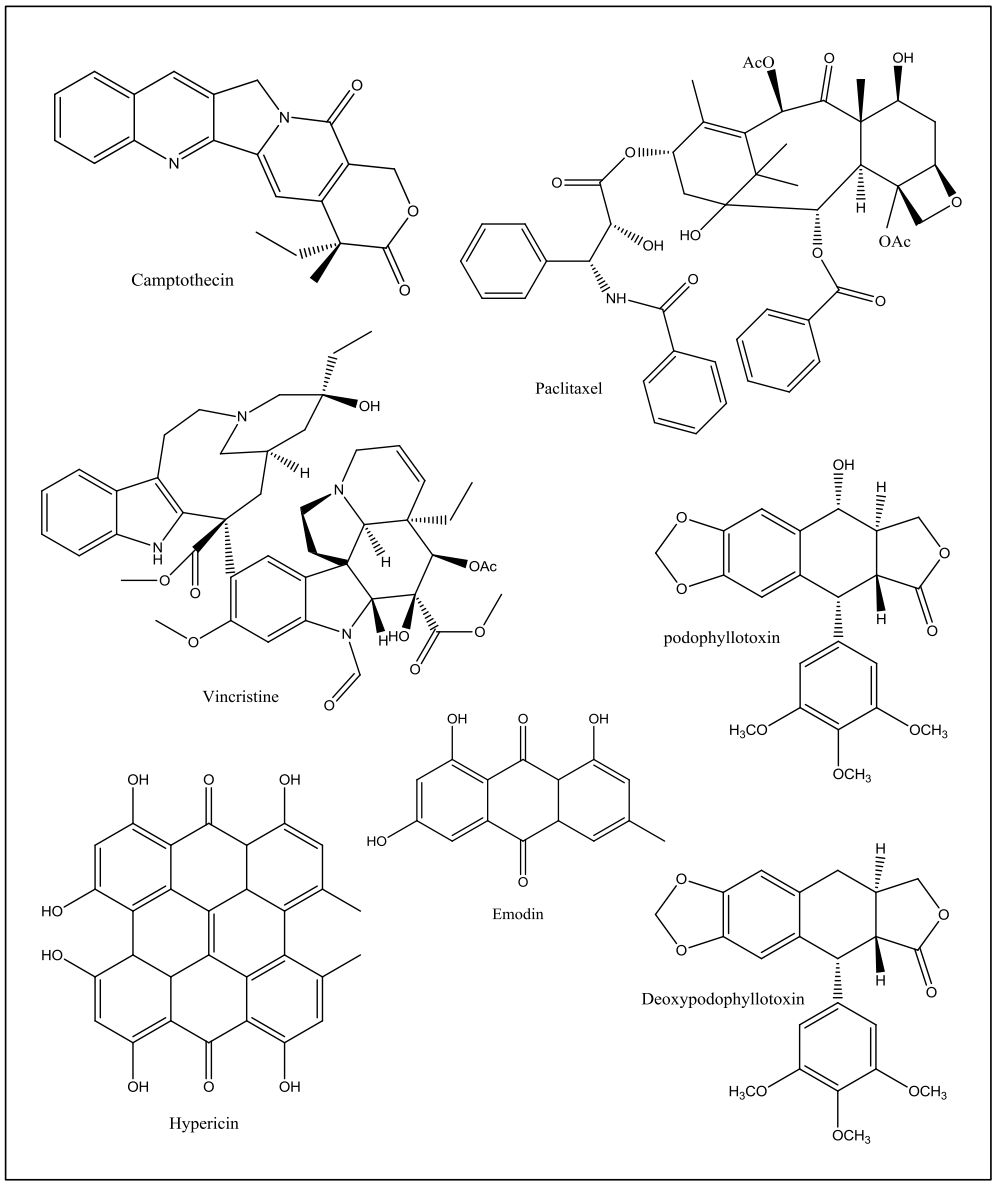


Figure 1.3: Endophytic secondary metabolites that mimic their host compounds.

So, it is generally accepted that endophytes represent an important and largely untapped reservoir of unique chemical structures for drug discovery or as lead compounds for agriculture

(Alvin *et al.,* 2014). In addition, they are of interest as alternative source for secondary metabolites produced by their host plants. Current research on endophytes is aiming towards a better understanding of the dynamic endophyte–plant relationship with respect to ecological, biochemical and molecular contexts (Aly *et al.,* 2011; Kusari *et al.,* 2012; Verma *et al.,* 2014). This will not only help to optimize secondary bioactive metabolite production under laboratory conditions but also allow industrial scale of production of the desired compounds.

1.2.1. Endophyte host plant relationship

It is believed that many plant processes have been attributed to be shaped by endophytic fungal association. For example, endophytic fungi are suggested to play a major role in structuring plant communities and in shaping processes such as colonization, competition, coexistence and soil nutrient dynamics (Saikkonen *et al.,* 1998). Thus, endophytes form a symbiotic relationship with their plant host (Kogel *et al.,* 2006). Depending on the species involved, the outcome of a plant-endophyte interaction can range from antagonism to mutualism. This is why the distinct range of plant-endophyte interactions has been referred to as a continuum (Tan and Zou, 2001; Schulz and Boyle, 2005).

In case of mutualistic fungi, they contribute to fitness benefits which are responsible for plant adaptation to biotic and abiotic stress by increasing resistance to drought and water stress as well as tolerance to high temperature and high salinity (Redman *et al.,* 2002; Strobel and Daisy, 2003; Zhang *et al.,* 2006; Rodriguez *et al.,* 2009; Aly *et al.,* 2011; Selim *et al.,* 2012; Padhi *et al.,* 2013). It is noteworthy that in many cases the microbes function as the biological defense for the plant against foreign phytopathogens. The protection mechanisms of the endophytes are exerted directly by releasing metabolites to attack any antagonists or lyse affected cells and indirectly by either inducing host defense mechanisms or promoting growth. (Tan and Zou, 2001; Berg and Hallmann, 2006; Hartley and Gange, 2009; Aly *et al.,* 2011; Padhi *et al.,* 2013; Alvin *et al.,* 2014).

For instance, one of the recently suggested protection mechanisms of the former microorganisms hypothesized that endophytes may enhance the stress tolerance of their host plants by scavenging the harmful reactive oxygen species (ROS) which are generated by the plant defense mechanisms in response to environmental stress (White and Torres, 2010). In addition, the vast variety of antioxidant compounds produced by endophytes may also contribute

to the enhancement of stress tolerance in their hosts (Herrera-Carillo *et al.,* 2009; Torres *et al.,* 2009) and in return, the covalent relationship benefits the endophytic fungi itself. It is also possible that the host plant may provide compounds which are critical for the completion of the life cycle of the endophyte or essential for its growth or self-defense (Metz *et al.,* 2000; Strobel, 2002b; Rudgers *et al.,* 2004).

Interestingly, during the long periods of this co-evolution the coordinated association forced both partners to evolve strategies for mutual adaptation as indicated by the lack of plant-defense reactions following microbial infection (Christensen *et al.,* 2002). Consequently, some endophytic fungi have developed an ability to produce the same or similar bioactive substances as those originating from the host plants. Some recent reports suggest that the production of these substances is a result of a complex system of communication involving genetic cross link between endophytes and their respective host plants during the establishment of the endophytic association (Kusari *et al.,* 2012; Kozyrovska, 2013).

One of these concepts which have long been hypothesized is the horizontal gene transfer (HGT) between the plant host and its endophytes although so far this process has only been shown to occur between microbial endophytes (Taghavi *et al.,* 2005). It has been strongly suggested that interactions between endophytes and their respective plant host contribute to the co-production of these bioactive molecules (Heinig *et al.,* 2013). For example, in the case of paclitaxel it has been recently shown that the endophyte genomes do not contain any sequences with significant homology to the paclitaxel biosynthetic genes from *Taxus* species. (Heinig *et al.,* 2013) indicating that paclitaxel biosynthesis in endophytes might have developed independently from its plant host. However, the mechanism still remain enigmatic (Verma *et al.,* 2014).

The plant–endophyte interactions would provide some useful insights into the complex host-endophyte relationship. Recent observations suggest that the type of interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environment (Moricca and Ragazzi, 2008). It seems that many factors in the host as related to the season, age, environment and location may influence the biology of the endophyte. This indicates that bioprospecting for endophyte natural products should be host plant based rather than fungal taxon based (Strobel and Daisy, 2003).

1.2.2. Rationale for plant selection from endophytic fungal diversity

Considering the enormous number and the diversity of plants, ingenious strategies should be utilized to narrow the search for endophytes which are producing plant compounds. Investigating the secondary metabolites from the plants growing in unique environmental settings, plants with an unconventional biology as well as plants with established ethnobotanic values might be the best setting for plant-endophyte interactions (Strobel *et al.,* 2004). Endophytic fungi associated with traditionally used medicinal plants especially those of the tropical regions would be more promising sources of endophytes which are producing novel biochemicals (Kaul *et al.,* 2012; Wang *et al.,* 2012; Baraban *et al.,* 2013; Alvin *et al.,* 2014; Almeida *et al.,* 2014). In addition, plants of the tropical rain forests, those growing in harsh habitats such as hot and cold deserts, saline and acidic soils and marine habitats have to be screened for bioactive metabolite-producing endophytes (Rateb and Ebel, 2011; Debbab *et al.,* 2012; Ebada and Proksch, 2013; Debbab *et al.,* 2013). Briefly, testing endophytes isolated from different tissues of plant hosts rather than those isolated from the same organ of different plant hosts and from plants growing in unusual and less-studied habitats would be far more productive (Hyde and Soytong, 2008).

1.2.3. Novel metabolites production in cultured endophytes

Plant-endophytic fungi have been found to produce novel bioactive metabolites with a wide range of medicinal applications such as antibiotics, immunosuppressants, antiparasitics, and anticancer agents. Therefore, exploring the as-yet untapped natural products from the uninvestigated endophytes increases the chances of finding novel compounds (Alvin *et al.,* 2014). Meanwhile, gene sequencing studies of fungal genomes revealed the presence of many genes encoding enzymes that produce secondary metabolites as compared to those producing the known chemicals isolated from fungi. Thus, many genes which are responsible for the production of secondary metabolites are not expressed in the artificial laboratory environment (Pettit, 2009; Schroeckh *et al.,* 2009). So, the expression of such dormant genes in fungi is promising in order to obtain novel bioactive compounds (Chen *et al.,* 2013). Recently, a more precise identification and phylogenetic accommodation of sterile morphotypes and unculturable fungi was achieved by using highly discriminant DNA-based techniques (Tejesvi *et al.,* 2009; Tejesvi *et al.,* 2011; Jeewon *et al.,* 2013; Wang *et al.,* 2014).

Moreover, the recent 'genomics revolution' has given a momentum of considerable progress in the development of new technologies in endophytic fungi biosynthesis (Kusari *et al.,* 2012). Such researches showed that gene expression is controlled by various mechanisms such as nuclear transcriptional regulators ("epigenetic modifiers"). Moreover, recent studies revealed that histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors are useful for activating dormant biosynthetic genes (Wang *et al.,* 2010; Asai *et al.,* 2011; Chen *et al.,* 2013). Indeed, the manipulation of the HDAC and DNMT activities by chemical agents alters the gene expression patterns in fungi, resulting in the production of novel secondary metabolites (Chung *et al.,* 2013).

Production of secondary constituents by endophytes may further be influenced by different growth conditions like culture parameters such as composition of growth medium (carbon, nitrogen sources, phosphate, and trace elements), aeration, light, pH, and temperature. These factors can dramatically change the secondary metabolite profile and even induce the synthesis of several new metabolites (Bode *et al.,* 2002; Suryanarayanan *et al.,* 2009). In this context, systematic alteration of cultivation parameters of a given microorganism which is known as OSMAC (one strain many compounds) approach was found to greatly increases the likeability of finding new metabolites from a single strain (Bode *et al.,* 2002).

Also, fungi are able to regulate the energetically expensive process of secondary metabolite production according to environmental conditions and specific needs (e.g. competitive forces, host plant interaction and communication) (Pettit, 2009). In this context it should be pointed out that many recent studies on natural products from endophytes have so far been conducted using co-cultivation experiments by adding cell lysates (from other fungi or from bacteria) as elicitors of secondary metabolism to cultivation media (Ola *et al.,* 2013; Fernanda *et al.,* 2013; Marmann *et al.,* 2014; Kusari *et al.,* 2014).

1.3. Hypersaline ecosystems

1.3.1. Fungi in hypersaline ecosystem

Hypersaline environments are those whose salt concentrations are above that of sea water (3.3% total dissolved salts). Based on their origin, they can be classified into thalassohaline and athalassohaline environments. The former type is characterized by salt concentration similar to that of sea water where Na^+ and Cl^- are the dominant ions and the pH is near neutral to slightly alkaline as these environments result from evaporation of sea water. Athalassohaline environments are those to have ionic composition differing hugely from that of seawater (Das Sarma and Arora, 2001). Typical examples of athalassohaline waters that have been studied in more detail are the Dead Sea, Great Salt Lake, some cold hypersaline lakes in Antarctica or alkaline lakes (East African lakes) like Lake Magadi or the lakes of Wadi Al-Natrun (Kladwang *et al.,* 2003; Butinar *et al.,* 2005; Mansour *et al.,* 2011; Gonçalves *et al.,* 2012; Oren and Cinerman, 2012).

Hypersaline lakes are considered as extreme environments for microbial life because of the effects of salt on water activity (Oren, 1999). Until a decade ago, it was a general belief in microbial ecology that fungi cannot inhabit natural hypersaline environment and in general, microbial diversity decreases with increased salinity; until it was shown that few fungi contaminating food or other substrates were recognized as "domestic extremophiles" with a general xerophilic phenotype that was determined by the water potential of the medium rather than the chemical nature of the solute (Hocking, 1993; Northolt *et al.,* 1995; Pitt and Hocking, 1997; Gunde-Cimerman *et al.,* 2000). Various subsequent reports revealed that fungi are the active inhabitants of hypersaline lakes around the world (Gunde-Cimerman *et al.,* 2000; Butinar *et al.,* 2005b; Zalar *et al.,* 2007; Sepcic *et al.,* 2011).

Halophilic and halotolerant fungi differ in halophilic behavior from the majority of halophilic prokaryotes. With few exceptions, halophilic fungi do not require salt for viability because they can grow and adapt to the salinity range from freshwater to almost saturated NaCl solutions (Plemenitas *et al.,* 2008). Phosphorous and nitrogen, are two main nutrients which consider most important variables influencing their presence and numbers in nature, followed by dissolved oxygen, water activity and pH (Cantrell *et al.,* 2011).

Studies of fungi in hypersaline ecosystems as a distinctive ecological group have been initiated only recently and these organisms still remain poorly known. To date, most of the

halophilic and halotolerant fungi described from natural hypersaline environments have been identified either as known foodborne species with previously unrecognized natural niches or as species that were not known and consequently were newly described (Cantrell *et al.,* 2011) because the isolation and identification of fungi from these habitats have been frequently hindered due to their slow growth and low ability to compete (Gunde-Cimerman and Plemenitas, 2006).

Mycologists have recognized that most of the fungi found in extreme environments belong to the imperfect stage of the Ascomycota (Zalar *et al.,* 2007). Within Ascomycota the salt tolerant black yeast *A. pullulans* (which produces exopolymer pullulan that has a broad spectrum use in the food and pharmaceutical industries (Leathers, 2003; Singh *et al.,* 2008)) and the extremely halotolerant black yeasts *T. salinum* and *H. werneckii* have been shown to produce extracellular hydrolytic enzymes that are active at high salt concentrations and could therefore exhibit important roles in different industries (Zalar *et al.,* 2005). Indeed, many halophilic and halotolerant fungi synthesize specific bioactive metabolites under stress conditions particularly at increased salt concentrations (Sepcic *et al.,* 2011).

1.3.2. Halophilic fungi from sediments of hypersaline lakes

In fact, most species during both dry-hot and freezing-cold seasons stay in a dormant stage in the bottom sediments. The rest could even last for years if suitable conditions do not return (Canfield *et al.,* 2004). For example, a small portion of the biodiversity is expressed in progressively rising salinity while the majority of species being temporarily resting as cysts in the sediments (Caumette, 1993).

A recent study demonstrated that the number of halophilic organisms and their metabolites in the sediment of a salt lake were higher than those of the organisms present in the water (Mansour *et al.,* 2011).

Studies of the chemistry of fungi from sediments of hypersaline lakes have only recently been initiated by a very limited number of research groups (Xin *et al.,* 2007). To date, no review articles covering fungal metabolites from sediments of athalassohaline environments have yet been published.

Marine fungi have proved to be a rich and promising source of novel compounds. A review of marine-derived fungi in 2011 reported that the total number of new natural products

from marine-derived fungi including isolates from sediments currently exceeds 1000. Many of these compounds have novel skeletons and display antitumor, antibiotic and antidiabetic activities (Rateb and Ebel, 2011). As an environment with much resemblance to hypersaline sediment habitats, some secondary metabolites including polyketides, terpenoids, alkaloids, peptides and dikitopiperazines were produced by fungi derived from marine sediments with close structural relationships to the fungal metabolites from hypersaline ecosystems reported to date.

A sediment-borne isolate of *Gliocladium sp.* from China was found to produce the new diketopiperazines, **gliocladride** (Yao *et al.,* 2007) and the **isomeric PJ147** and **PJ157** of which isomeric PJ147 displayed a marked cytotoxicity towards the human A375-S2 melanoma cell line (Huang *et al.,* 2007b).

Aspergiolide A, a novel anthraquinone derivative with naphtho [1,2,3-de]chromene-2,7-dione skeleton was isolated from cultures of the marine-derived fungus *Aspergillus glaucus*. The fungal strain was obtained from the marine sediment collected from mangrove roots in Fujian province, China. The compound selectively inhibited the proliferation of A549, HL-60, BEL-7402 and P388 cancer cell lines (Du *et al.,* 2007; Sun *et al.,* 2009).

Bioassay-guided investigation of the culture broth obtained from the marine-derived fungus *Cosmospora* sp. SF-5060 which was isolated from an inter-tidal sediment collected at Gejae Island, Korea yielded the known compound **aquastatin A**. The compound exhibited potent inhibitory activity against protein tyrosine phosphatase 1B (PTP1B). Studies demonstrated that PTP1B, an intracellular non-receptor type PTP, negatively regulates insulin- and leptin-receptor mediated signaling pathways. Thus, its inhibition may represent an outstanding novel therapy for type 2 diabetes and obesity (Seo *et al.,* 2009).

The fungus *Trichoderma* sp. which was isolated from marine sediment in the South China sea and found to produce a new cyclopentenone, **trichoderone**. This compound displayed selective cytotoxicity towards six cancer cell lines (You *et al.,* 2010).

Chloctanspirones A and **B**, two novel chlorinated polyketides with unprecedented skeleton, were isolated from marine sediment derived fungus *Penicillium terrestre*. The chloctanspirone A was active against both HL-60 and A549 (Lia *et al.,* 2011).

Recently, two new secondary metabolites namely, **pinodiketopiperazine A** and **6, 7-dihydroxy-3-methoxy-3-methylphthalide**, were isolated from a natural source for the first time but have been previously synthesized and found to be characterized from the marine sediment-

derived fungus *Penicillium pinophilum* SD-272. These compounds displayed potent brine shrimp (*Artemia salina*) lethality with LD_{50} 11.2 µM (Wang *et al.,* 2013).

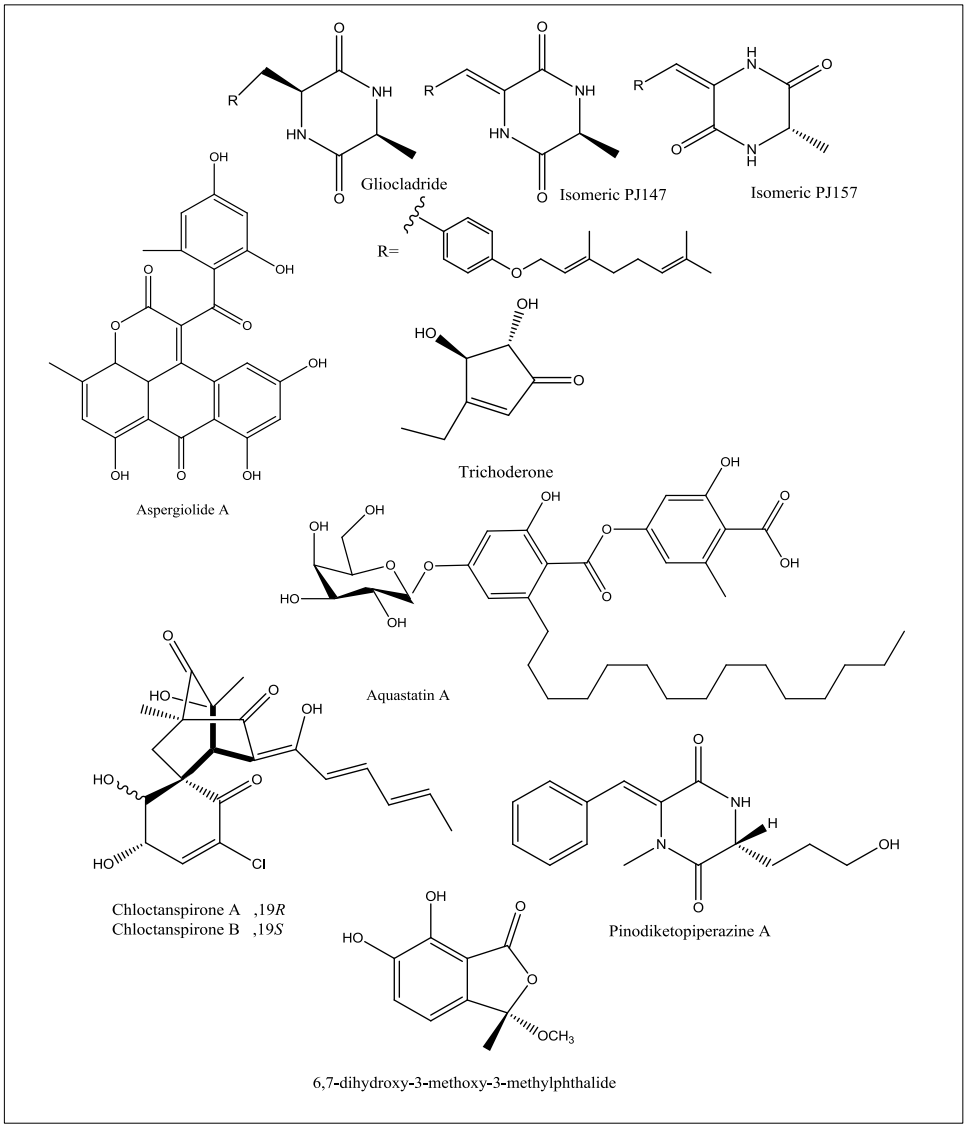


Figure 1.4: Structures of secondary metabolites isolated of fungi derived from marine sediments.

In general, halophilic fungi represent a wide source of yet undiscovered compounds that besides their unprecedented chemical structures often possess interesting biological activities.

These could be used in medicine, pharmaceutics, industry and other fields of human activities (Wang *et al.,* 2009; Sepcic *et al.,* 2011).

1.4. Aim and scope of the study

Different environmental factors including diverse physical conditions and biological situations in nature may change the behavior of fungi and favor the production of diverse range of secondary metabolites. Thus, the search for alternatively unexplored ecological niches has been targeted in this study. The aim of this study was to continue improving the efficiency of the selection, screening, dereplication, isolation and structure elucidation followed by preliminary evaluation of the pharmaceutical potential of the metabolites produced by some unexplored ecosystems which are exemplified by the following fungal isolates; the endophytic fungus *Alternaria* sp., an endophyte was originated from the seeds of *Ziziphus jujube* and the fungus *Penicillium* sp., a fungus which has been isolated from the sediment of the Wadi Al-Natrun (a hypersaline lake located in Egypt).

2. Materials and Methods:

2.1. Materials

2.1.1. Biological materials

2.1.1.1. Plant material

Seeds of *Ziziphus jujube* were collected from Uzbekistan in September 2010 by Elena Kamilova. The seeds were placed in a plastic bag after any excess moisture was removed. Every attempt was made to store the materials at 4° C until isolation procedures could be instituted.

2.1.1.2. Sediment material

The sediment samples were collected in December 2012 by Cong-Dat Pham and Hendrik Niemann from Wadi El-Natron hyper saline lakes located in Egypt 80 Km northwest of Cairo (Figure 2.1). All samples were immediately stored at 4° C until isolation procedures could be established.

Figure 2.1: Sediment sampling area (Wadi El-Natrun).

2.1.2. Media

2.1.2.1. Composition of malt agar (MA) medium

MA medium was used for short term storage of fungal cultures or fresh seeding for preparation of liquid cultures.

Agar-agar	15.0 g
Malt extract	15.0 g
Distilled water	to 1000 mL
pH	7.4 - 7.8 (adjusted with NaOH/HCl)

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.

2.1.2.2. Composition of 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)

2.1.2.3. Composition of rice medium for solid cultures

Rice	100 g
Distilled water	100 mL

Water was added to the rice and kept overnight before autoclaving.

2.1.2.4. Composition of Luria Bertani (LB) medium

This medium was used to conduct antibacterial assays.

Peptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	To 1000 mL
pH	7.0 (adjusted with NaOH/HCl)

To prepare the agar plates, 15.0 g agar were added to 1 L broth media.

2.1.2.5. Composition of yeast medium

This medium was used to perform bioassays using *Saccharomyces cerevisiae*.

Peptone	5.0 g
Yeast extract	3.0 g
Malt extract	3.0 g
Glucose	10.0 g
Distilled water	To 1000 mL

To prepare the agar plates, 15.0 g agar were added to 1 L broth media.

2.1.2.6. Composition of fungal medium for bioassay

Mannitose	50.0 g
Saccharose	50.0 g
Succinic acid	5.4 g
Yeast extract	3.0 g
KH_2PO_4	0.1 g
$MgSO_4$	0.3 g
$FeSO_4$	10.0 mg
$ZnSO_4$	10.0 mg
Distilled water	To 1000 mL
pH	5.4 (adjusted with NaOH/HCl)

2.1.2.7. Composition of potato dextrose agar (PDA) medium for bioassay

Potato infusion (see below)	1000 mL
Dextrose	20.0 g
Agar	15.0 g

Potato infusion: The potatoes (200 g) were first washed and cut into small pieces, then boiled in 1000 mL distilled water for 1 hour and filtered to get the potato infusion.

2.1.2.8. Composition of trypticase soy broth (TSB)

Peptone from casein	17.0 g
Peptone from soymeal	3.0 g

Glucose	2.5 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Distilled water	To 1000 mL
pH	7.3 (adjusted with NaOH/HCl)

2.1.3. Chemicals

2.1.3.1. General laboratory chemicals

Anisaldehyde (4-methoxybenzaldehyde)	Merck
(-)-2-Butanol	Merck
Dimethylsulfoxide	Merck
Formaldehyde	Merck
L-(+)-Ascorbic acid	Merck
Hydrochloric acid	Merck
Potassium hydroxide	Merck
Pyridine	Merck
Concentrated sulphuric acid	Merck
Trifloroacetic acid (TFA)	Merck
Concentrated ammonia solution	Fluka
Acetic anhydride	Merck
Ortho-phosphoric acid 85% (p.a.)	Merck
Sodium hydrogen carbonate	Sigma
Trifluroacetic acid (TFA)	Merck

2.1.3.2. Chemicals for culture media

Agar-agar	Galke
Chloramphenicol	Sigma
Glucose	Caelo
Malt extract	Merck
NaCl	Merck

Peptone	BD
Streptomycin	Sigma
Yeast extract	Sigma

2.1.3.3. Chemicals for agarose gel electrophoresis

Agarose	Serva
TBE-buffer	Merck
Ethidium bromide	Serva
Standards	NEB

2.1.4. Chromatography

2.1.4.1. Stationary phases

Pre-coated TLC plates, Silica Gel 60 F_{254}, layer thickness 0.2 mm	Merck
Silica Gel 60, 0.04 - 0.063 mm mesh size	Merck
Pre-coated TLC plates, RP-18, F_{254} S, layer thickness 0.25 mm	Merck
RP-18, 0.04 - 0.063 mm mesh size	Merck
Sephadex LH 20, 0.25 - 0.1 mm mesh size	Merck
Diaion HP20	Supelco

2.1.4.2. Spray reagents

The reagents were stored in amber-colored bottles and kept refrigerated until use. TLC was used to monitor the identity of each of the fractions and the qualitative purity of the isolated compounds. It was also utilized to optimize the solvent system that would be applied for column chromatography.

Anisaldehyde/H_2SO_4 Spray Reagent

Methanol	85 mL
Glacial acetic acid	10 mL
Conc. H_2SO_4	5 mL (added slowly)
Anisaldehyde	0.5 mL

Vanillin/H_2SO_4 Spray Reagent

Methanol	85 mL
Conc. H_2SO_4	15 mL (added slowly)
Vanillin	1 g

2.1.5. Solvents

2.1.5.1. General solvents

Acetone, acetonitrile, dichloromethane, ethanol, ethyl acetate, n-hexane and methanol were used. The solvents were purchased from the Institute of Chemistry, University of Duesseldorf. They were distilled before using and special grades were used for spectroscopic measurements.

2.1.5.2. 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).

2.1.5.3. Solvents for optical rotation

Chloroform	Spectral grade (Sigma)
Methanol	Spectral grade (Sigma)
Water	Spectral grade (Fluka)

2.1.5.4. Solvents for NMR

Acetone-d_6	Uvasol, Merck
Chloroform-d	Uvasol, Merck
DMF-d_7	Uvasol, Merck
DMSO-d_6	Uvasol, Merck
Methanol-d_4	Uvasol, Merck
Pyridine-d_5	Uvasol, Merck

2.2. Methods

2.2.1. Purification of fungal strains

The seeds of the Plant material were washed with sterilized demineralized water and then thoroughly surface treated with 70% ethanol for 1-2 minutes and ultimately air dried under a laminar flow hood. This is done in order to eliminate surface contaminating microbes. Furthermore, the outer tissues were removed from the seeds using a sterile scalpel, and the inner tissues were carefully dissected under sterile conditions. In the case of sediment samples, the dilution plating technique has been used (Singh *et al*., 2012). Then both samples were placed onto malt agar plates containing antibiotic (see section 2.1.2.1). After 3-4 weeks of incubation at room temperature, hyphal tips of the fungi were removed and transferred to fresh malt agar medium. Plates are prepared in duplicates to eliminate the possibility of contamination. Pure strains were isolated by repeated inoculation.

2.2.2. Cultivation of pure fungal strains

2.2.2.1. Cultivation for short term storage

Fungi were grown on malt agar medium under room temperature for several days. When fungal hyphae almost cover the surface of the MA plate, cultures were stored at 4º C for a maximum period of 6 months, and then re-inoculated onto fresh MA media.

2.2.2.2. Cultivation for screening and isolation of secondary metabolites in different media:

Mass growth of pure fungi for screening as well as isolation and identification of secondary metabolites was carried out by transferring fresh fungal cultures into Erlenmeyer flasks (1L each) containing 300 mL of Wickerham medium for liquid cultures or 100 g rice for solid cultures. The cultures were then incubated at room temperature (no shaking) for 21 and 30 days, respectively. Large scale cultivation was carried out using 30 and 10 1L Erlenmeyer flasks for liquid and solid rice cultures, respectively.

2.2.3. Extraction of secondary metabolites

2.2.3.1. Extraction of Wickerham liquid cultures

2.2.3.1.1. Total extraction method

To each 1L Erlenmeyer about 250 mL EtOAc were added and left overnight to stop cell growth. Culture media and mycelia were then extracted in the Ultraturrax for 10 min for cell destruction, followed by vacuum filtration using Buchner. The mycelium residue was discarded while culture filtrates were collected and extracted with EtOAc and *n*-BuOH till exhaustion. The combined EtOAc phases were washed with distilled water and then taken to dryness. The dry residue was then partitioned between *n*-hexane and 90% MeOH. The extraction scheme is described in (Figure 2.2).

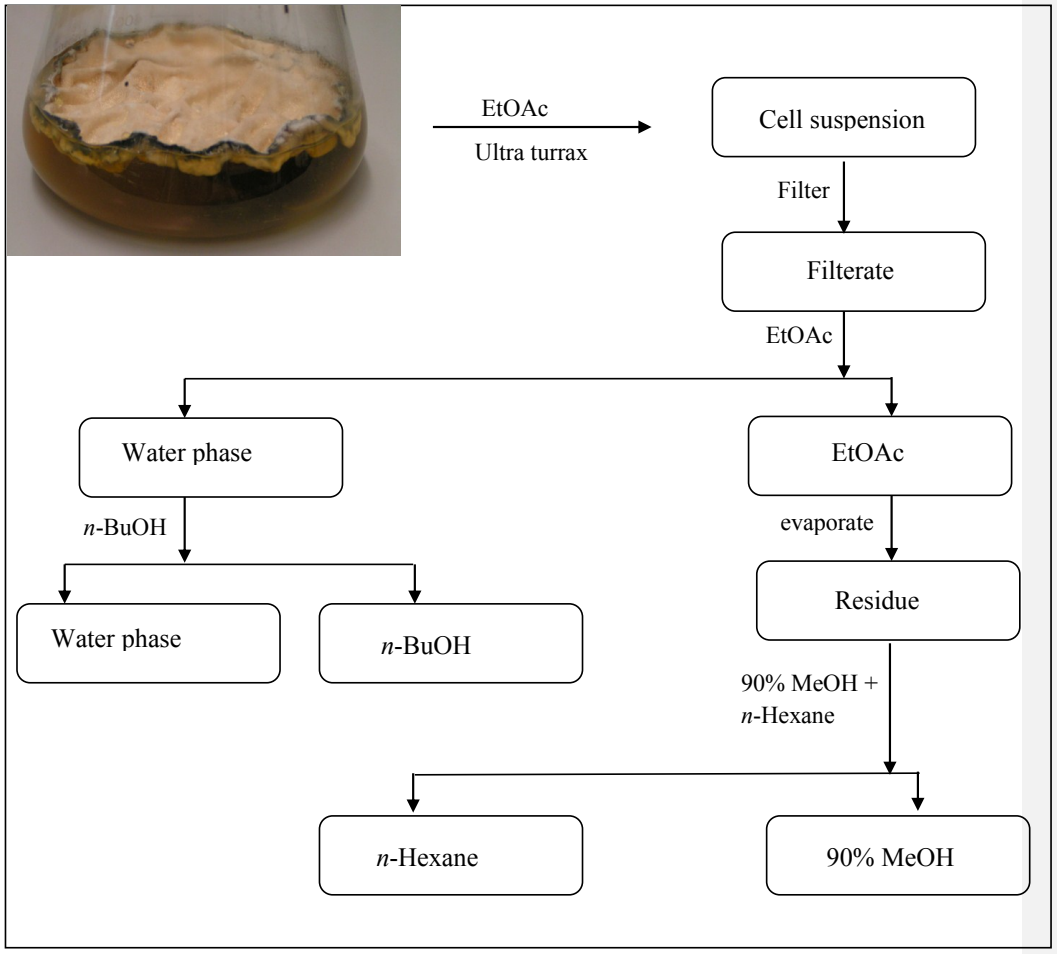


Figure 2.2: Total extraction of culture media and mycelia.

2.2.3.1.2. Separate extraction of culture media and mycelia

This taking place by separation of fungal mycelia from culture media and left in MeOH overnight. Using Ultraturrax cells were destructed and extracted for 10 min, followed by filtration and repeated extraction till exhaustion. The culture media were extracted in the same manner as described above in 2.2.3.1.1 to obtain the EtOAc extract. The extraction scheme is described in (Figure 2.3).

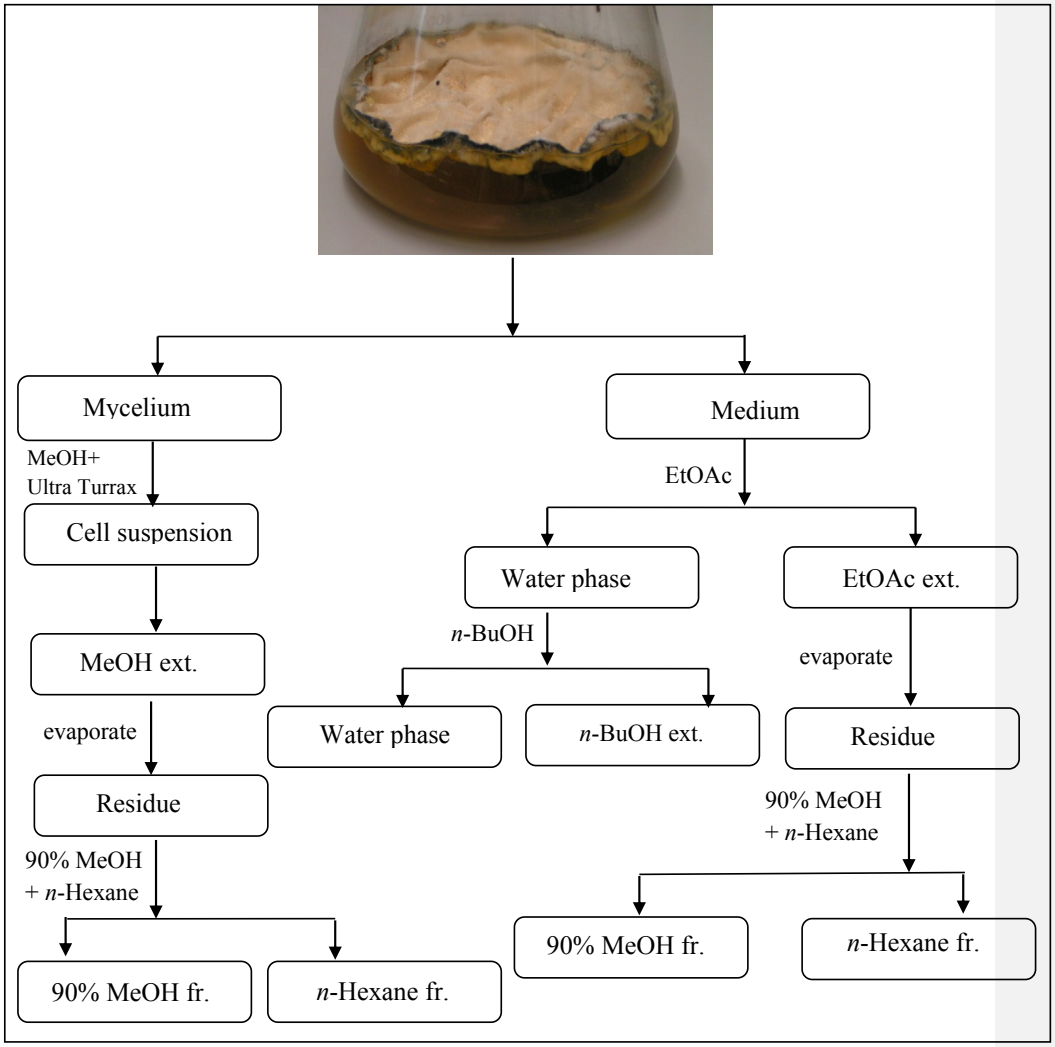


Figure 2.3: Separate extraction of culture media and mycelia.

2.2.3.2. Extraction of solid rice cultures

To each 1L Erlenmeyer about 250 mL EtOAc were added to the cultures and left overnight. Culture media were then cut in pieces to allow complete extraction and left for 3–5 days. Then filtration was done followed by repeated extraction with EtOAc and MeOH till exhaustion. The combined EtOAc phases were washed with distilled water and then taken to dryness. The dry residues obtained from EtOAc and MeOH extracts were partitioned between *n*-hexane and 90% MeOH. See (Figure 2.4).

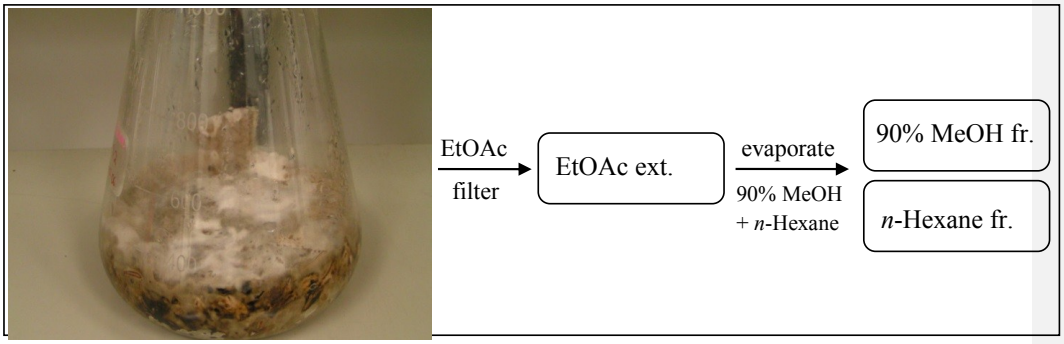


Figure 2.4: Extraction of solid rice media

2.2.3.3. Solvent-solvent extraction

Solvent-solvent extraction is a widely employed technique to separate organic compounds from a mixture. It involves the separation of compounds into two immiscible solvents. Since the technique is based upon an unequal distribution of solutes between two solvents with different polarities, the solutes will be more soluble in one solvent compared to the other. The distribution of a component A between two phases can be expressed by the distribution coefficient (K):

$$\text{Phase K} = \frac{[A]_{\text{top phase}}}{[A]_{\text{lower phase}}}$$

Where, [A] is the concentration of solute A.

The following general principles should be considered in choosing the solvents:

- The solvents involved in the extraction must be immiscible
- The solvents must not react with the components that will be separated
- The solvents should be easily removed by evaporation after the process

In this study, solvent-solvent extraction was the first step in the separation process. It was meant to "clean" the ethyl acetate extract from salts and other undesirable polar constituents by water-ethyl acetate extraction. Subsequently, the methanol-*n*-hexane extraction was applied to remove fatty acids and other undesirable non polar components.

2.2.4. Identification of fungal strains and their taxonomy

2.2.4.1. Fungal identification

Fungal strains were identified using a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region. This was carried out by Lena Hammerschmidt, Sergi Akone and the author at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf.

DNA isolation

Fungal DNA isolation has been achieved by using DNeasy® Plant Mini Kit (QIAgen). The lyophilized fungal mycelia were pulverized and disrupted with the help of glass beads. Then cell lysis was carried out by addition of lysis Buffer AP-1 and RNAse-A solution followed by incubation of the mixture at 65º C. The remaining detergent, protein and polysaccharide were precipitated by addition of Buffer AP-2 to the lysate. The lysate was then applied to the Qiashredder™ Mini Spin Column and centrifuged to remove the cell debris and other remaining precipitates. The lysate was then transferred to a new tube. An adequate volume of ethanolic Buffer AP3/E was added to the lysate and the mixture was then applied to DNeasy Mini Spin Column. After centrifugation, the filtrate was discarded. The column was washed by addition of ethanolic Buffer AW followed by centrifugation. Another portion of Buffer AW was added to the column and centrifuged at maximum speed to dry the membrane in the column from residual ethanol.

Fungal DNA, which is incorporated into the membrane, was eluted by addition of Buffer AE directly to the membrane in the DNeasy column. The column was then incubated at room temperature for 5 minutes and then centrifuged to collect the filtrate, which was the fungal DNA dissolved in Buffer AE.

The amplification of DNA

After the isolation process, the isolated DNA was then amplified by Polymerase Chain Reaction (PCR). The PCR was carried out using HotStarTaq Master Mix Kit (QIAgen). The Master Mix contains HotStarTaq®DNA polymerase, PCR buffer (with $MgCl_2$) and dNTPs.

ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) and ITS 4 (with base sequences TCCTCCGCTTATTGATATGC) (Invitrogen), as primers, were mixed with Hotstar Taq Master Mix Kit and DNA template. Thus, each PCR reaction mixture contained 5- 10 ng of genomic DNA, 1 µM each of the primers ITS 1 and ITS 4, and 1 U of Hot start Taq-Polymerase (Invitrogen) in a total volume of 50 µL. The mixture was then applied to the thermal cycler (BioRad) using the programmed PCR cycle as outlined below:

- Initial activation step in 95º C for 15 minutes to activate HotStarTaq® DNA polymerase
- Cycling steps which were repeated 35 times:

  Denaturing: 1 minute at 95º C, annealing: 1 minute at 56º C, extension: 1 minute at 72º C
- Final extension for 10 minutes in 72º C

Purification of PCR products and DNA sequencing

The PCR product was purified using 2% Agarose-Gel-Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 500 bp stained DNA fragment was then excised from the agarose gel.

The next step of PCR product purification was performed using Perfectprep® Gel Cleanup Kit (Eppendorf). The binding buffer was mixed to the PCR product and incubated at 50º C for 10 minutes in an eppendorf thermomixer at 1000 rpm. The mixture was mixed with a volume of isopropanol and then centrifuged. The filtrate was discarded and the column was washed with wash buffer twice followed by centrifugation.

Amplified fungal DNA (PCR product), which was incorporated into the column, was eluted by addition of elution buffer or molecular biology grade water to the center of the column. The column was then centrifuged to collect the filtrate, which was the fungal DNA dissolved in elution buffer. The amplified fungal DNA was then submitted for sequencing by a commercial service and the base sequence was compared with publicly available databases such as GenBank with the help of Blast-Algorithmus.

2.2.4.2. Taxonomy

***Alternaria* sp.**

The fungus *Alternaria* sp. was isolated from the seeds of *Ziziphus jujube* (see Figure 2.5). The plant seeds were collected in September 2010 from Uzbekistan.

Taxonomy

Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Dothideomycetes
Order	Pleosporales
Family	Pleosporaceae
Genus	*Alternaria*
Species	*Alternaria* sp.

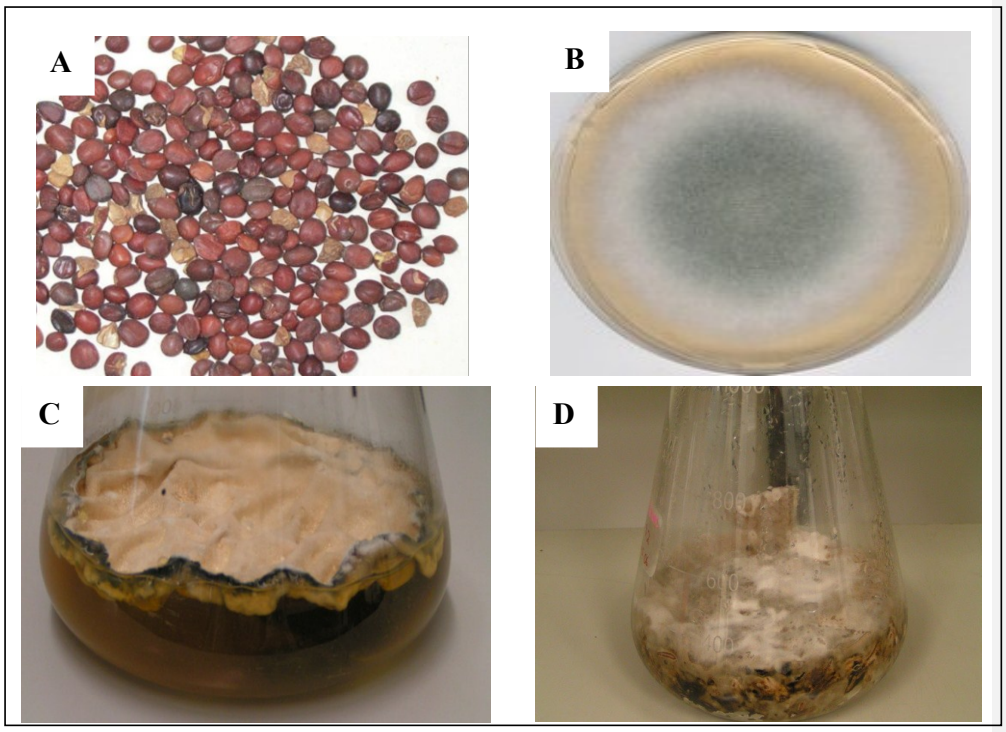


Figure 2.5: *Alternaria* sp. (**A**: Seeds of *Ziziphus jujube*. **B**: Pure strain on malt agar plate. **C**: Liquid culture in Wickerham medium. **D**: Rice culture).

***Penicillium* sp.**

The fungus *Penicillium* sp. was isolated from the sediment of hypersaline lake (see Figure 2.6). The sediment was collected in December 2012 from Wadi El-Natrun, Egypt.

Taxonomy

Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Euascomycetes
Order	Eurotiales
Family	Trichomaceae
Genus	*Penicillium*
Species	*Penicillium* sp.

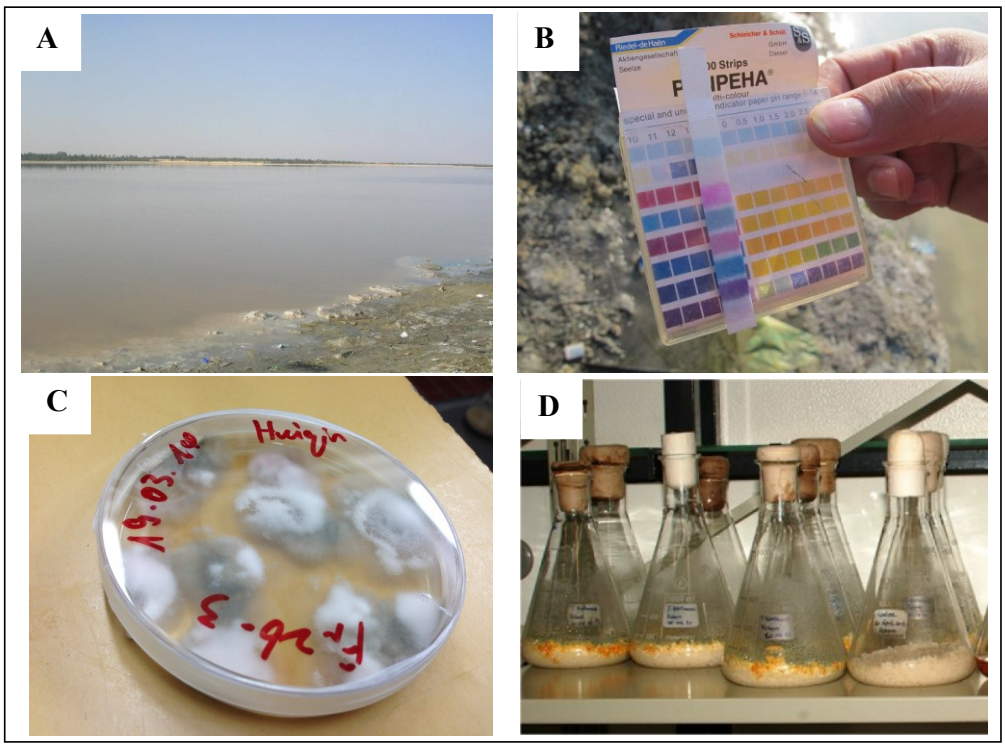
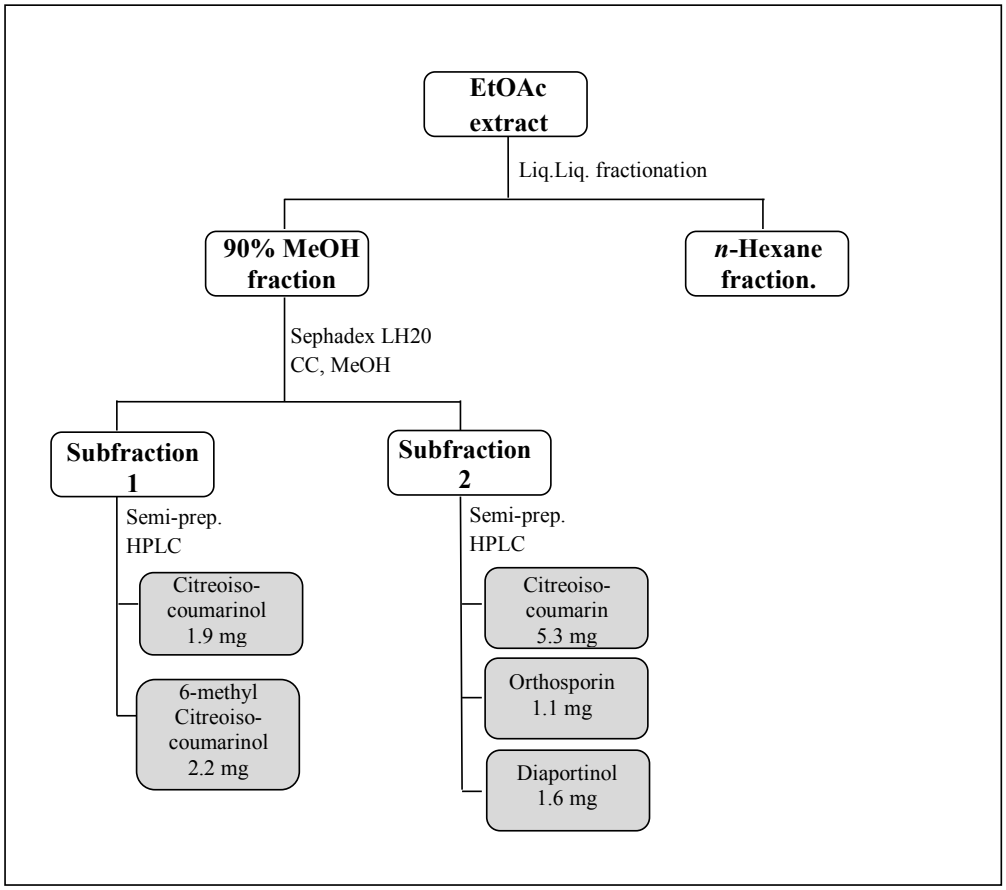


Figure 2.6: *Penicillium* sp. (**A**: A lake in Wadi El-Natrun, Egypt. **B**: Hyper salinity of the lake, PH=10. **C**: Pure strain on malt agar plate. **D**: Rice culture).

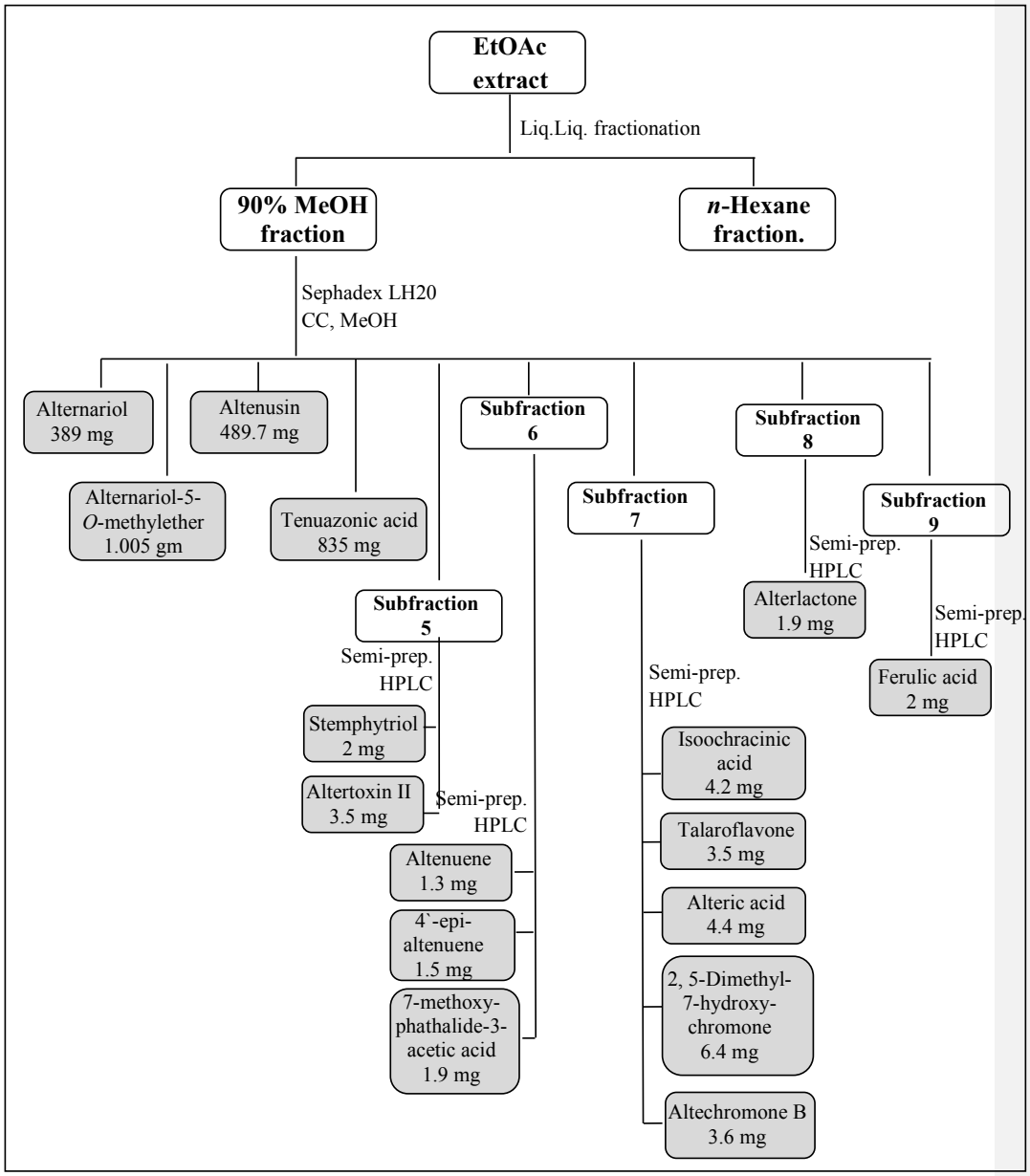
2.2.5. Isolation and purification of secondary metabolites

2.2.5.1. Isolation of the secondary metabolites from *Alternaria* sp.

2.2.5.1.1. Secondary metabolites isolated from small scale of *Alternaria* sp.

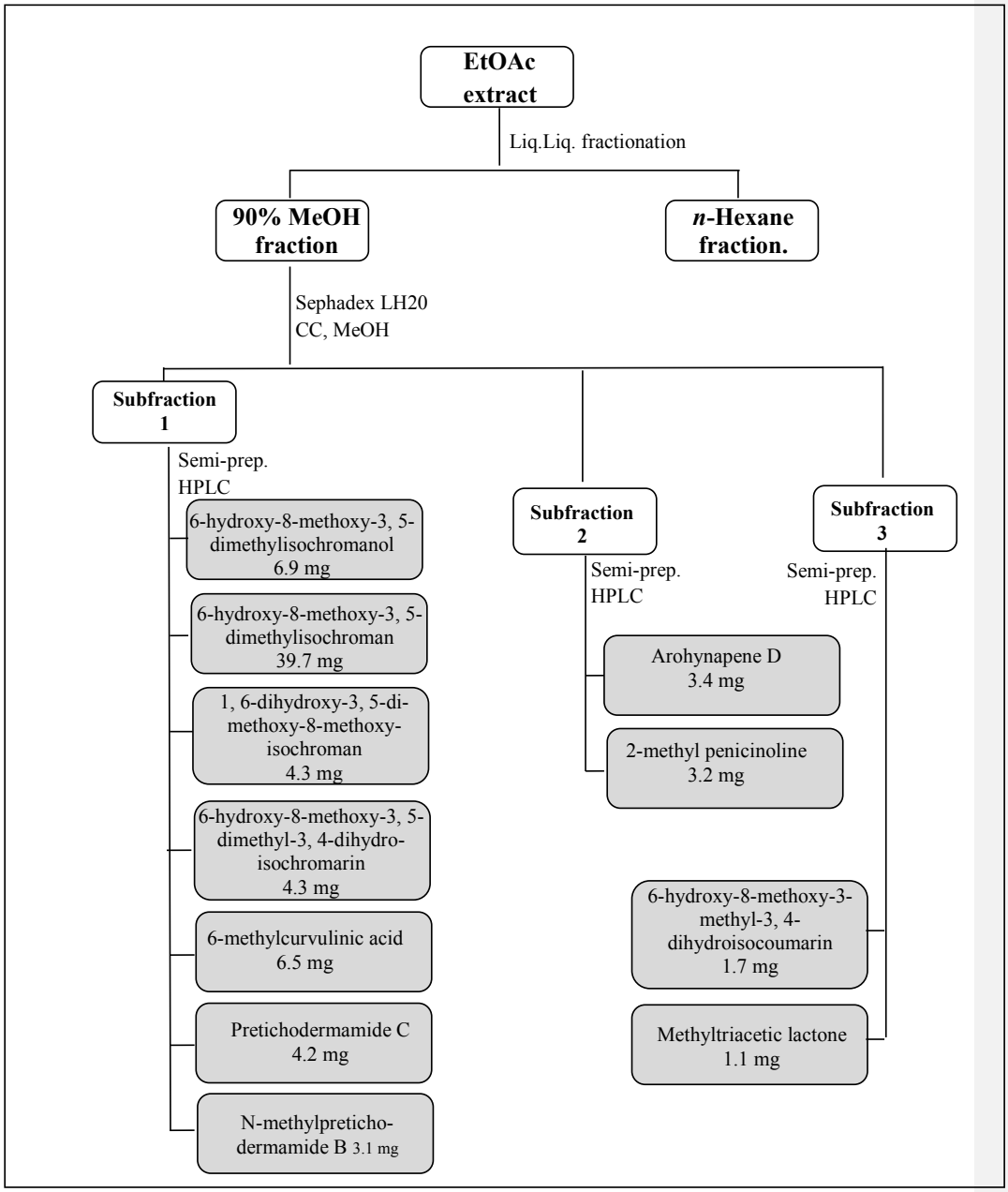


2.2.5.1.2. Secondary metabolites isolated from large scale of *Alternaria* sp.



2.2.5.2. Isolation of the secondary metabolites from *Penicillium* sp.

2.2.5.2.1. Secondary metabolites isolated from rice cultures of *Penicillium* sp.



2.2.5.3. Chromatographic methods

2.2.5.3.1. Thin layer chromatography (TLC)

Chromatography refers to any separation method in which the components are distributed between stationary phase and mobile phase. The separation occurs because sample components have different affinities for the stationary and mobile phases and therefore move at different rates along the TLC plates and the column. TLC was performed on pre-coated TLC plates with silica gel 60 F_{254} (layer thickness 0.2 mm, E. Merck**,** Darmstadt, Germany**)** with the following eluents:

For polar compounds

EtOAc: MeOH: H_2O (30:5:4, 30:6:5 and 30:7:6)

For semi-polar compounds

DCM:MeOH (95:5, 90:10, 85:15, 80:20 and 70:30)

DCM:MeOH:EtOAc (90:10:5 and 80:20:10)

For non-polar compounds

n-Hexane:EtOAc (95:5, 90:10, 85:15, 80:20 and 70:30)

n-Hexane:MeOH (95:5 and 90:10)

TLC on reversed phase RP18 F_{254} (layer thickness 0.25 mm, Merck, Darmstadt, Germany) was used for polar substances and using the different solvent systems of MeOH: H_2O (90:10, 80:20, 70:30 and 60:40). The band separation on TLC was detected under UV lamp at 254 and 366 nm, followed by spraying the TLC plates with anisaldehyde/H_2SO_4 or vaniline/H_2SO_4 reagent and subsequent heating at 110 °C.

2.2.5.3.2. Column chromatography

Fractions derived from liquid –liquid fractionation between n-hexane and 90% MeOH and 10% H_2O. The last polar fraction were subjected to repeated separation through column chromatography using appropriate stationary and mobile phase solvent systems previously determined by TLC. Size exclusion separation system were used involves separations based on molecular size of compounds being analyzed. The stationary phase consists of porous beads

(Sephadex LH-20). The larger compounds will be excluded from the interior of the bead and thus will elute first. The smaller compounds will be allowed to enter the beads and elute according to their ability to exit from the small sized pores they were internalized through. Elution was performed using MeOH.

2.2.5.3.3. Semi-preparative high pressure liquid chromatography (HPLC)

This process was used for purification of compounds from fractions previously separated using column chromatographic separation. The most appropriate solvent system was determined before running the HPLC separation. The mobile phase combination was MeOH and nanopure H_2O with or without 0.01 % TFA or 0.1 % formic acid, pumped in gradient or isocratic manner depending on the compounds retention time. Each injection consisted of 1-3 mg of the fraction dissolved in 1 mL of the solvent system. The solvent system was pumped through the column at a rate of 5 mL/min. The eluted peaks were detected by the online UV detector and collected separately in Erlenmeyer flasks. The separation column (125 × 4 mm, ID) was pre-filled with Eurospher C18 (Knauer, Berlin, Germany).

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.

2.2.5.3.4. Analytical high pressure liquid chromatography (HPLC)

Analytical HPLC was used to identify the distribution of peaks either from extracts or fractions, as well as to evaluate the purity of isolated compounds. The solvent gradient used started with MeOH: nanopure H_2O (10:90), adjusted to pH 2 with phosphoric acid, and reached to 100 % MeOH in 35 minutes. The autosampler injected 20 µL sample. All peaks were detected by UV-VIS photodiode array detector. In some cases, special programs were used. HPLC instrument consists of the pump, the detector, the injector, the separation column and the reservoir of mobile phase. The separation column (125 × 2 mm, ID) was pre-filled with Eurospher-100 C18 (5 µm), with integrated pre-column (Knauer, Berlin, Germany).

LC/UV system specifications are described as follows:

-Pump	Dionex P580A LPG
-Detector	Dionex Photodiode Array Detector UVD 340S
-Column thermostat	STH 585
-Autosampler	ASI-100T
HPLC Program	Chromeleon (V. 6.3)
Column	Knauer (125 × 4 mm, ID), pre-packed with Eurosphere 100-5 C18, with integrated pre-column.

2.2.6. Structure elucidation of the isolated secondary metabolites

2.2.6.1. Mass spectrometry (MS)

Mass spectrometers use the difference in mass-to-charge ratio (*m/z*) of ionized molecules to separate them from each other. Mass spectrometry is therefore useful for quantification of atoms or molecules and also for determination of chemical and structural information of molecules. A mass spectrometer consists of an ion source, ion detector and mass-selective analyzer. The output of mass spectrometers shows a plot of relative intensity vs. the mass-to-charge ratio (*m/z*).

2.2.6.1.1. Electrospray ionization mass spectrometry (ESI-MS)

A mass spectrometer is an analytical instrument used to determine the molecular weight of a compound. Basically, mass spectrometers are divided into three parts; ionization source, analyzer and detector, which should be maintained under high vacuum conditions in order to maintain the ions travel through the instrument without any hindrance from air molecules. Once a sample was injected into the ionization source, the molecules are ionized. The ions were then passed and extracted into the analyzer. In the analyzer, the ions were separated according to their mass (*m*) to charge (*z*) ratio (*m/z*). Once the separated ions flow into the detector, the signals are transmitted to the data system where the mass spectrum is recorded.

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 H_2O/MeOH mixtures and injected to HPLC/ESI-MS set-up. For standard MS/MS measurements, a solvent gradient that started with acetonitrile: nanopure H_2O (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.

2.2.6.1.2.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.

HRESI-MS was measured on a Micromass Qtof 2 mass spectrometer at Helmholtz Centre for Infection Research, Braunschweig. The time-of-flight analyzer separates ions according to their mass-to-charge ratios (*m/z*) by measuring the time it takes for ions to travel through a field free region known as the flight.

2.2.6.2. Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic

field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. It is used to study physical, chemical, and biological properties of matter. As a consequence, NMR spectroscopy finds applications in several areas of science. NMR spectroscopy is routinely used by chemists to study chemical structure using simple one dimensional technique. Two dimensional techniques are used to determine the structure of more complicated molecules.

NMR spectra were recorded at 300º K on a Bruker ARX-500 by Dr. Peter Tommes, and Burker AVANCE DMX 600 NMR spectrometer by Dr. Schaper, Klaus, Institut für Anorganische und Strukturchemie, Heinrich-Heine Universität, Düsseldorf. All 1D and 2D spectra were obtained using the standard Bruker software. The samples were dissolved in different solvents, the choice of which was dependent on the solubility of the samples. Residual solvent signals were used as internal standards (reference signal). The observed chemical shift (δ) values were given in ppm and the coupling constants (J) in Hz.

2.2.6.3. Optical activity

Optically active compounds contain at least one chiral centre. Optical activity is a microscopic property of a collection of these molecules that arises from the way they interact with light. Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter. The substance was stored in a 0.5 mL cuvette with 0.1 dm length. The angle of rotation was measured at the wavelength of 546 and 579 nm of a mercury vapour lamp at room temperature (25º C). The specific optical rotation was calculated using the expression:

$$[\alpha]_D^T = \frac{[\alpha]_{579} \times 3.199}{4.199 - \frac{[\alpha]_{579}}{[\alpha]_{546}}}$$

With $[\alpha]_D^T$= the specific rotation at the wavelength of the sodium D-line, 589 nm, at certain temperature T.

$[\alpha]_{579}$ and $[\alpha]_{546}$= the optical rotation at wavelengths 579 and 546 nm, respectively, calculated using the formula:

$$[\alpha]_\lambda = \frac{100\ \alpha}{l \times c}$$

Where α = the measured angle of rotation in degrees,

l = the length in dm of the polarimeter tube,

c = the concentration of the substance expressed in g/100 mL.

2.2.7. Testing the biological activity

Finding biologically important compounds from fungi, endophytic or aquatic is only achieved if, and when, assay systems have been devised that will allow for successful biologically guided fractionation of the culture extracts.

2.2.7.1. Antimicrobial serial dilution assay

This test was conducted under aseptic conditions using microtiter 96 well plates.

Microorganisms

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

Gram-positive bacteria	*Streptococcus pneumonia*
	Multi resistant *Staphylococcus aureus* (MRSA)
	Enterococcus faecalis
Gram-negative bacteria	*Klebsiella, pneumonia*
	Esherichia coli
	Pseudomonas aeruginosa
Fungi	*Aspergillus fumigatus*
	Aspergillus faecalis
	Candida albicans
	Candida krusei

Antimicrobial screening assay

Test samples were dissolved in 0.2 mL DMSO followed by dilution in 800 μL cell culture water. For further use, pure compounds were diluted from 250 to 62.5 μg/mL and extracts from 1250 to 312 μg/mL in MHB medium for bacterial screening and in Sabauroud

medium, for fungal screening. Afterwards the substance/extract solution was overlaid with the microbes (10^4 CFU/mL). Then plates were incubated at 35°C for 24 h and 48 h to allow bacterial and fungal growth, respectively. As negative control an antibiotic/antimycotic mix was used in addition to a non-treated infected control (positive).

The test was analysed by checking the microbial growth with the visible eye and by measurement of the turbidity at 650 nm. All procedures were done under aseptic conditions in a sterile laminar air flow according to good laboratory practice.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is defined as the least concentration of an antimicrobial required to inhibit or control the growth of a micro-organism. Substances with an activity around 125 μg/ml and extracts with 625 μg/ml were considered as possible candidates for further antimicrobial screening. With these positive candidates, MIC assays were performed to determine their exact values. Therefore the substances/extracts were diluted from 125 μg/mL to 0.24 μg/mL and screened in the same manner as in the primary screening. MICs were distinguished as the least dilutions of the substances/extracts that revealed no microbial growth visibly or by measurement of the turbidity at 650 nm.

2.2.7.2. Cytotoxicity test

2.2.7.2.1. Microculture tetrazolium (MTT) assay

Cytotoxicity tests were carried out by Prof. Dr. W. E. G. Müller, Institut fürPhysiologische 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 *et al.*, 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 microtiter 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 3 h 45 min at 37° C in a humidified incubator with 5% CO2, 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 microtiter-well spectrophotometer. The colour intensity is correlated with the number of healthy living cells. Cell survival was calculated using the formula:

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

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. Results:

3.1. Compounds isolated from the endophytic fungus *Alternaria* sp.

In this study, we investigated an endophytic fungal strain of the genus *Alternaria*, which was isolated from the healthy seeds of *Ziziphus jujube* growing in Uzbekistan. The pure fungal strain was cultivated first on small scale liquid Wickerham medium and both the antibiotic and the cytotoxic properties of the extract were evaluated. The EtOAc extract was subjected to further chromatographic methods to yield compound (**3**) citreoisocoumarin as a major substance in HPLC screening (see Figure 3.1 A). In addition, four components, *viz*, citreoisocoumarinol (**1**), 6-methyl-citreoisocoumarin (**2**)**,** orthosporin (**4**) and diaportinol (**5**) have been isolated from the same culture.

Furthermore, a large scale solid rice medium had been done. Interestingly, chemical screening using HPLC indicated a clear difference between *Alternaria* extracts obtained from small scale liquid medium and the large scale solid one. Investigation of the large scale rice cultures revealed altenusin (**8**) and alternariol (**6**) as main components with no traces of citreoisocoumarin (**3**) (see Figure 3.1 B). In addition, fourteen substances have been isolated from this large scale; *vis*, alternariol-5-*O*-methyl ether (**7**), tenuazonic acid (**9**), stemphytriol (**10**), altertoxin II (**11**), altenuene (**12**), 4`-epialtenuene (**13**), isoochracinic acid (**14**), 7-methoxyphthalide-3-aacetic acid (**15**), talaroflavone (**16**), alteric acid (**17**), alterlactone (**18**), 2, 5-dimethyl-7-hydroxychromone (**19**), altechromone B (**20**), and ferulic acid (**21**).

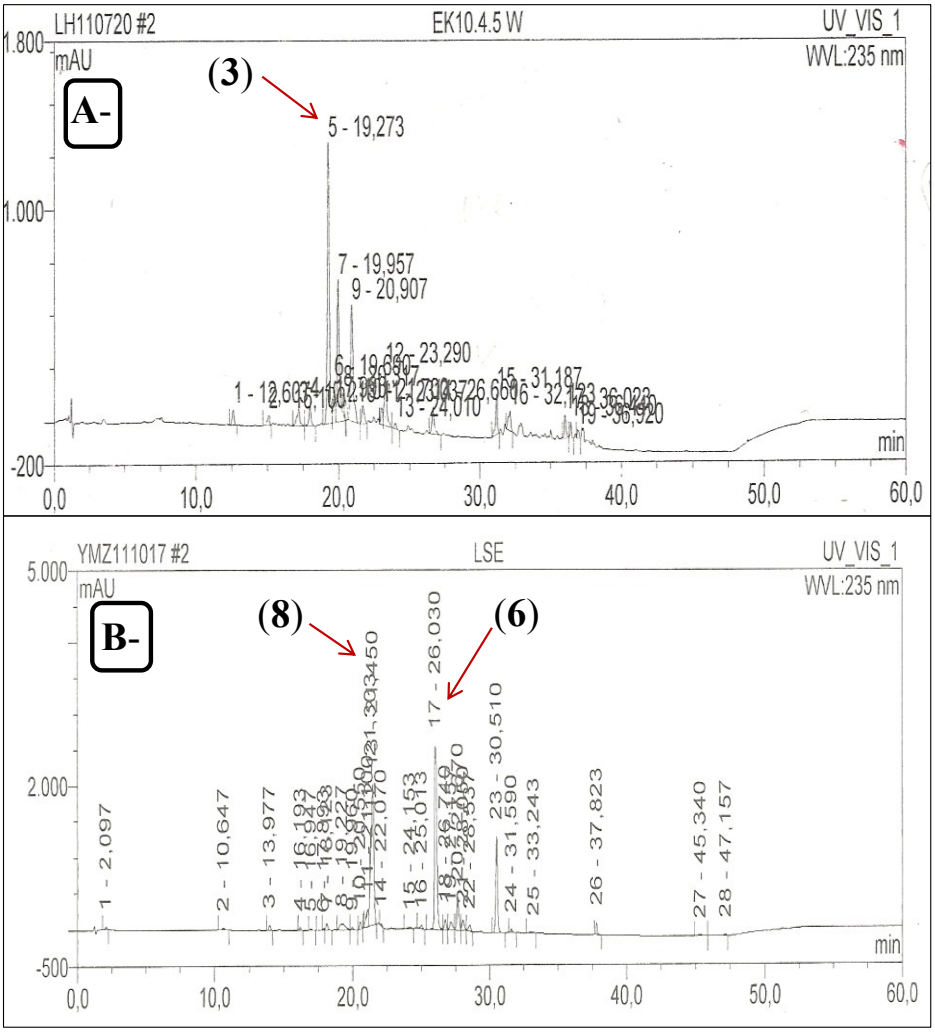
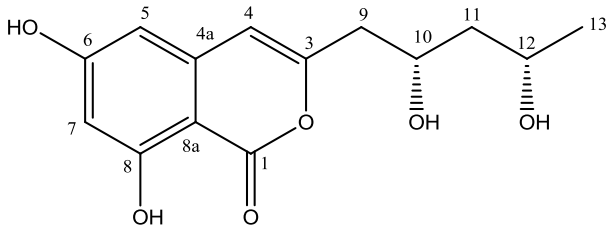
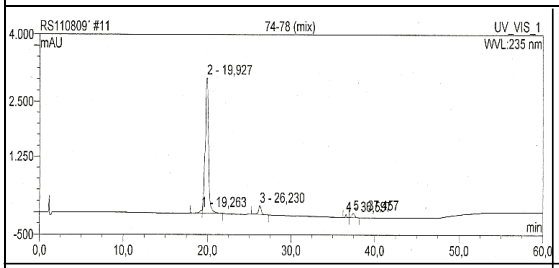


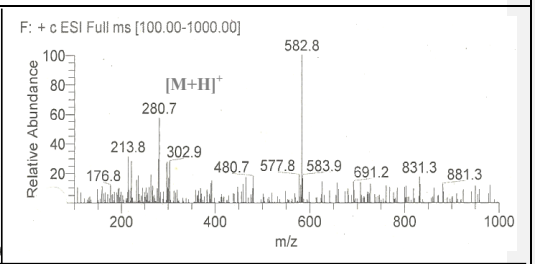
Figure 3.1 A-B: HPLC chromatograms of EtOAc extracts of *Alternaria* sp. cultures. **A:** Small scale in liquid medium (Wickerham medium) **B:** Large scale on rice medium. **(3):** Citreoisocoumarin. **(6):** Alternariol. **(8):** Altenusin.

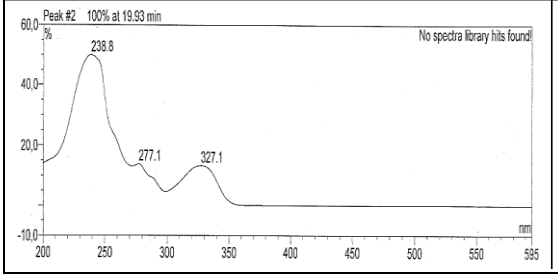
3.1.1. (+) - Citreoisocoumarinol (1, known compound)

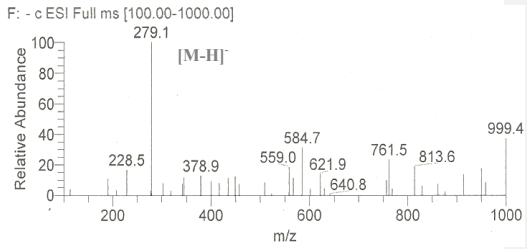
(+)-Citreoisocoumarinol	
Synonym(s)	3- (10,12-Dihydroxypentyl)-6,8-dihydroxy-isocoumarin
Sample code	74-78(mix)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.9 mg
Physical Description	Brown solid
Molecular Formula	$C_{14}H_{16}O_6$
Molecular Weight	280 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 15 (*c* 0.12, MeOH)
Retention time HPLC	19.9 min (standard gradient)











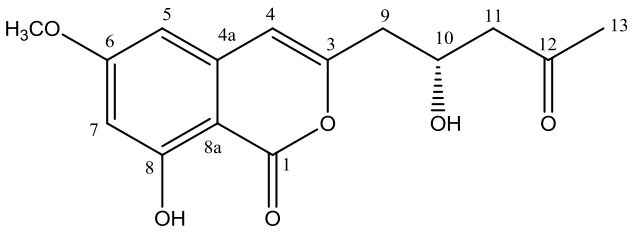
Compound (**1**), was isolated as a brown solid from the EtOAC extract of the small scale liquid cultures of *Alternaria* sp. Positive and negative ESIMS showed molecular ion peaks at *m/z* 280.7 $[M+H]^+$ (base peak) and *m/z* 279.1 $[M-H]^-$ (base peak), respectively indicating a molecular weight of 280 g/mol. Compound (**1**) was likely to be an isocoumarin derivative based on its characteristic UV absorption maxima at 238.8, 277.1 and 327.1 (Chong and Huang, 1986).

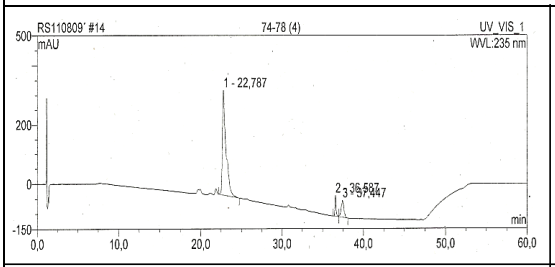
The proposed isocoumarin structure of this compound was further supported by its ^1H NMR and ^{13}C NMR data (Table 3.1). The ^1H NMR spectrum displayed characteristic downfield signals which are attributed to two overlapped aromatic signals which appear as a singlet at δ_H 6.33 ppm for H-5 and H-7, and an olefinic singlet at δ_H 6.39 ppm for H-4. Moreover, the up ^1H NMR spectrum showed a doublet at δ_H 1.20 ppm for CH_3-13, two geminal-coupled methylene protons; CH_2-9 (at δ_H (2.70 ppm [dd (*J*=4.1, 14.7 Hz)] and δ_H 2.57 ppm [dd (*J*=8.3, 14.6 Hz)]) and CH_2-11 at (δ_H 1.70 ppm [ddd (*J*=13.9, 8.5, 7.8 Hz)] and δ_H 1.60 ppm [*ddd* (*J*=13.9, 5.3, 4.4 Hz)]) and two downfieled multiplets at δ_H 4.15 and δ_H 4.00 ppm for two methine-groups H-10 and H-12, respectively. The ^{13}C NMR spectrum revealed the presence of 14 carbon atoms, eight aromatic carbons, a carbonyl function C-1 (δ_C 167.2 ppm), two oxygenated methine groups C-10 and C-12 (δ_C 67.1 and 68.7 ppm), respectively, two methylene groups C-9 and C-11 (δ_C 42.5 and 46.4 ppm), respectively and a methyl carbon C-13 (δ_C 22.5 ppm) .

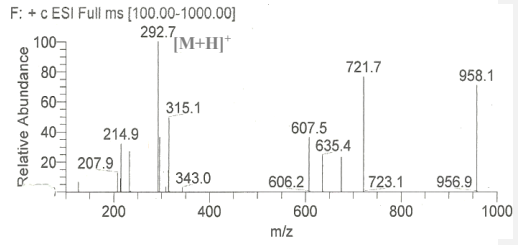
The ^1H-^1H-COSY spectrum (Figure 3.2) of compound (**1**) showed two spin systems, the first one is CH_3(13) CH (12) CH_2(11) CH (10) CH_2(9) and the second one is CH(7) CH(5). The $[\alpha]_D^{20}$ value was in full agreement with the published data of citreoisocoumarinol (Sheng *et al*., 1991). (+)-Citreoisocoumarinol was originally isolated from *Penicillium* sp. (Sheng *et al*., 1991). It is worth to mention that in this study the known compound (**1**) has been isolated for the first time from *Alternaria* sp.

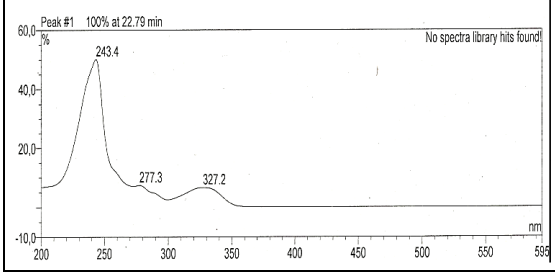
3.1.2. (+)-6-Methyl-citreoisocoumarin (2, known compound)

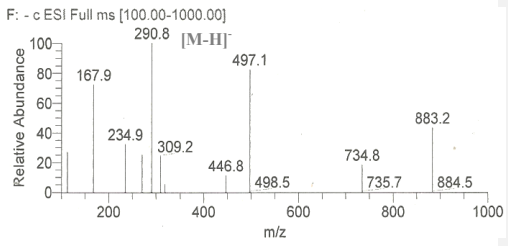
(+)-6-Methyl-citreoisocoumarin	
Synonym(s)	3- (10-Hydroxypentyl,12-ketone)-6-methoxy,8-hydroxy-isocoumarin
Sample code	74-78(4)
Biological source	Small scale *Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	2.2 mg
Physical Description	Brown solid
Molecular Formula	$C_{15}H_{16}O_6$
Molecular Weight	292 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 5.6 (*c* 0.23, $CHCl_3$)
Retention time HPLC	22.7 min (standard gradient)











[M+H]+

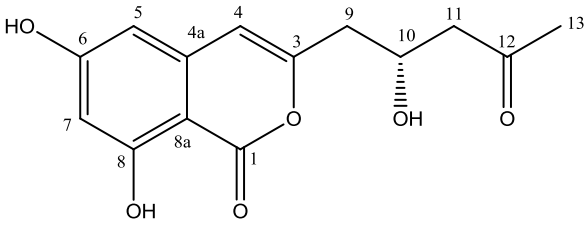
Compound (**2**)**,** was isolated from the EtOAC extract of the small scale liquid cultures of *Alternaria* sp. as a brown solid. Its UV spectrum showed λ_{max} (MeOH) at 243.4, 277.3 and 327.2 nm which are closely similar to compound (**1**). Positive and negative ESI-MS showed molecular ion peaks at *m*/*z* 292.7 $[M+H]^+$ and *m/z* 290.8 $[M-H]^-$ (base peak), respectively indicating that the molecular weight is 292 g/mol.

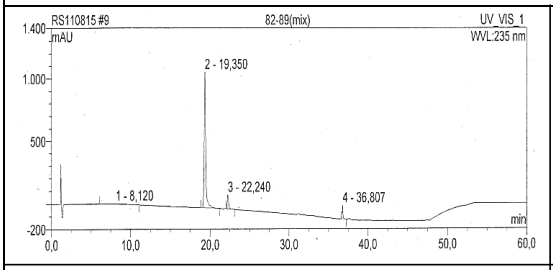
The ^1H NMR spectrum (Table 3.1) suggested an isocoumarin derivative which is very closely related to the previous compound (**1**), except for the appearance of an extra methoxyl signal at δ_H 3.88 ppm and the disappearance of the methine group signal H-12 which is present in compound (**1**). This suggestion was further supported by the downfield appearance of CH_3-13 as a singlet at δ_H 2.18 ppm confirming the attachment of this methyl group to a sp^2 carbon rather than sp^3 carbon as in (**1**). The aromatic region showed two *meta*-coupled protons at δ_H 6.50 [d (*J*=2.3 Hz)] and δ_H 6.47 [d (*J*=2.3 Hz)] for H-5 and H-7, respectively and a singlet olefinic proton which is resonated at δ_H 6.31 ppm for H-4.

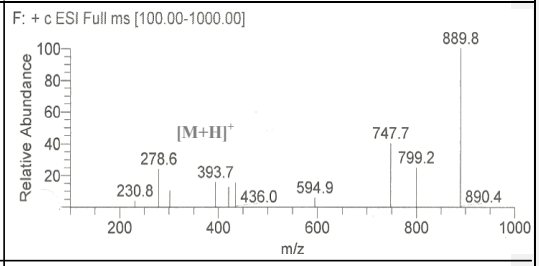
The ^1H-^1H-COSY spectrum (Figure 3.3) showed two spin systems, the first one is CH_2 (9)CH(10)CH_2(11) and the second one is CH(7)CH(5). Compound (**2**) was further confirmed by comparison of UV, $[\alpha]_D$,^1H NMR and mass spectral data with published data reported for (+)-6-methyl-citreoisocoumarin (George *et al.*, 1978). (+)-6-Methyl-citreoisocoumarin was previously isolated from the cultures of *Penicillium nalgiovense* (Thomas and Jens, 1999). To the best of our knowledge, compound (**2**) is isolated for the first time from *Alternaria* sp.

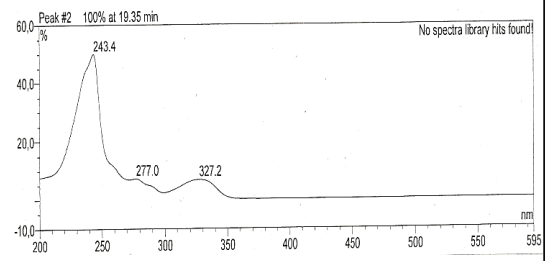
3.1.3. (+)-Citreoisocoumarin (3, known compound)

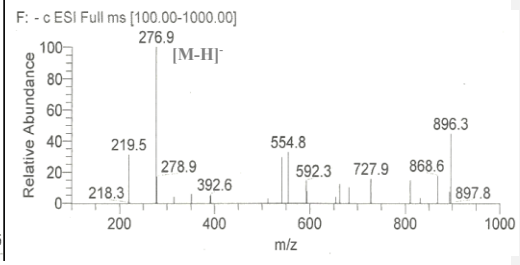
(+)-Citreoisocoumarin	
Synonym(s)	3-(10-Hydroxypentyl-12-ketone)-6,8 dihydroxy -isocoumarin
Sample code	82-89(mix)
Biological source	Small scale *Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	5.3 mg
Physical Description	viscous yellowish oil
Molecular Formula	$C_{14}H_{14}O_6$
Molecular Weight	278 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 25 (*c* 0.1, MeOH)
Retention time HPLC	19.3 min (standard gradient)







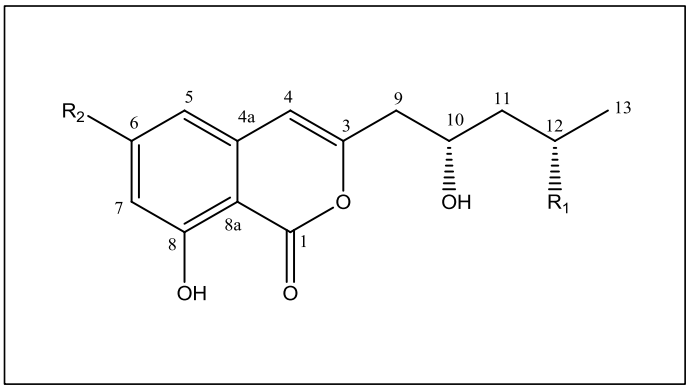




Compound (**3**), was isolated as viscous yellowish oil from the EtOAC extract of the small scale liquid cultures of *Alternaria* sp. It was supposed to be as an isocoumarin derivative from its UV absorption at λ_{max} (MeOH) 243.4, 277.3 and 327.2 nm (Chong and Huang, 1986) as in compounds (**1**) and (**2**). The LCMS of compound (**3**) revealed the appearance of a peak at *m/z* 278.6 $[M+H]^+$ in its ESI-MS spectrum indicating a molecular weight of 278 g/mol which is less by 2 amu than (**1**) suggesting that (**3**) has a higher degree of unsaturation than (**1**).

The ^{1}H NMR spectrum (Table 3.1) was in agreement with the isocoumarin substructure through the presence of two singlet aromatic protons, at δ_H 6.32 and 6.32 ppm for H-5 and H-7, respectivaly and one olefinic signal at δ_H 6.39 ppm for H-4. The $–CH_2$-CHOH-CH_2-CO-CH_3 aliphatic chain is proved to be present by the presence of the same signals which are present in (**2**) namely, a deshileded methyl singlet at δ_H 2.19 ppm, the two geminal coupled methylene protons resonating at (δ_H 2.62 ppm [dd (*J*= 14.4,8.8 Hz)], δ_H 2.67 ppm [dd (*J*= 14.4, 4.1 Hz)]) for CH_2-9 and at (δ_H 2.71 ppm [d (*J*=6.2 Hz)]) for CH_2-11and the multiplet methine proton at δ_H 4.49 ppm for H-10. The presence of the deshielded methyl singlet and the absence of 12-CH-OH signal in (**3**) confirm the suggestion of the oxidation of 12-CH-OH in (**1**) to a carbonyl group in (**3**) and explain the difference in the molecular weight between (**1**) and (**3**).

The ^{1}H-^{1}H-COSY (Figure 3.4 and Table 3.1) of compound (**3**) showed two spin systems, the first one is CH(4)CH_2(9)CH(10)CH_2(11) and the second one is CH(7)CH(5). These data together with the $[\alpha]_D^{20}$ were in full agreement with published data for (+)-citreoisocoumarin (Sheng *et al*., 1991; Watanabe *et al.*, 1998). Compound (**3**) has been previously isolated from *Ampelomyces* sp. (Hassan, 2007), *Aspergillus* (Watanabe *et al.*, 1998; Watanabe *et al.*, 1999) and *Penicillium* species (Sheng *et al*., 1991). This compound has not been reported before from *Alternaria* sp.



Nr.	**Compound**	**R_1**	**R_2**
1	Citreoisocoumarinol	OH	OH
2	6-Methyl-Citreoisocoumarin	=O	OCH_3
3	Citreoisocoumarin	=O	OH

Table 3.1: ^{1}H NMR and COSY data for compound (**1**), (**2**) and compound (**3**) at 500 (^{1}H) and 125 (^{13}C) MHz:

#	Compound (**1**)				Compound (**2**)			Compound (**3**)		
	δ_H(MeOD)	COSY	δ_C($CDCl_3$)[c]	δ_H(CD_3OD)[a]	δ_H(MeOD)	COSY	δ_H($CDCl_3$)[b]	δ_H(MeOD)	COSY	δ_H(CD_3OD)[a]
1			167.2							
3			156.0							
4	6.39,*s*		107.4	6.30 ,*s*	6.31, *s*		6.33,*s*	6.39 ,*s*	9b	6.33 ,*s*
4a			141.3							
5	6.33,*s*	7	102.6	6.37,*s*	6.50,*d* (2.3)	7	6.50,*d* (2.0)	6.32 ,*s*	7	6.39 ,*d* (2.2)
6			167.4							
7	6.33,*s*	5	101.1	6.30 ,*s*	6.47,*d* (2.3)	5	6.40,*d* (2.0)	6.32 ,*s*	5	6.32 ,*d* (2.2)
8			164.8							
8a			96.0							
9	2.70,*dd* (4.1,14.7) 2.57,*dd* (8.3,14.6)	9a, 10 9b	42.5	2.69,*dd* (4.4,14.4) 2.55,*dd* (8.3,14.4)	2.63,*d* (8.1) 2.60,*d* (8.1)	10, 11	2.65,*d* (5.5)	2.67 ,*dd* (14.4,4.1) 2.62,*dd* (14.4, 8.8)	9b,10 9a,10	2.67 ,*d* (6.3)
10	4.15 ,*m*	11a,11 b,9b	67.1	4.13,*m*	4.45,*m*	9a,9b, 11	4.45 ,*m*	4.49,*m*	9a,9b, 11	4.47 ,*m*
11	1.70,*ddd (*13.9, 8.5,7.8) 1.60,*ddd* (13.9, 5.3,4.4)	10, 12 10	46.4	1.70,*ddd* (13.7, 8.3,7.3) 1.60,*ddd* (13.7, 5.4,4.4)	2.71,*d* (6.4)	9a,9b, 10	2.69 ,*d (*5.5)	2.71,*d* (6.2)	10	2.71 ,*d* (6.3)
12	4.00,*m*	13-CH_3, 11a	68.7	3.98,*m*						
CH_3-13	1.20 ,*d* (6.2)	11a,12	22.5	1.19,*d* (5.9)	2.18,*s*			2.19,*s*		2.23 ,*s*
OCH_3-6					3.88,*s*		3.88,*s*			

(a) Sheng *et al*., 1991. (b) George *et al*., 1978. (c) Derived from HSQC

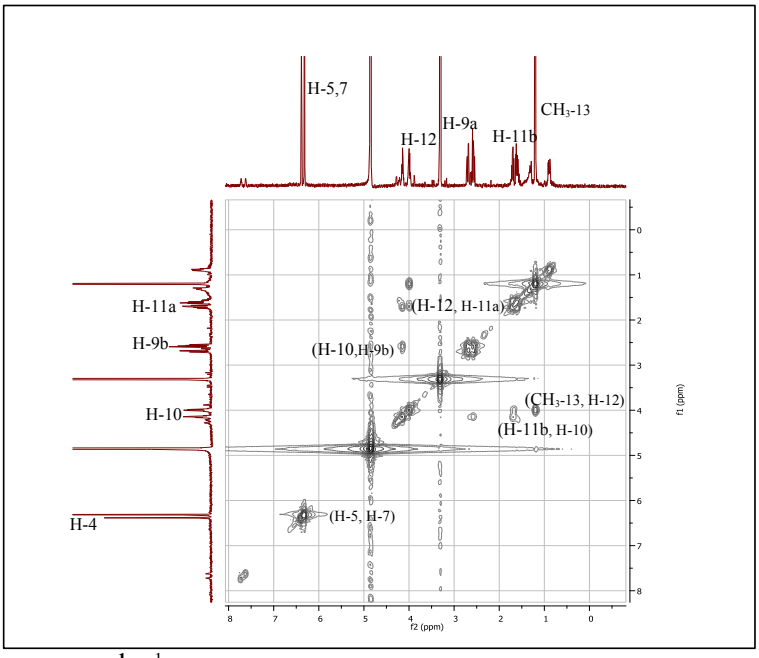


Figure 3.2: ^{1}H-^{1}HCOSY spectrum of compound (**1**)

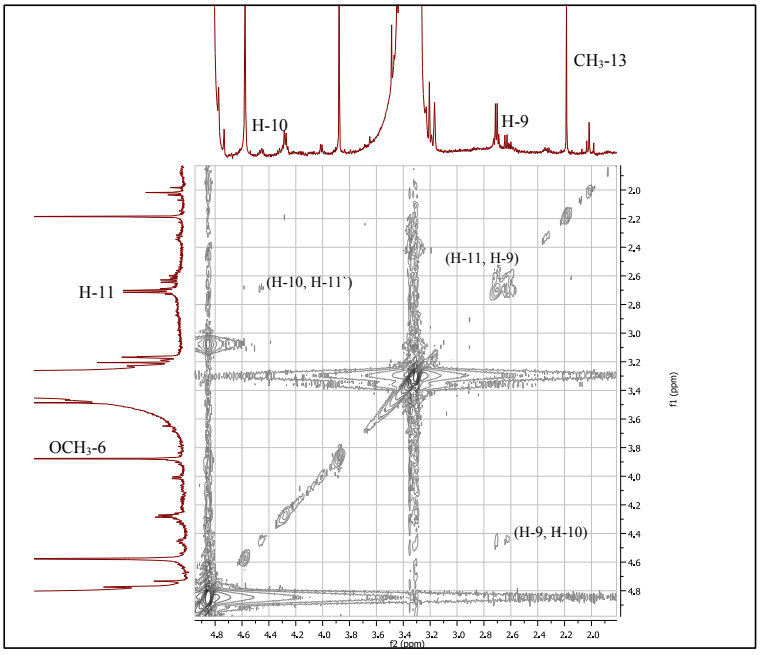


Figure 3.3: Expansion of ^{1}H-^{1}HCOSY spectrum of compound (**2**)

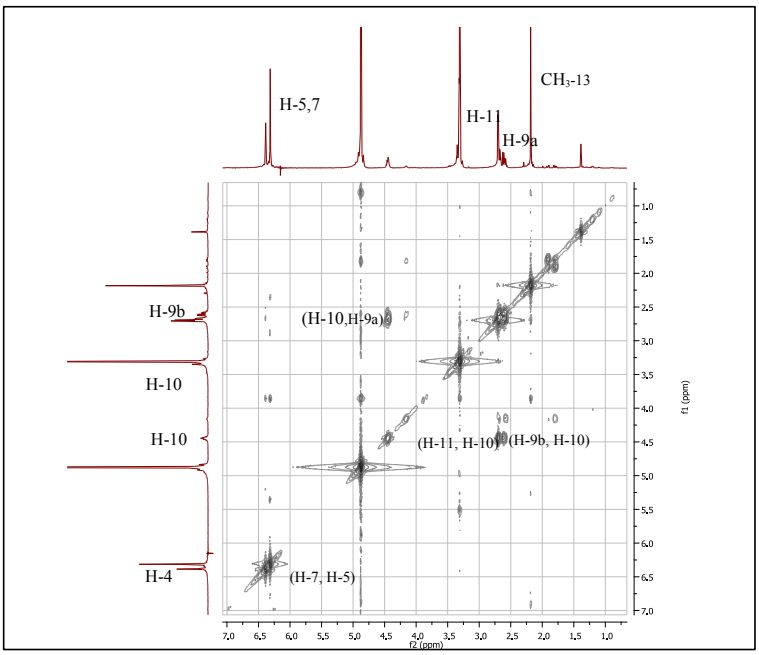
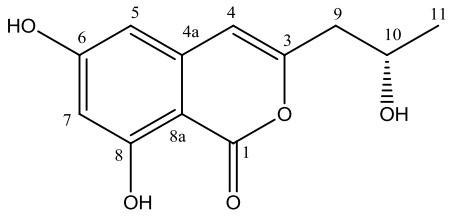
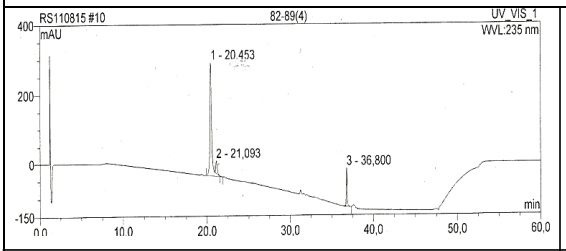


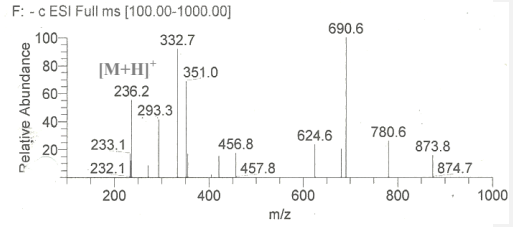
Figure 3.4: ^{1}H-^{1}HCOSY spectrum of compound (**3**)

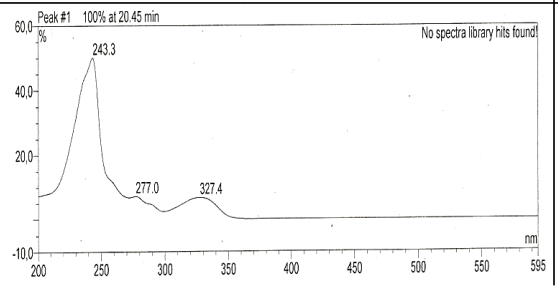
3.1.4. (+)-Orthosporin (4, known compound)

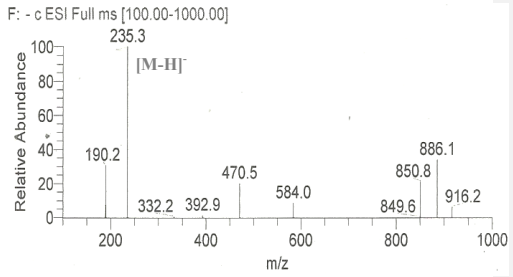
(+)-Orthosporin	
Synonym(s)	3-(10-Hydroxypropyl)-6,8 dihydroxy isocoumarin
Sample code	82-89(4)
Biological source	Small scale *Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.1 mg
Physical Description	Yellow solid
Molecular Formula	$C_{12}H_{12}O_5$
Molecular Weight	236 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 58.7 (*c* 1.0, CH_3OH)
Retention time HPLC	20.4 min (standard gradient)











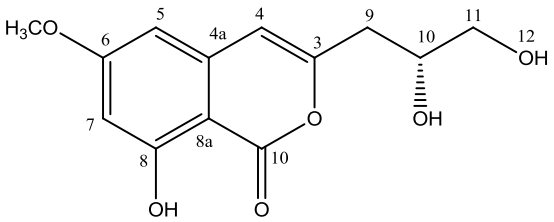
Compound (**4**), was isolated as a yellow solid from the EtOAC extract of the small scale liquid cultures of *Alternaria* sp. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 236.2 $[M+H]^+$ (base peak) and *m/z* 235.3 $[M-H]^-$ (base peak), respectively indicated a molecular weight of 236 g/mol. It showed UV absorption at λ_{max} (MeOH) 243.0, 277.0 and 327.4 nm which revealed the presence of the isocoumarin moiety as in (**1**-**3**) (Chong and Huang, 1986).

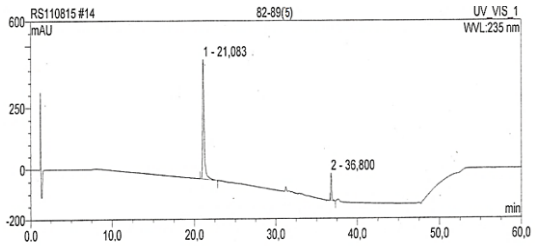
The ^{1}H NMR spectrum (Table 3.2) exhibited signals at δ_H 1.26 [d (*J*=6.25 Hz)], 2.59 (*brs*), 2.61 [d *(J*=4.6 Hz)] and 4.15 (*m*) ppm for CH_3-11, CH_2-9 and CH-10, respictively. In addition, sp^2 signals *viz*, an overlapped two aromatic singlets resonate at δ_H 6.32 ppm for H-5 and H-7 and an olefinic signal at δ_H 6.38 ppm for H-4 were observed. The ^{1}H-^{1}H COSY spectrum (Figure 3.5) showed two spin systems, the first one is CH_2(9)CH(10)CH_3(11) of the the 2-hydroxypropyl side chain substition and the second spin system is CH(7)CH(5).

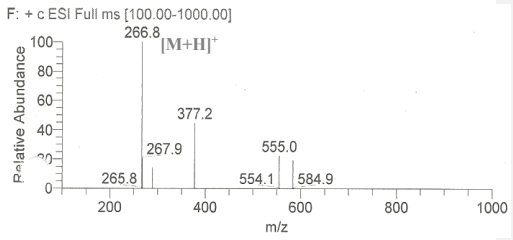
Comparison of UV, ^{1}H NMR (Table 3.2) and mass spectral data with literature data indicated that (**4**) is the known compound orthosporin. The absolute configuration was derived from the obtained $[\alpha]_D^{20}$ value which was found to have an identical sign as that measured for the same compound (Ichihara *et al*., 1989). The isolation of (+)-orthosporin has been known from several fungi such as *Drechslera siccans* (Hallock *et al.*, 1988) and from the fruiting body of *Daldinia concentrica* (In-Kyoung *et al*., 2006) but it was obtained here for the first time from *Alternaria* sp.

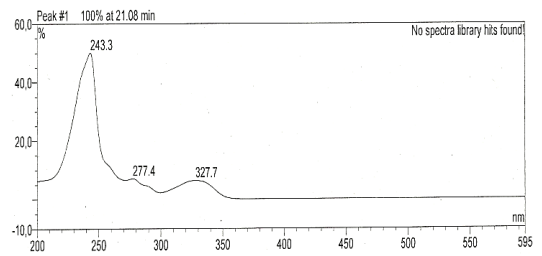
3.1.5. (+)-Diaportinol (5, known compound)

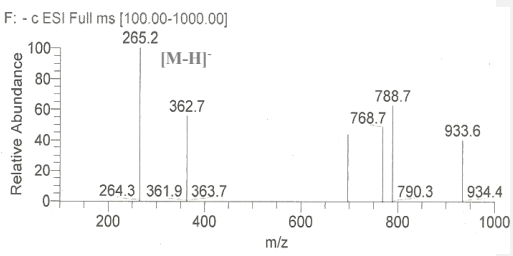
(+)-Diaportinol	
Synonym(s)	3- (10- Hydroxybutanol)-6-methoxy,8-hydroxy-isocoumarin
Sample code	82-89(5)
Biological source	Small scale *Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.6 mg
Physical Description	Yellowish solid
Molecular Formula	$C_{13}H_{14}O_6$
Molecular Weight	266 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 23° (*c* 0.07, CH_3OH)
Retention time HPLC	21.0 min (standard gradient)







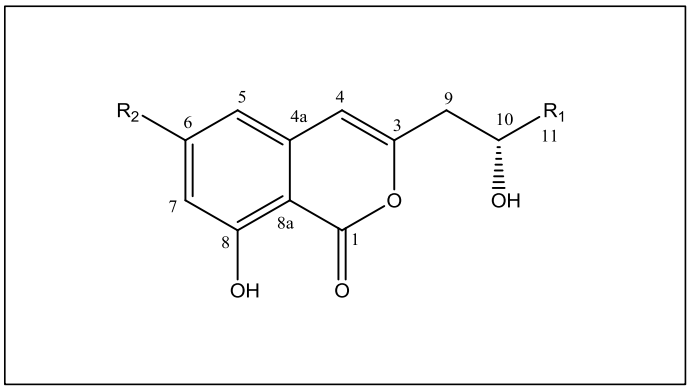




Compound (**5**), was isolated as a yellowish solid from the EtOAC extract of the small scale liquid cultures of *Alternaria* sp. In ESI mass spectrum, it revealed *pseudo*-molecular ion peaks at *m/z* 266.8 $[M+H]^+$ and 265.2 $[M-H]^-$ (base peak), respectively revealing a molecular weight of 266 g/mol. It showed UV absorption at λ_{max} (MeOH) 243.3, 277.4 and 327.7 nm, indicative for an isocoumarine chromophore similar to the previous compounds (Chong and Huang, 1986).

The ^{1}H NMR spectrum of (**5**) (Table 3.2) was quite similar to that of (**4**) except for the appearance of an extra methoxyl group at δ_H 3.88 ppm, the absence of the doublet assigned for CH_3-11 and the appearance of a hydroxymethyl signal at δ_H 3.56 [d (*J*=5.4 Hz)]. Moreover, the aromatic region demonstrated protons for the trisubstituted isocoumarin ring system through the appearance of two *meta*-coupled doublets at δ_H 6.47 (*J*= 2.3 Hz) and 6.50 (*J*= 2.3 Hz) for H-5 and H-7, respectively and the singlet olefinic proton H-4 at δ_H 6.48 ppm. The remaining substitution pattern, -CH_2-CHOH-CH_2OH apears in the up region in which a methylene group adjacent to the the iscoumarin ring CH_2-9 resonates at δ_H 2.79 [dd (*J*=14.7,4.2 Hz)] and δ_H 2.56 [dd (*J*=14.7,8.8 Hz)] while the terminal hydroxy methylene group CH_2-11 appeared at δ_H 3.56 [d (*J*=5.4 Hz)] and the oxgenated methine proton H-10 appears as a multiplet at δ_H 4.03 ppm. The ^{1}H-^{1}H COSY spectrum showed two spin systems, the first one is CH_2(9)CH(10)CH_2(11) and the second one is CH(5)CH(7).

The literature (Thomas and Jens, 1999) described diaportinol from *Penicillium nagiovense* with the same structure as for compound (**5**). In addition, the $[\alpha]_D^{20}$ value agreed with published data (Ichihara *et al*., 1989). It is worth to mention that the known compound (**5**) is isolated for the first time from *Alternaria* sp.



Nr.	**Compound**	**R_1**	**R_2**
4	Orthosporin	CH_3	OH
5	Diaportinol	CH_2OH	OCH_3

Table 3.2: ^{1}H NMR and COSY data for compound (**4**) and compound (**5**) at 500 (^{1}H) and 125 (^{13}C) MHz:

#	Compound (**4**)				Compound (**5**)		
	δ_H(MeOD)	COSY	δ_H (acetone_d_6)[a]	δ_H($CDCl_3$)[b]	δ_H(MeOD)	COSY	δ_H $(CD_3)_2CO$[c]
1							
3							
4	6.38 ,*s*		6.41,*s*	6.25,*s*	6.48,*s*		6.46,*s*
5	6.32, *s*	7	6.36 ,*d* (2.1)	6.14 ,*d* (1.7)	6.47,*d* (2.3)	7	6.53,*s*
6							
7	6.32 ,*s*	5	6.40 ,*d* (2.1)	6.18 ,*d* (1.7)	6.50,*d* (2.3)	5	6.53,*s*
8							
8a							
9	2.59 ,*brs* 2.61 ,*d* (4.6)	9b,10 9a,10	2.56, *dd* (14.3,7.6) 2.61, *dd* *(*14.3,5.8)	2.60 ,*d* (6.5)	2.79,*dd* (14.7,4.2) 2.56,*dd* (14.7,8.8)	9a,10 9b,10	2.79,*dd* (14.7,3.4) 2.56,*dd* (14.7,8.8)
10	4.15 ,*m*	9	4.17,*m*	4.15,*m*	4.03,*m*	9a,9b,11	4.04,*m*
11	1.26,*d* (6.25)		1.22 ,*d* (6.1)	1.20 ,*d* (6.2)	3.56,*d* (5.4)	10	3.58 ,*d* (5.4)
OCH_3-6					3.88 ,*s*		3.91,s

a) Ichihara *et al*., 1989.
b) In-Kyoung *et al*., 2006.
c) Thomas and Jens, 1999.

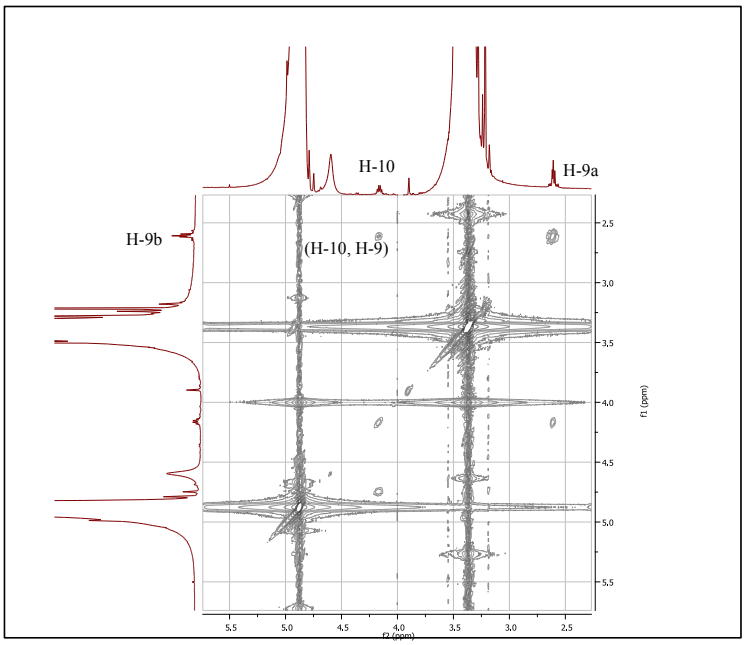


Figure 3.5: Expansion of ^{1}H-^{1}HCOSY spectrum of compound (**4**)

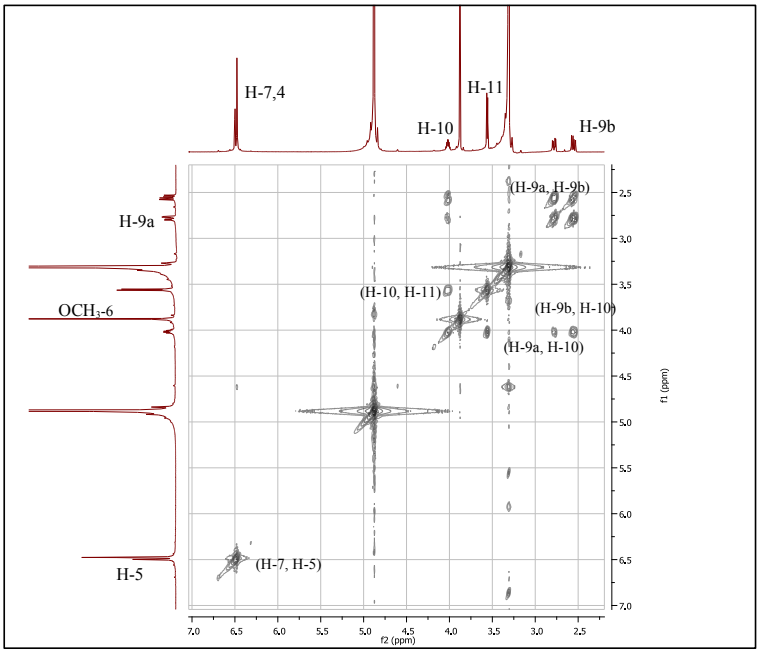
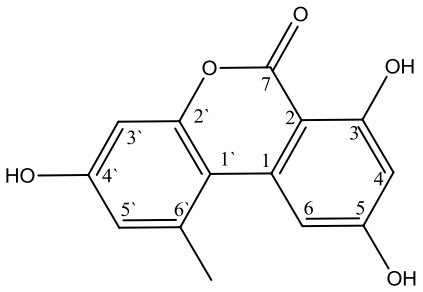
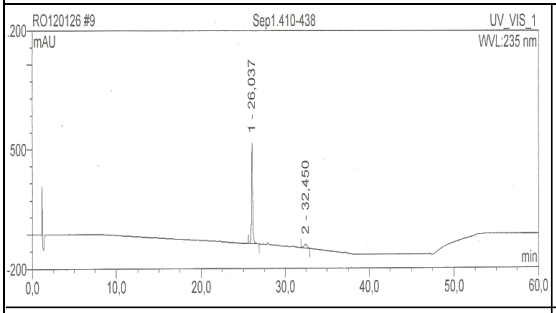


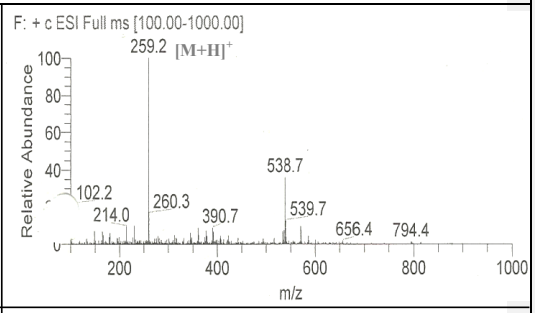
Figure 3.6: ^{1}H-^{1}HCOSY spectrum of compound (**5**)

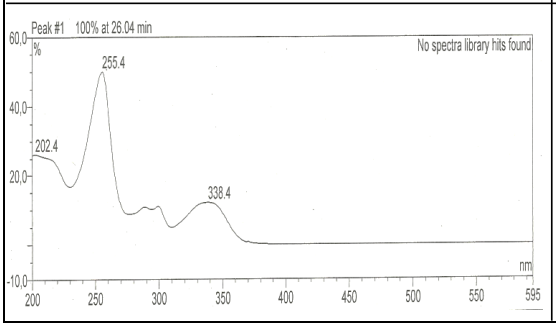
3.1.6. Alternariol (6, known compound)

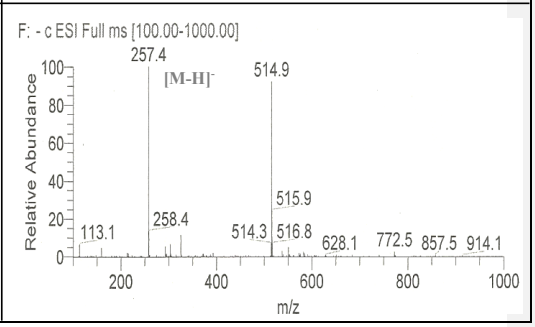
Alternariol	
Synonym(s)	3,7,9-Trihydroxy-1-methyl-6H-dibenzo[b,d]pyran-7-one
Sample code	Sep.1,410-438
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	389.4mg
Physical Description	reddish needles
Molecular Formula	$C_{14}H_{10}O_5$
Molecular Weight	258 g/mol
Retention time HPLC	26 min (standard gradient)











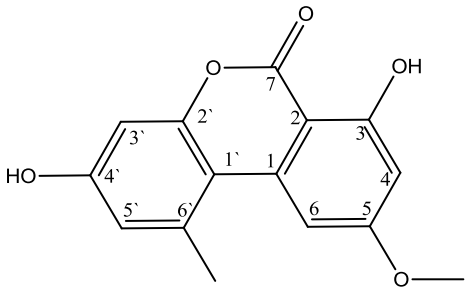
Compound (**6**), was isolated from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. as reddish needles. The molecular weight of compound (**6**) was established by the ESI-mass measurement, which provided a quasimolecular ions at m/z 259.2 $[M^{+}+H]^{+}$ in positive mode and m/z 257.4 $[M-H]^{-}$ in negative mode, suggesting the molecular weight of 258 g/mol. The UV spectrum λ_{max} (MeOH) showed absorbances at 202.4, 255.4 and 338.4 nm which are similar to the UV spectra typical for alternariol derivatives (Freeman, 1965).

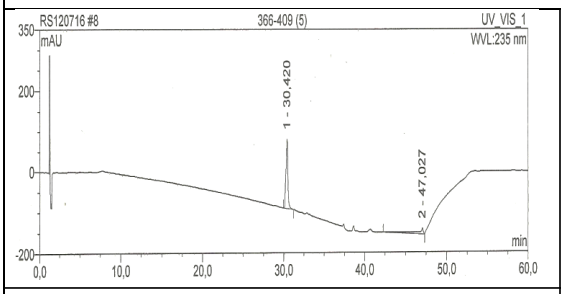
The ^{1}H NMR spectrum (Table 3.3) of compound (**6**) demonstrated the presence of one aromatic methyl group resonating at δ_H 2.74 ppm for CH_3-6` together with four aromatic proton signals. The coupling constants observed for the aromatic ring protons indicated the presence of two aromatic rings each bearing a pair of *meta*-coupled protons at δ_H 6.36 (*J*=2.0 Hz), 7.24 (*J*=2.0 Hz), 6.59 (*J*=2.6 Hz) and 6.68 (*J*=2.6 Hz) assigned for H-4, H-6, H-3` and H-5`, respectively.

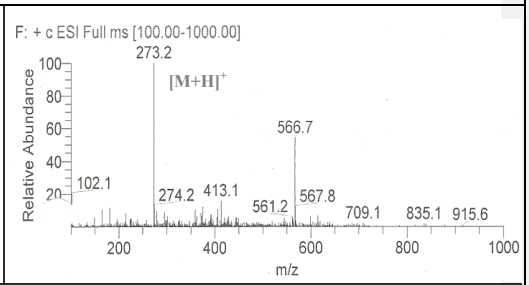
The ^{1}H-^{1}H-COSY spectrum (Figure 3.7 and Table 3.3) showed two spin systems, the first one is CH(4)CH(6) and the second one is CH(3`)CH(5`)CH_3(6`). UV, ^{1}H NMR (see Table 3.3) and mass spectral data of compound (**6**) were found to be identical with those reported for alternariol isolated from several *Alternaria* species (Stinson *et al.*, 1986; Freeman, 1965; Coombe *et al*., 1970).

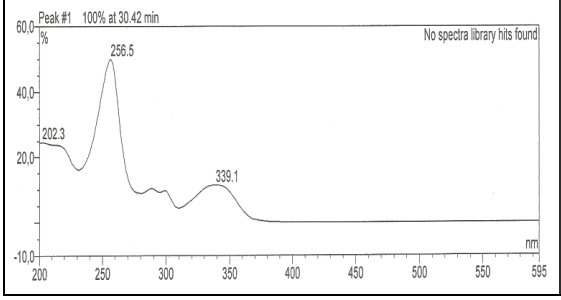
3.1.7. Alternariol-5-*O*-methyl ether (7, known compound)

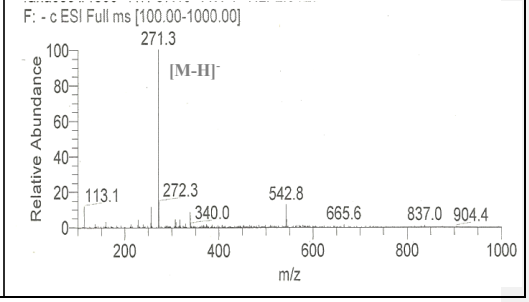
Alternariol-5-*O*-methyl ether	
Synonym(s)	3,7-Dihydroxy-9-methoxy-1-methyl-6H dibenzo[b,d] pyran-6-one, Djalonensone
Sample code	366-409 (5)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.005gm
Physical Description	reddish needles
Molecular Formula	$C_{15}H_{12}O_5$
Molecular Weight	272 g/mol
Retention time HPLC	30.4min (standard gradient)







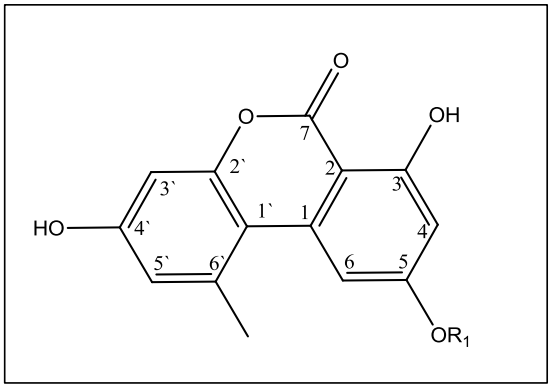




Compound (**7**) was isolated from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. as reddish needles; UV absorption shows three bands at λ_{max} (MeOH) 202.3, 256.5 and 339.1 nm which is similar to (**6**). Positive and negative ESI-MS showed molecular ion peaks at *m*/*z* 273.2 $[M+H]^+$ and *m*/*z* 271.3 $[M-H]^-$ (base peak), respectively indicating that the molecular weight is 272 g/mol. The molecular weight of compound (**7**) differs from that of (**6**) by 14 amu indicating the possible presence of an extra methyl group in (**7**).

The ^1H NMR spectrum (Table 3.3) was in agreement with the above suggestion through the presence of an extra methoxyl group signal at δ_H 3.92 ppm. In addition, a singlet for the aromatic methyl group at δ_H 2.77 ppm for CH_3-6` was also observed together with the two pairs of *meta*-coupled protons as in (**6**), each appears as a doublet at $\delta_H$ 6.55 (*J*=2.1 Hz), 7.29 (*J*=2.1 Hz), 6.61 (*J*=2.5 Hz) and 6.70 (*J*=2.5 Hz) corresponding to H-4, H-6, H-3` and H-5`, respectively.

The ^1H-^1H-COSY spectrum (Figure 3.8 and Table 3.3) showed two spin systems similar to those observed for compound (**6**), the first one is CH(4)CH(6) and the second one is CH(3`)CH(5`)CH_3(6`) indicated a close structure resemblance of (**6**) and (**7**). Careful review of the literature confirmed that the spectral data of compound (**7**) were in full agreement with those reported for the known alternariol-5-*O*-methyl ether (Freeman, 1965**;** Onocha *et al.*, 1995; Coombe *et al.*, 1970).



Nr.	**Compound**	**R_1**
6	Alternariol	H
7	Alternariol-5-*O*-methyl ether	CH_3

Table 3.3: ^{1}H NMR and COSY data of compounds (**6**) and (**7**) at 500 (^{1}H) and 125 (^{13}C) MHz:

	Compound (**6**)			Compound (**7**)		
Position	δ_H(MeOD)	COSY	δ_H(MeOD)[a]	δ_H(MeOD)	COSY	δ_H(MeOD)[a]
4	6.36,*d* (2.0)	6	6.32,*d* (2.0)	6.55,*d* (2.1)	6	6.54,*d* (1.8)
6	7.24,*d* (2.0)	4	7.20,*d* (2.0)	7.29,*d* (2.1)	4	7.28,*d* (1.8)
3`	6.59,*d* (2.6)		6.55,*d* (2.5)	6.61, *d* (2.5)		6.61,*d* (2.5)
5`	6.68,*d* (2.6)	CH_3-6`	6.65,*d* (2.5)	6.70, *d* (2.5)	CH_3-6`	6.70,*d* (2.5)
CH_3-6`	2.74, *s*	5`	2.71,s	2.77,*s*	5`	2.76, *s*
OCH_3-5				3.92,*s*		3.91, *s*

(a) Onocha *et al.*, 1995.

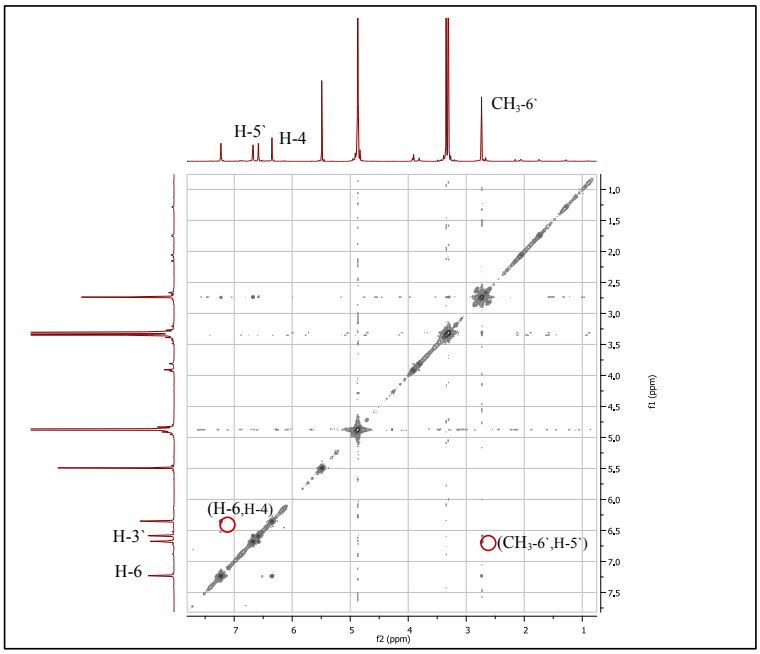


Figure 3.7: ^{1}H-^{1}HCOSY spectrum of compound (**6**)

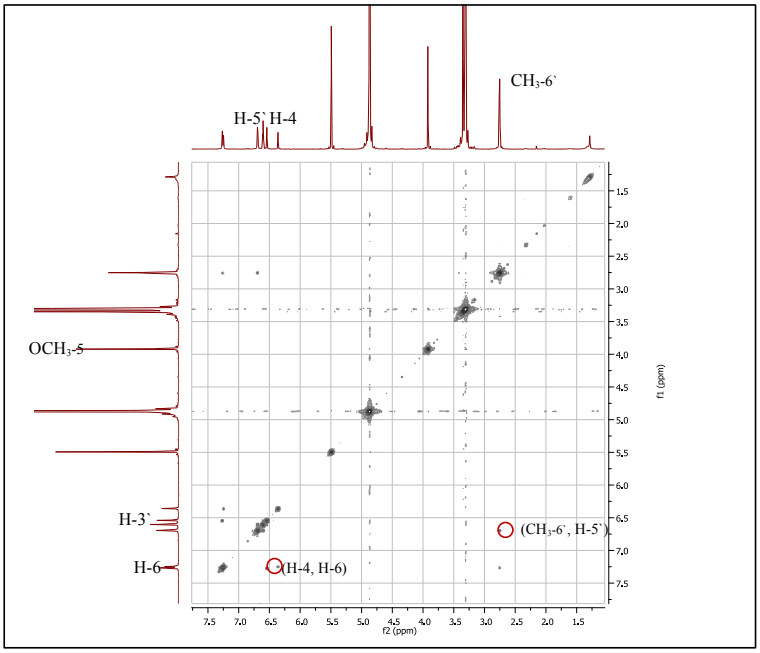
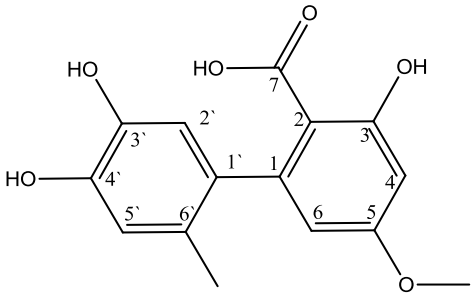
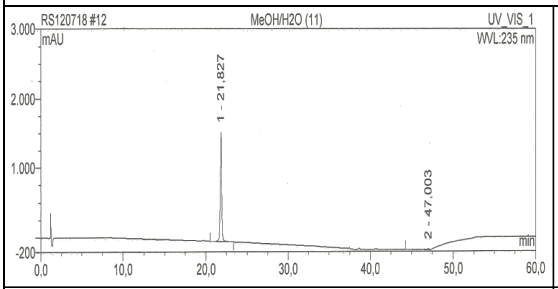


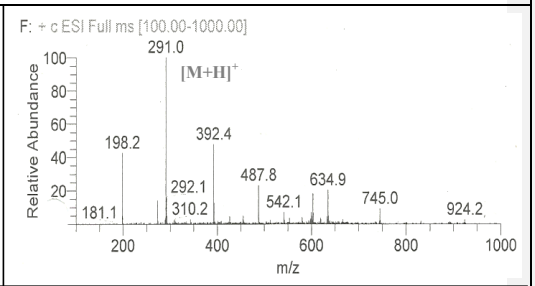
Figure 3.8: ^{1}H-^{1}HCOSY spectrum of compound (**7**)

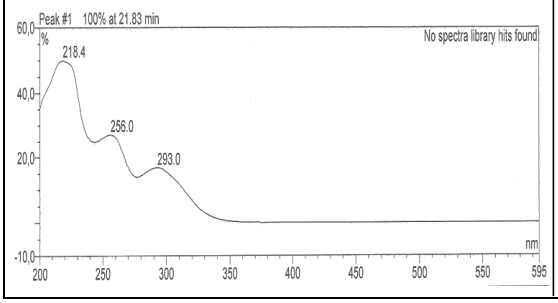
3.1.8. Altenusin (8, known compound)

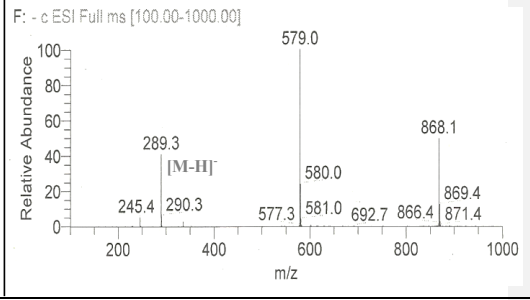
Altenusin	
Synonym(s)	3,4',5'-Trihydroxy-5-methoxy-2'-methyl[1,1'-biphenyl]-2-carboxylic acid
Sample code	MeOH/H_2O (11)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	498.7mg
Physical Description	reddish crystals
Molecular Formula	$C_{15}H_{14}O_6$
Molecular Weight	290 g/mol
Retention time HPLC	21.82 min (standard gradient)







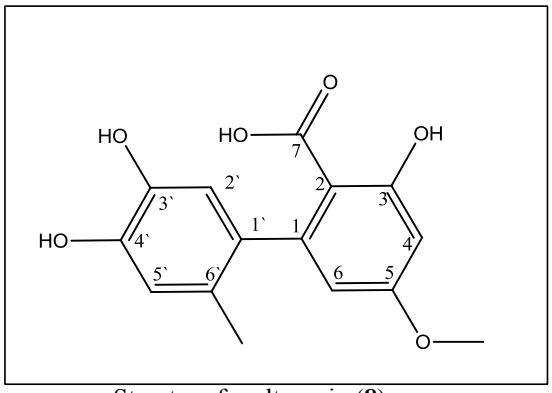




Compound (**8**), was isolated as reddish crystals from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It displayed UV absorbances at λ_{max} (MeOH) 218.4, 256 and 293nm, having the typical pattern of alternariol derivatives (Freeman, 1965). The molecular weight of compound (**8**) was established by the ESI-mass measurement which provided quasimolecular ions at m/z 291.0 $[M^{+}+H]^{+}$ in positive mode and m/z 289.3 $[M-H]^{-}$ in negative mode, suggesting the molecular weight of 290 g/mol.

This structure was then confirmed by ^{1}H NMR analysis (Table 3.4). The ^{1}H NMR spectrum showed two singlets which appeared at δ_H 1.91 ppm for CH_3-6` and at $\delta_H$ 3.81 ppm assigned for $OCH_3$-5. Furthermore, two *meta*-coupled aromatic protons at $\delta_H$ 6.43 and 6.17 (*J*=2.6 Hz) for H-4 and H-6, respectively and two aromatic singlets resonate at $\delta_H$ 6.49 and 6.59 ppm assigned for H-2` and H-5`, respectively, were observed.

The ^{1}H-^{1}H-COSY spectrum (Figure 3.9 and Table 3.4) showed two spin systems, the first one is CH(4)CH(6) and the second one is CH(5`)$CH_3$(6`) confirming the planar structure of compound (**8**). The obtained UV, ^{1}H NMR (Table 3.4) and mass spectral data were found to be identical with the published data for the known altenusin (Statoshi *et al*., 1995), previously reported from *Alternaria* species (Coombe *et al*., 1970; Hassan, 2007).



Structure for altenusin (**8**)

Table 3.4: ^{1}H NMR and COSY data of compound (**8**) at 500 (^{1}H) and 125 (^{13}C) MHz:

Position	Compound(**8**)			
	δ_H(MeOD)	COSY	δ_H(MeOD)[a]	δ_H(DMSO-d_6)[b]
4	6.43,*d* (2.6)	6	6.42,*d* (2.5)	6.43,*d* (2.7)
6	6.17,*d* (2.6)	4	6.15,*d* (2.5)	6.10,*d (2.7)*
2`	6.49,*s*		6.47,*s*	6.42,*s*
5`	6.59,*s*		6.57,*s*	6.54,*s*
CH_3-6`	1.91,*s*	5`	1.89,*s*	1.86,s
OCH_3-5	3.81,s		3.79,*s*	3.76,s

(a) Hassan, 2007. (b) Statoshi *et al*., 1995.

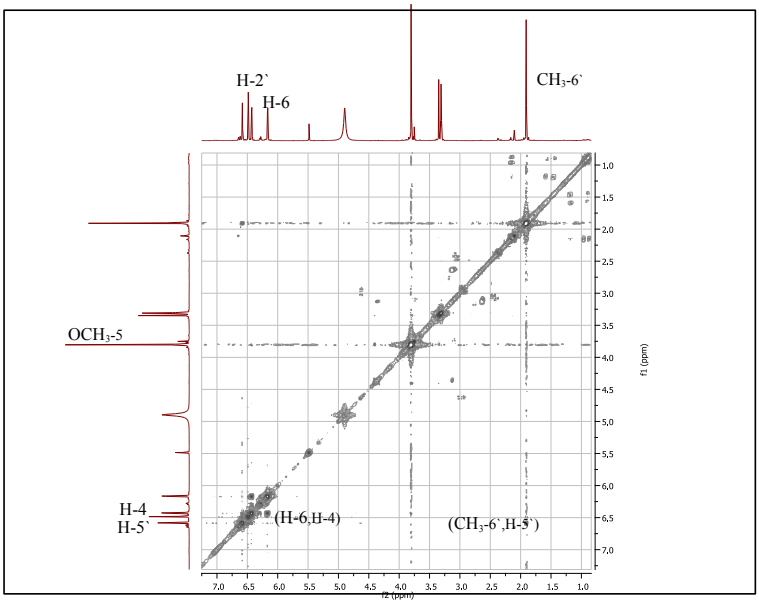
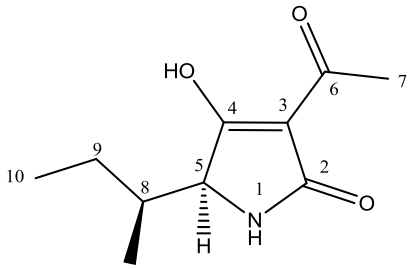
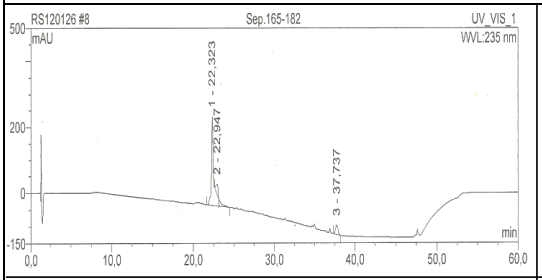


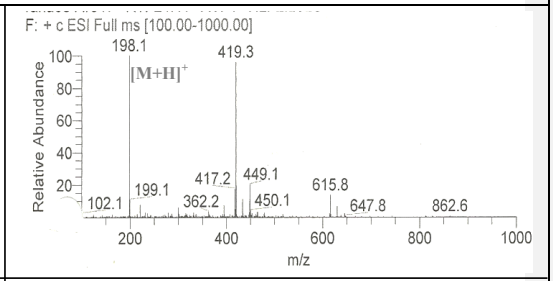
Figure 3.9: ^{1}H-^{1}H COSY spectrum of compound (**8**)

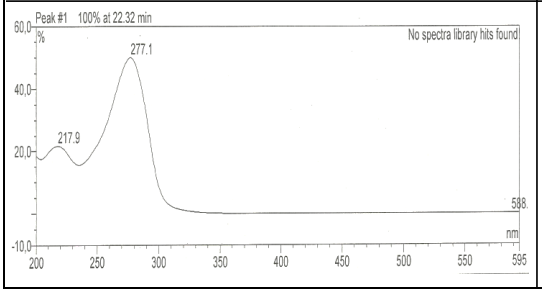
3.1.9. Tenuazonic acid (9, known compound)

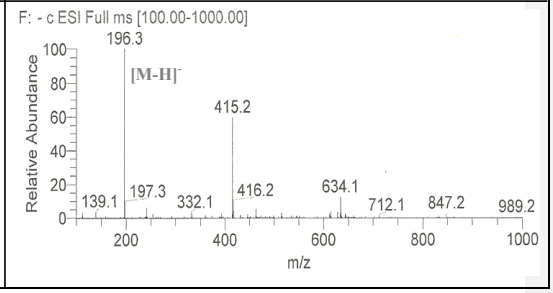
Tenuazonic acid	
Synonym(s)	3-Acetyl-1,5-dihydro-4-hydroxy-5-(1-methylpropyl)-2H-pyrrol-2-one
Sample code	Sep1.165-182
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	835mg
Physical Description	Viscous brown oil
Molecular Formula	$C_{10}H_{15}O_3N$
Molecular Weight	197 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 99 (*c* 0.2, $CHCl_3$)
Retention time HPLC	22.32 min (standard gradient)







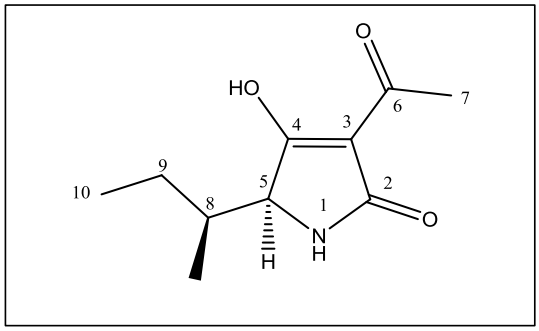




Compound (**9**), was obtained from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. as an oily viscous brown liquid. This compound was investigated by UV spectroscopy and showed strong absorption at λ_{max} 217.9 and 277.1 nm. In the ESI-MS, the *m/z* 198 was recorded as $[M+H]^+$ indicating the molecular weight of compound (**9**) is 197 g/mol.

The ^{1}H NMR spectrum (Table 3.5) exhibited a down-fielded methyl singlet at δ_H 2.42 ppm for CH_3-7, two signals at δ_H 0.91 [t (*J*= 7.4 Hz)] and at δ_H 0.99 [d (*J*= 6.9 Hz)] for CH_3-10 and CH_3-11, respectively, a methylene group resonates as multiplets at δ_H 1.38 and 1.25 ppm for CH_2-9 as well as two methine groups resonate at δ_H 1.91 (*brs*) and 3.84 ppm (*brs*) for H-8 and H-5, respectively. The ^{1}H-^{1}H COSY (Figure 3.10 and Table 3.5) spectrum showed a spin system, CH_3(10)CH_2(9)CH(8)CH_3(11)CH (5) confirming the 2-butyl side chain moiety.

Together with the optical rotation, compound (**9**) is suggested to be the known compound tenuazonic acid which has been isolated from several fungal species, as a vivotoxin from *Alternaria longipes* (Mikami *et al*., 1971), as a mycotoxin from *Alternaria tenuis* (Meronuck *et al.*, 1972), and as a magnesium and calcium complex from *Phoma sorghina* (Pieter and Christiaan, 1976).



Structure for tenuazonic acid (**9**)

Table 3.5: ^{1}H NMR and COSY data of compound (**9**) at 500 (^{1}H) and 125 (^{13}C) MHz:

Postion	Compound (**9**)				
	$\delta H_{(MeOD)}$	COSY	$\delta H_{(CDCl3)}$ [a]	$\delta H_{(DMSO-d6)}$ [b]	COSY [b]
5	3.84,*brs*		3.75	3.77, *d* (2.8)	8
8	1.91,*brs*	CH_3-11, 9b	1.92	1.78, *m*	5,9a,9b,11
9a	1.38,*m*	CH_3-10, CH_3-11, 9b	1.30	1.24, *m*	8,9b,10 8,9a,10
9b	1.25,*m*	CH_3-10, CH_3-11, 9a, 8		1.09, *m*	
CH_3-7	2.42,*s*		2.43	2.33, *s*	
CH_3-10	0.91, *t* (7.4)	9a, 9b	0.89	0.80, *t* (7.2)	9a,9b
CH_3-11	0.99, *d* (6.9)	9a, 9b,8	1.00	0.90, *d* (6.9)	8
NH				8.74,*brs*	
OH				11.10,*brs*	

a) Nolte *et al*., 1980. b) Hassan, 2007.

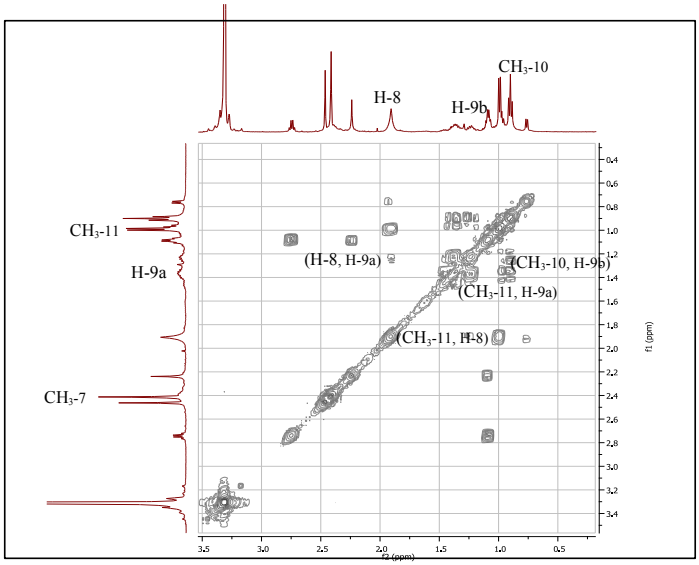
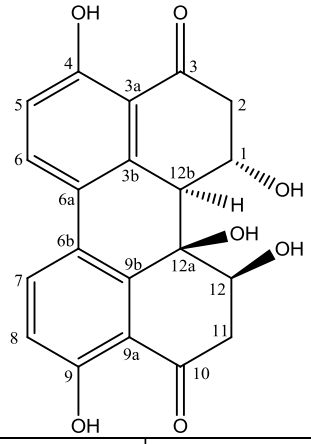
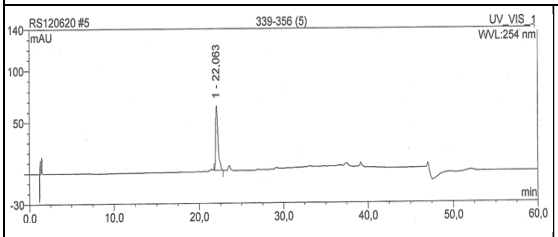


Figure 3.10: ^{1}H-^{1}H COSY spectrum of compound (**9**)

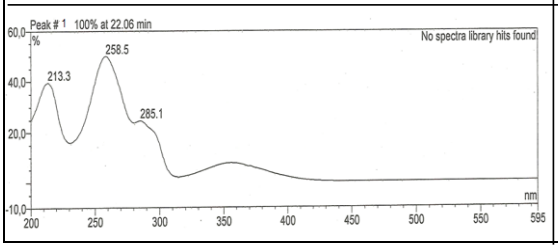
3.1.10. Stemphytriol (10, known compound)

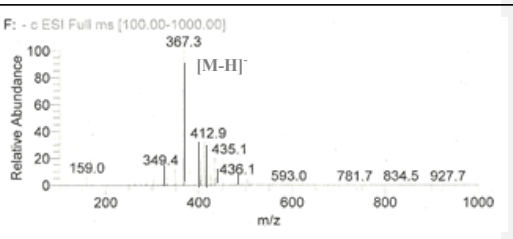
Stemphytriol	
Synonym(s)	1,4,9,12,12a-Pentahydroxy-3,10-dioxo-1,2,3,10,11,12,12a,12b-octahydroperylene
Sample code	339-356 (5)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	2 mg
Physical Description	Yellow solid
Molecular Formula	$C_{20}H_{16}O_7$
Molecular Weight	368 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 340 (*c* 0.07, MeOH)
Retention time HPLC	22.06 min (standard gradient)





(+)-ESI-MS: No ionization





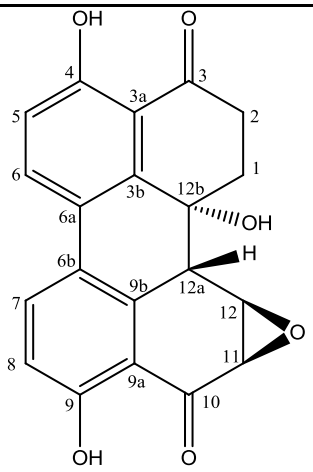
Compound (**10**), was isolated from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. as a yellow solid, Compound (**10**) showed UV absorbances at λ_{max} (MeOH) 213.3, 258.5, 285.1 nm. The molecular weight of compound (**10**) was established by the ESI-mass measurement which provided a quasimolecular ion at m/z 367.3 $[M-H]^-$ in the negative mode indicating a molecular weight of 368 g/mol.

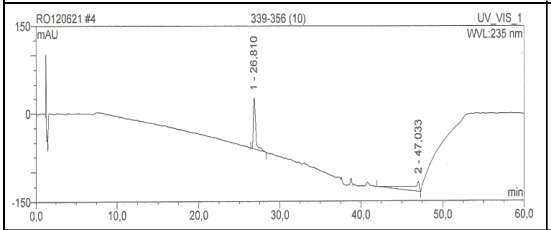
The analysis of the ^{1}H NMR data (Table 3.6) showed the presence of two aliphatic methylene groups, one at δ_H 2.47 ppm [Hα: dd (*J*=2.8, 9.0 Hz)] and 3.14 ppm [Hβ: d (*J*=9.0 Hz)]) assigned for CH_2-2 and the other at (δ_H 2.65 ppm [Hα: d (*J*=16.5 Hz)] and 3.06 ppm [Hβ: d (*J*=16.5 Hz)]) for CH_2-11. In addition, three methine groups at δ_H 4.42 ppm [d (*J*=10.4 Hz)], δ_H 4.37 ppm [d (*J*=9.1)] and δ_H 3.23 ppm (*m*) for H-1, H-12 and H-12b, respectively and two pairs of *ortho*-coupled protons (AX-system) at δ_H 7.01 ppm [d (*J*=9.0 Hz)], 7.97 ppm [d (*J*=9.0 Hz)], 8.01 ppm [d (*J*=9.0 Hz)] and 7.06 ppm [d (*J*=9.0 Hz)] (for H-5, H-6, H-7 and H-8, respectively) were observed.

^{1}H-^{1}H COSY spectrum (Figure 3.11 and Table 3.6) revealed four spin systems; CH_2(2)CH(1)CH(12b),CH(12)CH_2(11),CH(5)CH(6), and CH(7)CH(8). The structure was further confirmed by comparing UV, ^{1}H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ value with published data for the known compound stemphytriol, previously reported from several fungi and represents a biologically highly active stemphyl-and altertoxins (Karsten *et al*., 1999; Cole, 1981). It is worth to mention that compound (**10**) is isolated for the first time from *Alternaria sp.*

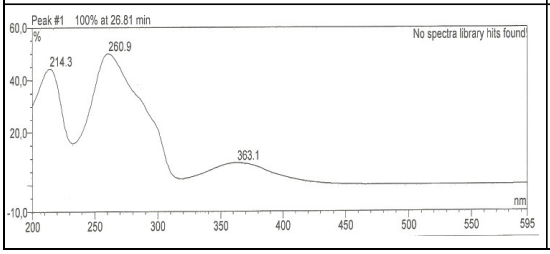
3.1.11. Altertoxin II (11, known compound)

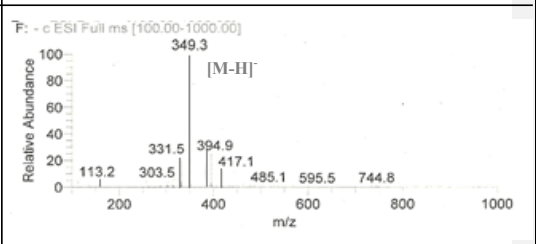
Altertoxin II	
Synonym(s)	Stemphyltoxin II, -4,9,12b-Tri-hydroxy-3,10-dioxo-1,2,3,10,11,12,12a,12b-octahydro-11,12-epoxyperylene
Sample code	339-356 (10)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	3.5 mg
Physical Description	Yellow solid
Molecular Formula	$C_{20}H_{14}O_6$
Molecular Weight	350 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 415 (*c* 0.11, MeOH)
Retention time HPLC	26.8 min (standard gradient)





(+)-ESI-MS:No ionization

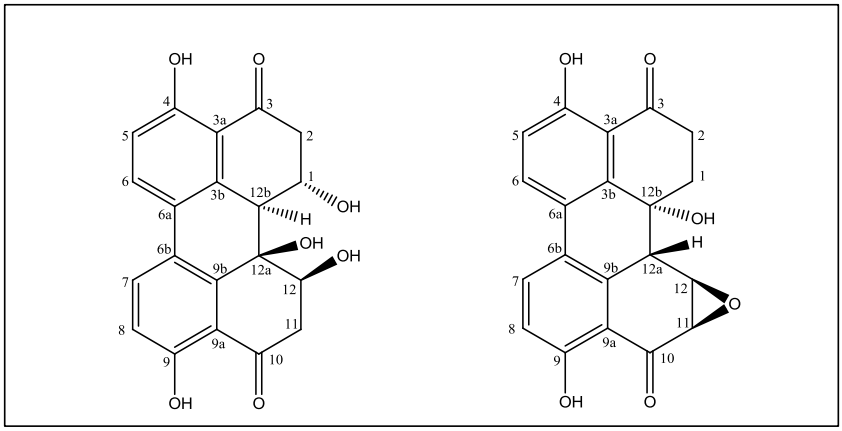




Compound (**11**)**,** was isolated as a yellow solid from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. Negative ESI-MS showed molecular ion peak at *m*/*z* 349.0 $[M-H]^-$ (base peak) indicating a molecular weight of 350 g/mol. The molecular mass showed 18 amu less than compound (**10**) suggesting that one molecule of H_2O is missing in (**11**). The UV spectra of compound (**11**) and compound (**10**) are similar indicating the presence of the same chromophore.

The ^1H NMR spectrum (Table 3.6) revealed the presence of two pairs of *ortho*-coupled aromatic protons at δ_H 7.07 ppm [d (*J*=8.9 Hz)], 8.08 ppm [d (*J*=8.9 Hz)], 8.00 ppm [d (*J*=8.8 Hz)] and 6.99 ppm [d (*J*=8.8 Hz)] assigned for H-5, H-6, H-7 and H-8, respectively. Moreover, the ^1H NMR spectrum showed signals at δ_H 2.87 ppm [Hα: ddd (*J*=13.4, 7.5, 2.5 Hz)] and 2.47 ppm [Hβ: td (*J*=12.5, 4.8 Hz)] for CH_2-1 and δ_H 2.77 ppm [Hα: ddd (*J*=17.7, 4.0, 2.5 Hz)] and 3.20 ppm [Hβ: ddd (*J*=17.7, 14.3, 5.0 Hz)] for CH_2-2. In addition, three methine protons resonate at δ_H 3.57 (*brs*) ppm, δ_H 3.68 ppm [d (*J*=3.0 Hz)] and 4.34 ppm [d (*J*=3.5 Hz)] for H-12a, H-11 and H-12, respectively. The appearance of the methine signal CH-12a in (**11**), the downfield chemical shifts of H-11 in comparison to H-11 in (10) together with the molecular weight of (**11**) confirm the presence of an epoxy bridge between C-11 and C-12 in (**11**).

The structure of compound (**11**) was further confirmed by interpretation of ^1H-^1H COSY spectrum (Figure 3.12 and Table 3.6) which showed the following four spin systems, $CH_2(2)CH_2(1)$, CH(11)CH(12)CH(12a), CH(5)CH(6) and CH(7)CH(8). The optical rotation of compound (**11**) is $[\alpha]_D^{20}$ + 415 which is comparable to that which is reported in literature (Cynthia *et al*., 1989; Stack *et al*., 1986). Thus, compound (**11**) is identified as the known altertoxin II which was previously reported by Arnone and co-workers from *Stemphylium botryosum* (Arnone *et al*., 1986) and by Stack and co-workers isolated from *Alternaria alternata* (Stack *et al*., 1986).



Structure of stemphytriol (**10**) and altertoxin II (**11**)

Table 3.6: ^{1}HNMR and COSY data for compound (**10**) and compound (**11**) at 500 (^{1}H) and 125 (^{13}C) MHz:

#	Compound (**10**)			Compound (**11**)		
	δ_H(MeOD)	COSY	δ_H (acetone-*d6*)[a]	δ_H(MeOD)	COSY	δ_H (acetone-*d6*)[b]
1	4.42,*d* (10.4)	12b	4.75,*m*			
1 α				2.87, *ddd* (13.4,7.5, 2.5)	1β	2.95,*ddd* (13.3,5,2.5)
1 β				2.47,*td* (12.5,4.8)	1α, 2α	2.59,*dd* (14.1,4.1) 3.25,*d* (17.6)
2 α	2.47,*dd* (2.8, 9.0)	2 β	2.99,*d* (2.8)	3.20, *ddd* (17.7,14.3,5)	1α, 1β,2 β	2.77, *d* (17.6)
2 β	3.14, *d* (9.0)	2 α	3.01,*d* (8.1)	2.77, *ddd* (17.7,4.0, 2.5)	1β,2 α	
5	7.01,*d* (9.0)	6	6.95,*d* (8.7,5)	7.07,*d* (8.9)	6	7.06,*d* (8.8)
6	7.97, *d* (9.0)	5	7.97,*d* (8.7)	8.08, *d* (8.9)	5	8.13,*d* (8.8)
7	8.01, *d* (9.0)	8	8.01,*d* (8.7)	8.00, *d* (8.8)	8	8.04,*d* (8.8)
8	7.06, *d* (9.0)	7	7.06,*d* (8.7)	*6.99, d (8.8)*	7	6.99,*d* (8.8)
11				*3.68,d (3.0)*	12	3.72,*dd* (3.7,0.9)
11α	2.65,*d* (16.5)	11 β	2.82,*dd* (4.5,16.3)			
11β	3.06,*d* (16.5)	11 α,12	3.17,*dd* (12.5,16.3)			
12	4.37,*d* (9.1)	11 β	4.67,*dd* (4.5,12.5)	4.34,*d* (3.5)	11	4.44,*d* (0.4)
12a				3.57, *brs*		3.62,*dd* (0.9, 0.4)
12b	3.23,*brs*	1	3.15,*d* (8.6)			

(a) Karsten *et al.,* 1999.

(b) Arnone *et al*., 1986.

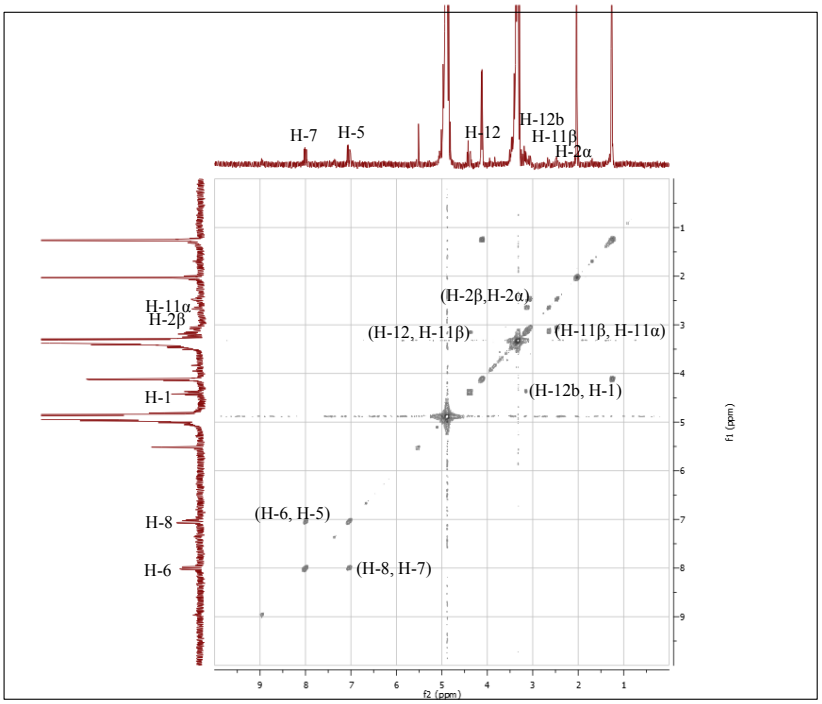


Figure 3.11: ^{1}H-^{1}HCOSY spectrum of compound (**10**)

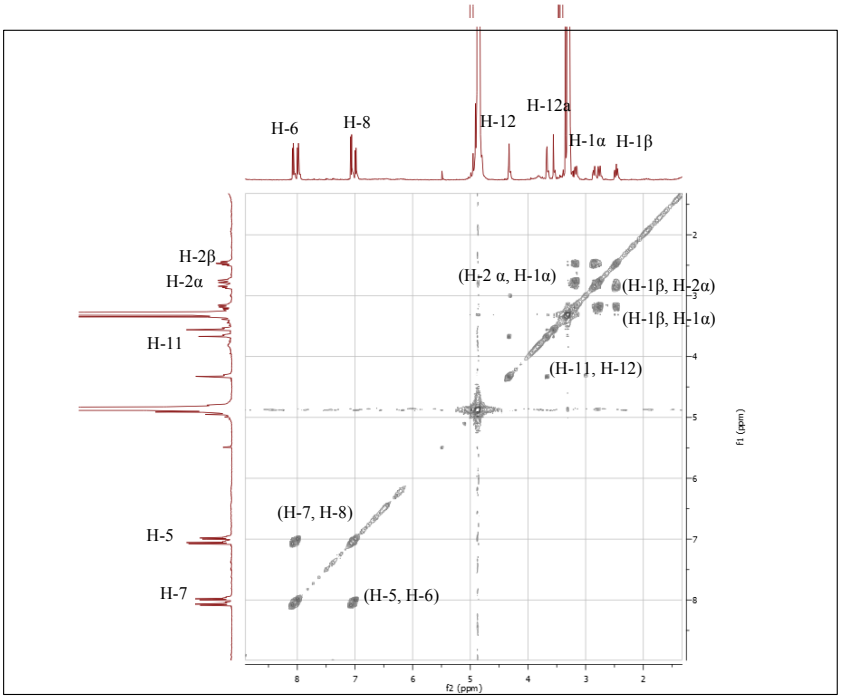
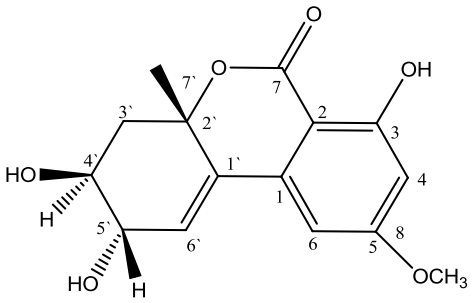
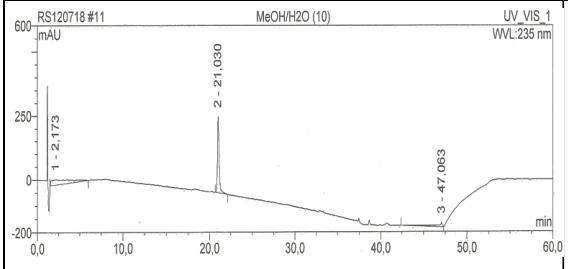


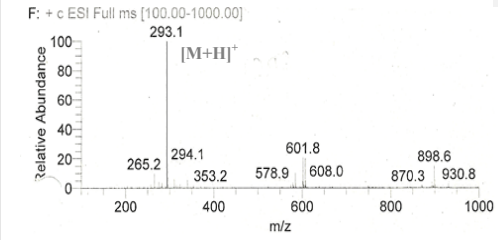
Figure 3.12: ^{1}H-^{1}HCOSY spectrum of compound (**11**)

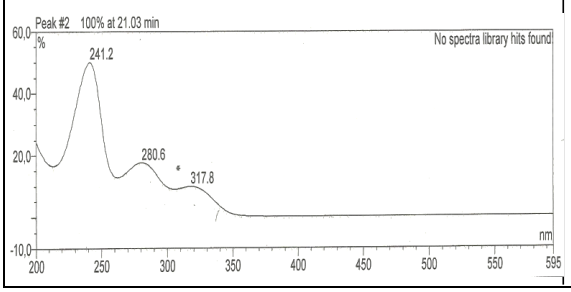
3.1.12. Altenuene (12, known compound)

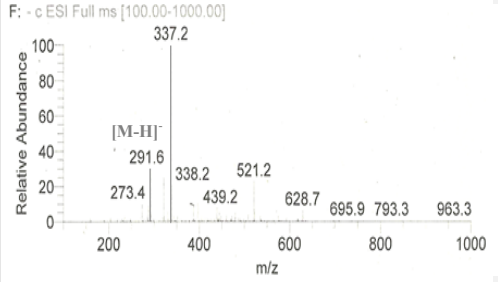
Altenuene	
Synonym(s)	2`,3`4`,5`-Tetrahydro-3,4`a,5`a-trihydroxy-5-methoxy-2`a-methyldibenzo[a]-pyrone
Sample code	MeOH/H_2O(10)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.3 mg
Physical Description	Viscous yellow oil
Molecular Formula	$C_{15}H_{16}O_6$
Molecular Weight	292 g/mol
Optical Rotation $[\alpha]_D^{20}$	-16 (*c* 0.205, $CHCl_3$)
Retention time HPLC	21.0 min (standard gradient)











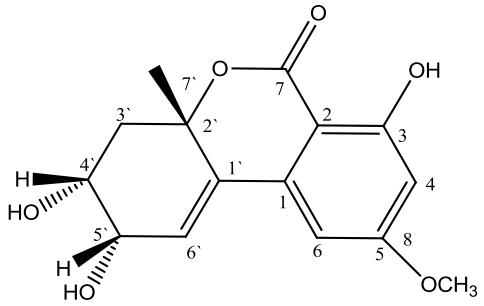
Compound (**12**), was isolated as viscous yellow oil from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. The ESI/MS of compound (**12**) showed a positive and negative *pseudo*-molecular ion at *m/z* 293 $[M+H]^+$ (base peak) and at *m/z* 291.6 $[M-H]^-$ (base peak), respectively indicating a molecular weight of 292 g/mol. The UV spectrum of (**12**) showed absorbances at λ_{max} (MeOH) 241.2, 280.6, 317.8 nm, which are characteristic for the alternariol derivatives (Freeman, 1965).

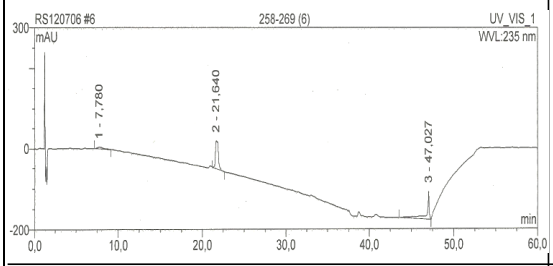
The ^{1}H NMR spectrum (Table 3.7) of compound (**12**) showed *meta*-coupled aromatic protons signals at δ_H 6.49 [d (*J*=2.3 Hz)] and 6.68 [d (*J*=2.3 Hz)] for H-4 and H-6, respectively, a singlet at δ_H 3.88 ppm assigned for an aromatic methoxyl group, a singlet methyl group appeared at δ_H 1.51 ppm, a doublet signal appeared at δ_H 6.24 (*J*=2.8 Hz) for an olefinic proton, two oxygenated methine protons appeared at δ_H 3.79 [ddd (*J*=9.0, 5.8, 3.8 Hz)] and 4.08 [dd (*J*=5.8, 2.8 Hz)] for H-4`and H-5`, respectively and methylene protons resonated at δ_H 2.42 [Hα: dd (*J*=14.4, 3.8 Hz)] and δ_H 1.99 [Hβ: dd (*J*=14.4, 9 Hz)] for CH_2-3`.

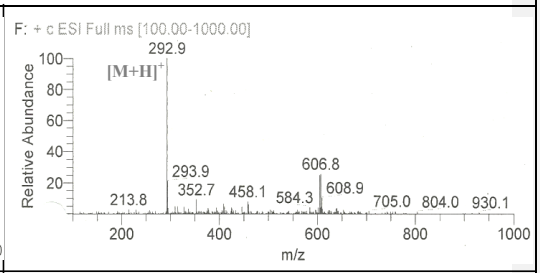
The ^{1}H-^{1}H COSY spectrum (figure 3.13 and Table 3.7) showed two spin systems, the first one is CH(4)CH(6) and the second one is CH_2(3`)CH(4`)CH(5`)CH(6`). The structure was further confirmed by comparing UV, ^{1}H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ value with published data for the known compound altenuene, previously reported from *Alternaria* species (Mcphail *et al.*, 1973; Bradburn *et al*., 1994).

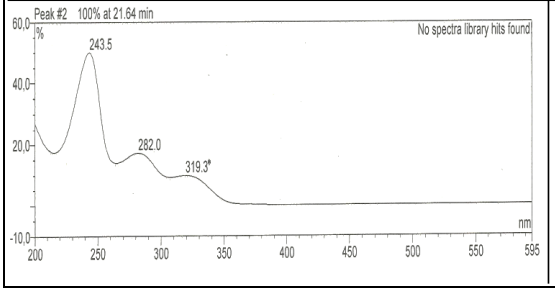
3.1.13. 4`-Epialtenuene (13, known compound)

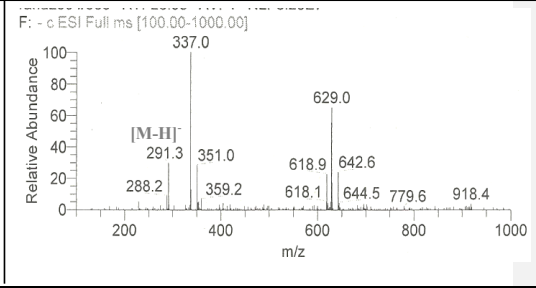
4` -Epialtenuene	
Synonym(s)	2,3,7-Trihydroxy-9-methoxy-4amethyl-4,4a-dihydro-2H-benzo[c]chromen-6 (3H)-one
Sample code	258-269 (6)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.5 mg
Physical Description	Viscous yellow oil
Molecular Formula	$C_{15}H_{16}O_6$
Molecular Weight	292 g/mol
Optical Rotation $[\alpha]_D^{20}$	+20 (*c* 0.185, $CHCl_3$)
Retention time HPLC	21.6 min (standard gradient)







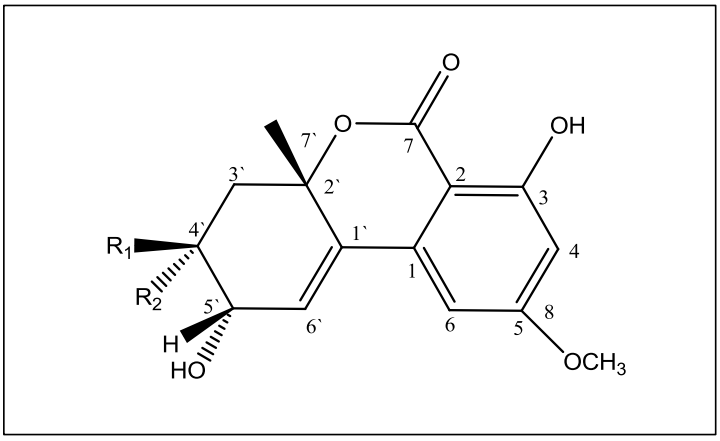




Compound (**13**)**,** was isolated as viscous yellow oil from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. Its ESI/MS spectrum showed the most intense peak at *m/z* 292.9 $[M+H]^+$ upon positive ionization indicating a molecular weight of 292 g/mol. Its UV showed absorbances at λ_{max} (MeOH) 243 nm with shoulders at 282 and 319.3 nm which is close to the UV spectrum of compound (**12**). Accordingly, compound (**13**) is suggested to be also an alternariol derivative (Bradburn *et al*., 1994).

The ^{1}H NMR spectrum of this compound exhibited a great similarity to the previous one (**12**) (Table 3.7) except that the spectrum exhibited that both H-3`(α and β) at ($\delta_H$ 2.17 [brt (*J*=12.3)], 2.28 [dd (*J*=12.0, 3.7)]) and H-4` at δ_H 3.82 [d (*J*=2.75)] resonate at significantly different chemical shifts and have different coupling constants in comparison to (**12**) which hints at a difference in configuration in both compounds. Similar to compound (**12**), the ^{1}H NMR data of compound (**13**) showed a *meta*-coupled aromatic protons at δ_H 6.50 [d (*J*=2.3 Hz)] and 6.66 [d (*J*=2.3 Hz)] for H-4 and H-6, respectively, an olefinic signal appears as doublet at δ_H 6.17 [d (*J*=2.5 Hz)] for H-6`, a singlet which is attributed to methoxy group resonates at $\delta_H$ 3.88 ppm, a methyl singlet resonates at $\delta_H$ 1.57 ppm for $CH_3$-7` and an oxygenated methine proton at δ_H 4.21 [dd (*J*= 8.0, 2.5 Hz)] for H-5`.

The ^{1}H-^{1}HCOSY spectrum (Figure 3.14) of (**13**) showed two spin systems, the first one is CH(4)CH(6) and the second one is CH_2(3`)CH(4`)CH(5`)CH(6`). The comparison of NMR data of (**13**) with those reported in the literature (Hassan, 2007) confirmed the axial orientation of H-4`. From the previous data and also by comparison of $[\alpha]_D^{20}$, compound (**13**) is identified as 4`-epialtenuene, a compound which was previously isolated from an endophytic *Alternaria* strain (Hassan, 2007). Interestingly, the original reports indicated that altenuene (**12**) and 4`-epialtenuene (**13**) were obtained as racemates (Hassan, 2007), and no optical rotation values for the isomers have been reported.



Nr.	**Compound**	**R_1**	**R_2**
12	Altenuene	OH	H
13	4`-Epialtenuene	H	OH

Table 3.7: ^{1}HNMR data for compound **(12)** and compound **(13)** at 500 (^{1}H) and 125 (^{13}C) MHz:

Position	Compound(**12**)			Compound (**13**)			
	$\delta_{H(MeOD)}$	COSY	$\delta_{H(MeOD)}$[a]	$\delta_{H(MeOD)}$	COSY	$\delta_{H(MeOD)}$[a]	COSY[a]
4	6.49,*d* (2.3)	6	6.45, *d* (2.2)	6.50,*d* (2.3)	6	6.46,*d* (2.2)	6
6	6.68, *d* (2.3)	4	6.64, *d* (2.2)	6.66,*d* (2.3)	4	6.63,*d* (2.2)	4
3`α	2.42, *dd* (14.4, 3.8)	3`β, 4`	2.40, *dd* (14.5, 3.7)	2.17,*brt* (12.3)	3`β,4`α	2.15,*brt* (12.3)	3`β,4`α
3`β	1.99,*dd* (14.4, 9.0)	3`α	1.96, *dd* (14.5, 9.1)	2.28,*dd* (12.0, 3.7)	3`α,4`α	2.25,*dd* (11.9, 3.7)	3`α,4`α
4`	3.79,*ddd* (9.0,5.8,3.8*)*	3`α,5`	3.77, *ddd* (9.1, 5.6, 3.7)	3.82,*d* (2.75)	3`α,β, 5`β	3.73,*ddd* (12.3,8.2,3.7)	3`α,β, 5`β
5`	4.08,*dd* (5.8,2.8)	4`	4.06, *dd* (5.6, 2.8)	4.21,*dd* (8.0 ,2.5)	4`α,6`	4.20,*dd* (8.2,2.5)	4`α,6`
6`	6.24, *d* (2.8)	5`	6.21, *d* (2.8)	6.17,*d* (2.5)	5`β	6.17,*d* (2.5)	3`α
CH_3-7`	1.51,*s*		1.49, *s*	1.57,*s*		1.54,*s*	
OCH_3-8	3.88,*s*		3.86,*s*	3.88,*s*		3.85,*s*	

(a) Hassan, 2007.

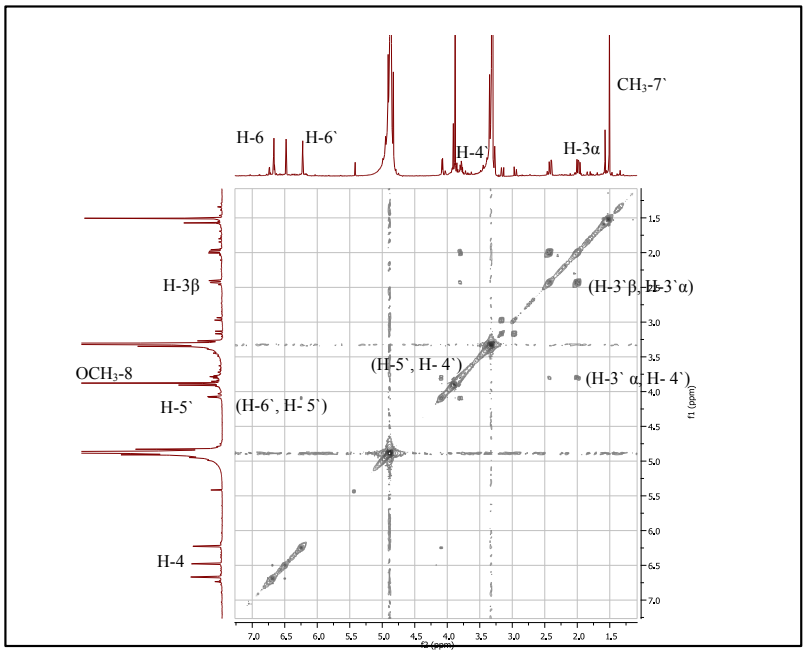


Figure 3.13: ^{1}H-^{1}HCOSY spectrum of compound (**12**)

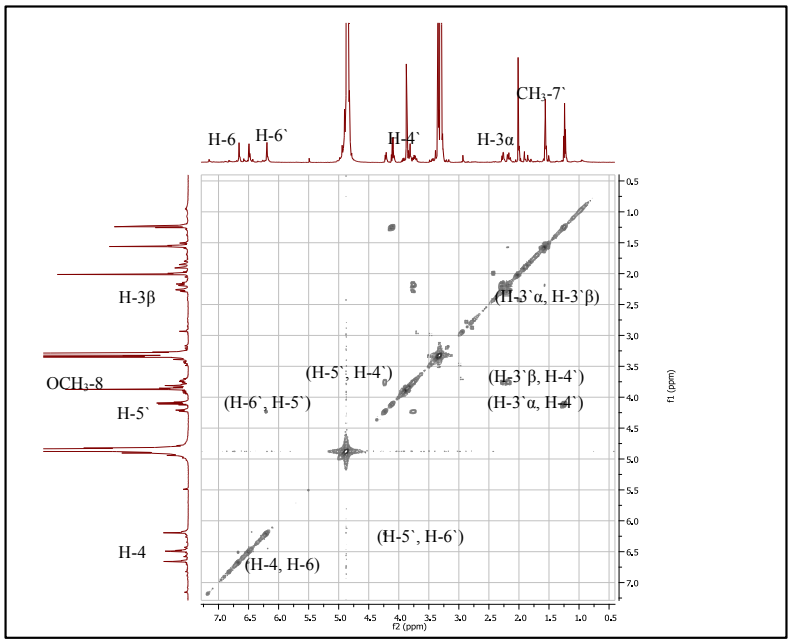
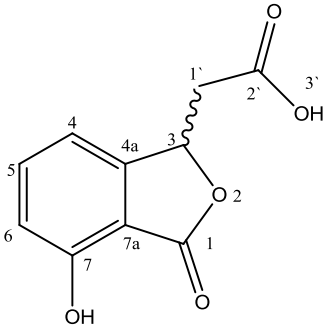
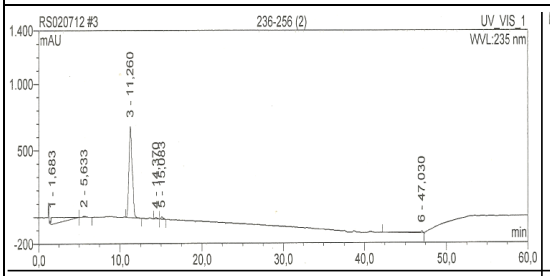


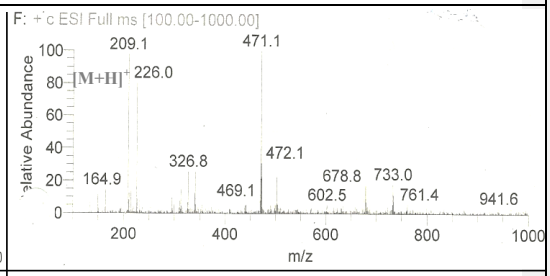
Figure 3.14: ^{1}H-^{1}HCOSY spectrum of compound (**13**)

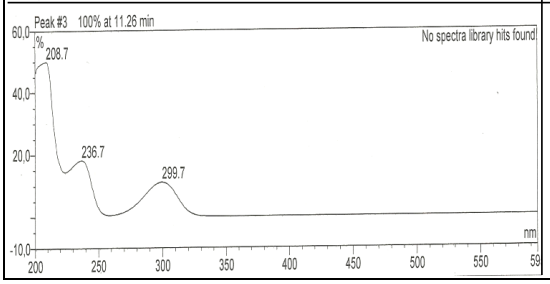
3.1.14. Isoochracinic acid (14, known compound)

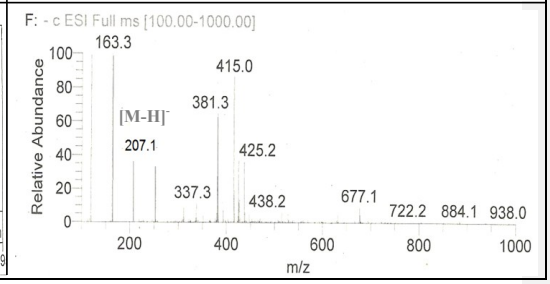
(±)-**Isoochracinic acid**	
Synonym(s)	3-Carboxymethyl-7-hydroxyphthalide
Sample code	236-256(2)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	4.2 mg
Physical Description	Yellow solid
Molecular Formula	$C_{10}H_8O_5$
Molecular Weight	208 g/mol
Optical Rotation $[\alpha]_D^{20}$	0° (*c* 0.3, MeOH)
Retention time HPLC	11.2 min (standard gradient)











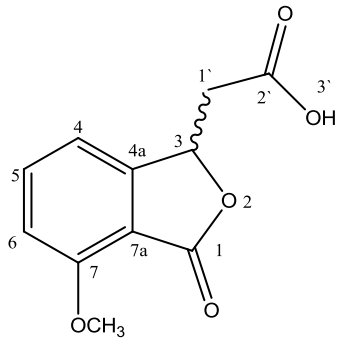
Compound (**14**), was isolated as a yellow solid from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It exhibited UV absorbances at λ_{max} (MeOH) 208.7, 236.7 and 295.2 nm. Positive and negative ESI-MS showed molecular ion peaks at *m*/*z* 209.1 $[M+H]^+$ and *m/z* 207.1 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 208 g/mol.

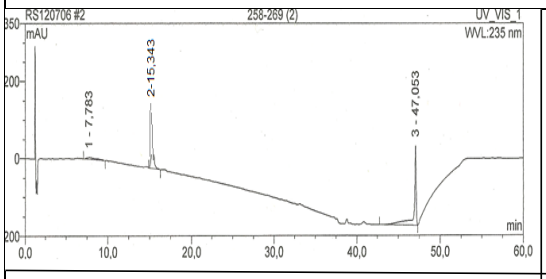
The ^1H NMR spectrum showed three adjacent protons for a tri-substituted benzene ring at δ_H 6.91 ppm [d (*J*=8.0 Hz)], 7.04 [d (*J*=8.0 Hz)] and 7.56 [t (*J*=8.0 Hz)] for H-6, H-4, and H-5, respectively, methylene protons CH_2-1` which resonate at δ_H 3.02 [dd (*J*=4.9, 16.6 Hz)] and 2.77 [dd (*J*=7.9, 16.6 Hz)]) and a methine proton H-3 resonates at δ_H 5.82 [dd (*J*=4.9, 7.9 Hz)]. Further confirmation was achieved from the ^1H-^1H-COSY spectrum (Figure 3.15). It showed two spin systems, the first one is CH_2(1`)CH(3) and the second one is CH(4)CH(5)CH(6) indicating the phthalide structure.

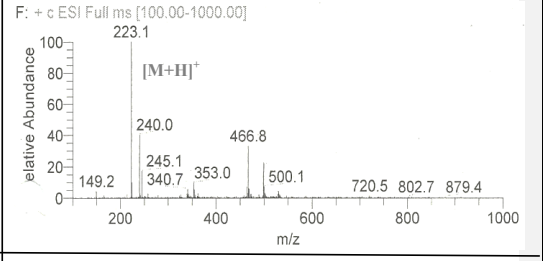
According to the aforementioned data (Table 3.8) and by comparison to the literature (Kiyoshi and Mitsuo, 1974), compound (**14**) was confirmed to be the known isoochracinic acid, which was first isolated from *Alternaria kikuchiana* and further has been synthesized by (Barry *et al.*, 1980).

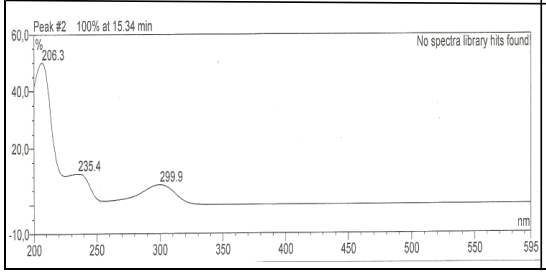
3.1.15. 7-Methoxyphthalide-3-acetic acid (15, new compound)

(±)-**7-Methoxyphthalide-3-acetic acid**	
Synonym(s)	3-Carboxymethyl-7-methoxyphthalide
Sample code	258-269 (2)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.9 mg
Physical Description	Yellowish brown solid
Molecular Formula	$C_{11}H_{10}O_5$
Molecular Weight	222 g/mol
Optical Rotation $[\alpha]_D^{20}$	0° (*c* 0.3, MeOH)
Retention time HPLC	15.3 min (standard gradient)







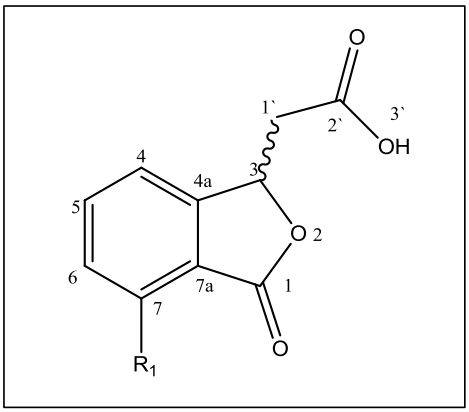


(-)-ESI-MS: No ionization

Compound (**15**) was isolated as a yellowish brown solid from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It exhibited UV absorbances at λ_{max} (MeOH) 206.3, 235.4 and 299.9 nm showing high similarity to the UV spectrum of isoochracinic acid (**14**). The difference in the molecular weight between (**14**) and (**15**) is 14 amu which indicates the possible presence of an extra methyl group in (**15**). This is confirmed by HRESI-MS which exhibited a strong peak at *m/z* 223.0604 $[M+H]^+$ indicating a molecular formula of $C_{11}H_{10}O_5$ (calculated 223.0601, Δ 0.0003).

The ^1H NMR spectrum (Table 3.8) of compound (**15**) was very similar to that of compound (**14**) with the only difference of the presence of an extra methoxyl signal at δ_H 3.71 ppm. Moreover, characteristic signals attributable to protons H-5, H-4 and H-6 appear at δ_H 7.55 [t (*J*=8.0 Hz)], 7.01 [d (*J*=8.0 Hz)] and 6.91 [d (*J*=8.0 Hz)], respectively. In addition, a methylene protons resonate at δ_H 3.08 [dd (*J*=4.6, 16.6 Hz)] δ_H 2.79 [dd (*J*=8.0, 16.6 Hz)] assigned for CH_2-1` and a methine proton at $\delta_H$ 5.82 [dd (*J*=4.6, 8.0 Hz)] for H-3. The $^1H$-$^1H$ COSY (Table 3.8) showed two spin systems, $CH_2$(1`)CH(3) and CH(4)CH(5)CH(6). The attachment of the side chain at C-3 was further confirmed by the HMBC correlation (Figure 3.16) from H-1`b to C-3 (δ_C 78.6). The position of the methoxy group at C-7 was secured by the HMBC correlation of the OCH_3 to C-7 (δ_C 159.4). Further inspection of HMBC spectrum indicated the correlation from H-4 to C-3 (δ_C 78.6), C-5 (δ_C 114.2) and C-6 (δ_C 117.3), H-5 to C-4a (δ_C 153.2) and C-7 (δ_C 159.4) and H-6 to C-4 (δ_C 112.8), C-5 (δ_C 114.2), C-7 (δ_C 159.4) and C-7a (δ_C 115.3).

Comparison of UV, ^1H, ^{13}C NMR, $[\alpha]_D$ and mass spectral data with literature data indicated the similarity of compound (**15**) to isoochracinic acid (**14**) in which the hydroxy group at C-7 is methoxylated (Kiyoshi and Mitsuo, 1974). 7-Methoxyphthalide-3-acetic acid (**15**) was previously known only as a synthetic compound (David, W. and David, P., 1977; Silva *et al*., 1992). To the best of our knowledge compound (**15**) was identified as a new natural product.



Nr.	Compound	R_1
14	Isoochracinic acid	OH
15	7-Methoxyphthalide-3-acetic acid	OCH_3

Table 3.8: ^{1}HNMR , COSY and HMBC data for compound (**14**) at 500 (^{1}H) and 125 (^{13}C) MHz and compound (**15**) at 600 (^{1}H) and 150 (^{13}C) MHz:

#	Compound (**14**)		Compound (**14**)[a]	Compound (**15**)			
	δ_H (MeOD)	COSY	δ_H ($CDCl_3$)	δ_H (MeOD)	COSY	δ_C (MeOD)[b]	HMBC
1						171.1	
2							
3	5.82,*dd* (4.9,7.9)	1`a, 1`b	5.91,*t* (7.0)	5.82,*dd* (4.6,8.0)	1`a, 1`b	78.6	
4	7.04,*d* (8.0)	5	7.12,*d* (8.0)	7.01,*d* (8.0)	5	112.8	3,5,6
4a						153.2	
5	7.56,*t* (8.0)	6,4	7.60, *t* (8.0)	7.55,*t* (8.0)	6,4	114.2	4a,7
6	6.91,*d* (8.0)	5	6.97,*d* (8.0)	6.91,*d* (8.0)	5	117.3	4,5,7,7a
7						159.4	
7a						115.3	
OCH_3-7				3.71,*s*		52.2	7
1`	a-3.02,*dd* (4.9, 16.6)	1`b, 3	2.95,*d* (7.0)	a-3.08,*dd* (4.6, 16.6)	1`b, 3	40.3	3,4a,2`
	b-2.77,*dd* (7.9,16.6)	1`a, 3		b-2.79,*dd* (8.0,16.6)	1`a, 3		2`
2`						172.1	
3`-OH			5.5-6.0, *brs*				

a) Kiyoshi and Mitsuo, 1974.
b) Confirmed from HMBC

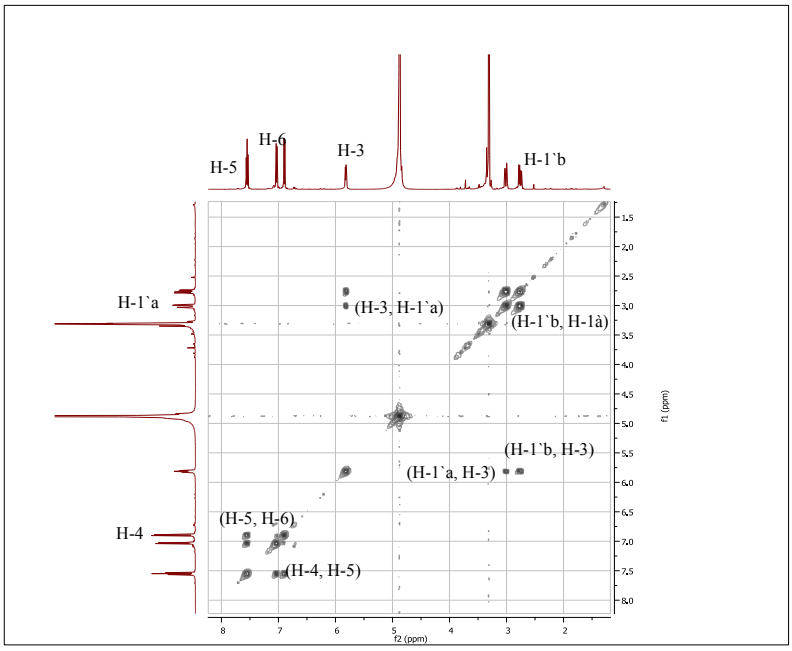


Figure 3.15: ^{1}H-^{1}HCOSY spectrum of compound (**14**)

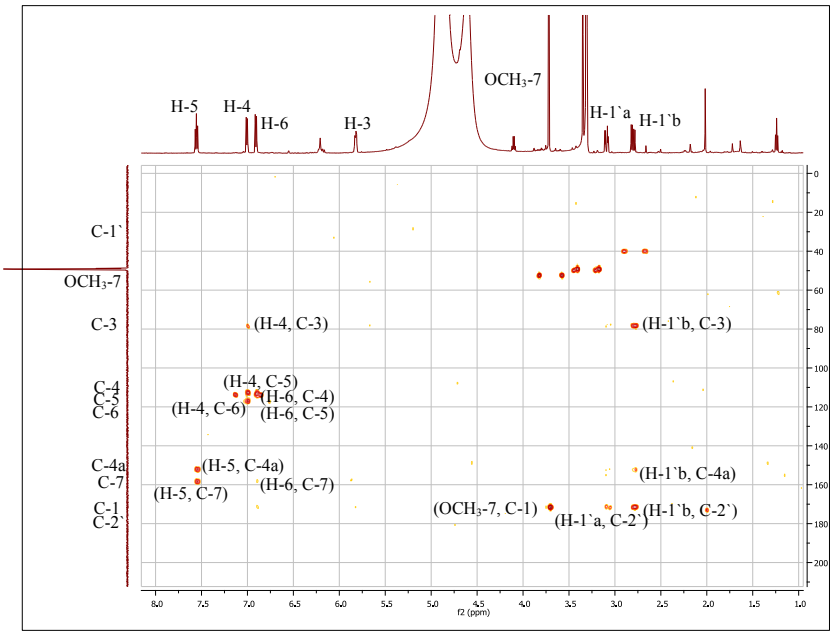
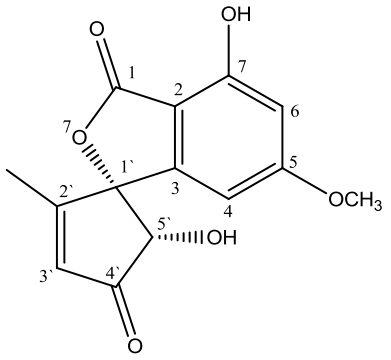
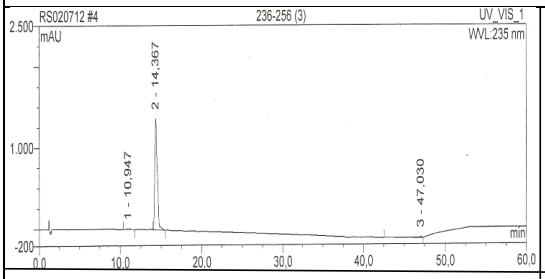


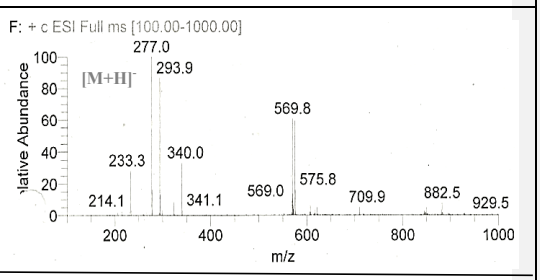
Figure 3.16: HMBC spectrum of compound (**15**)

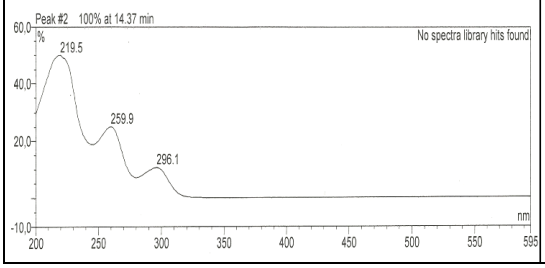
3.1.16. Talaroflavone (16, known compound)

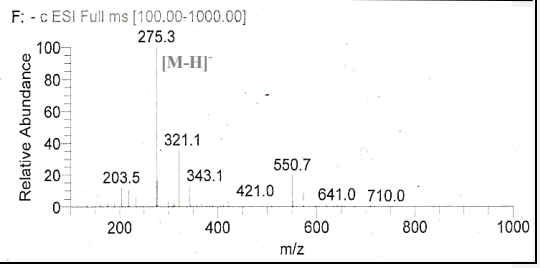
Talaroflavone	
Synonym(s)	5`-Hydroxy-2`-methyl-cyclopent-2`-ene-4`-one-1`,3,7-hydroxy-5-methoxy-1(3H)-isobenzofuranone
Sample code	236-256 (3)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	3.5 mg
Physical Description	white flakes
Molecular Formula	$C_{14}H_{12}O_6$
Molecular Weight	276 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 80 (*c* 0.7, MeOH)
Retention time HPLC	14.3 min (standard gradient)











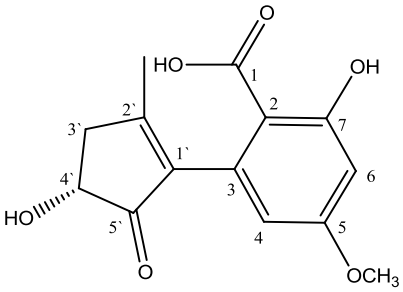
Compound (**16**), was isolated from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It crystalized from the eluent as white flakes. It exhibited UV absorbances at λ_{max} (MeOH) 219, 259 and 296 nm. Positive and negative ESIMS showed molecular ion peaks at m/z 277.0 $[M+H]^+$ (base peak) and m/z 275.0 $[M-H]^-$ (base peak), respectively indicating a molecular weight of 276 g/mol.

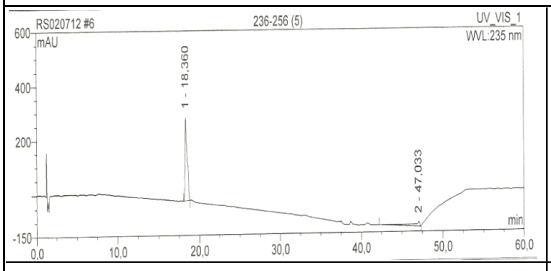
The ^{1}H NMR spectrum (Table 3.9) showed the presence of a doublet at δ_H 1.87 ppm [d, (*J*=1.4 Hz)] for CH_3-2`, a vinylic hydrogen H-3`at δ_H 6.36 [d (*J*=1.4 Hz)], a singlet at δ_H 4.76 ppm is consistent with a secondary alcohol in a pentacyclic lactone ring H-5`. Moreover, a *meta*-doublet at δ_H 6.08 and 6.47 ppm (*J*=1.9 Hz) for H-6 and H-4, respectively and a singlet methoxy group OCH_3-5 at δ_H 3.82 ppm were also detected. The ^{1}H-^{1}H-COSY spectrum (Figure 3.17 and Table 3.9) showed two spin systems; CH(4)CH(6) in the aromatic moiety and CH_3(2`)CH(3`) in a pentacyclic lactone ring.

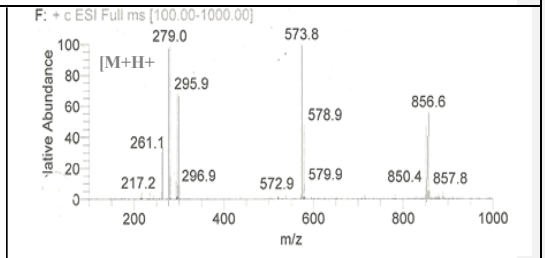
The obtained NMR data, the known mass, UV spectra and $[\alpha]_D^{20}$ for compound (**16**) in this study were in accordance with the known talaroflavone (Ayer and Racok, 1990; Hassan, 2007).

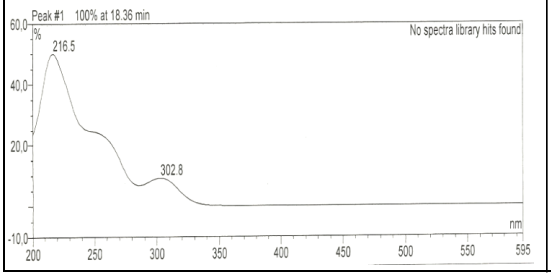
3.1.17. Alteric acid (17, known compound)

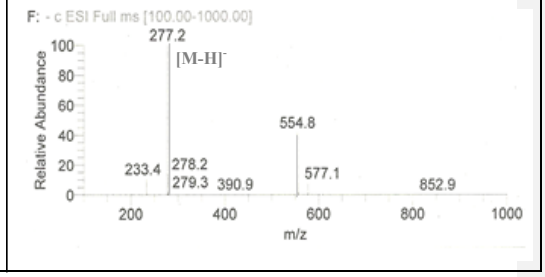
Alteric acid	
Synonym(s)	2-Hydroxy-6-(4-hydroxy-2-methyl-5-oxocyclopent-1-enyl)-4-methoxybenzoic acid
Sample code	236-256 (5)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	4.4 mg
Physical Description	viscous yellow oil
Molecular Formula	$C_{14}H_{14}O_6$
Molecular Weight	278 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 120 (*c* 1.0, MeOH)
Retention time HPLC	18.3 min (standard gradient)







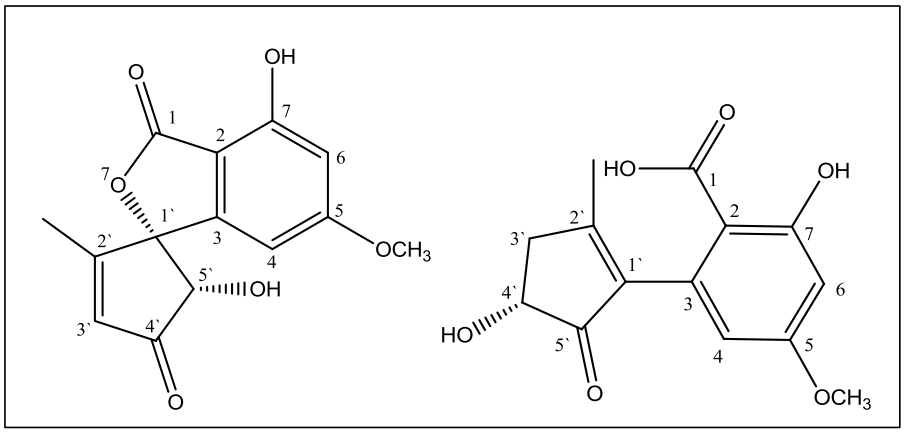




Compound (**17**) was isolated as viscous yellow oil (4.4 mg) from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It displayed UV absorbances at λ_{max} (MeOH) 216.5 and 302.8 nm. The positive and negative ESI-MS showed a *pseudo*-molecular ion peak at *m/z* 279.0 $[M+H]^+$ and 277.2 $[M-H]^-$ (base peak), respectively indicating a molecular weight of 278 g/mol.

The ^{1}H NMR spectrum (Table 3.9) showed the presence of a singlet at δ_H 2.02 ppm attributed to methyl group CH_3-2`, geminal-coupled methylene protons appeared at ($\delta_H$ 3.04 [*dd* (J=7.3, 17.5 Hz)], $\delta_H$ 2.55 [*d* (J=17.0 Hz)] ) assigned to $CH_2$-3`. Similar to talaroflavone (**16**) a singlet carbinolic proton H-4` in a pentacyclic lactone ring resonates at δ_H 4.31 ppm. Moreover, *meta*-coupled hydrogens at δ_H 6.16 and 6.49 ppm (each *brs*) corresponding to H-4 and H-6, respectively and a singlet methoxy group OCH_3-5 at δ_H 3.84 ppm were observed. The ^{1}H-^{1}H-COSY spectrum (Figure 3.18 and Table 3.9) showed two spin systems, the first one is CH_3(2`)$CH_2$(3`)CH(4`) and the second one is CH(4)CH(6).

Comparison of UV, ^{1}H NMR, mass spectral data, and the $[\alpha]_D$ value with literature data indicated the similarity of compound (**17**) to the known alteric acid (Hassan, 2007) which had previously been reported from culture filtrates of *Alternaria* sp. (Hassan, 2007).



Structure for talaroflavone (**16**) and alteric acid (**17**).

Table 3.9: ^{1}HNMR and data for compound (**16**) and compound (**17**) at 500 (^{1}H) and 125 (^{13}C) MHz:

Position	Compound (**16**)				Compound (**17**)		
	δ_H (MeOD)	COSY	δ_H (MeOD)[a]	δ_H (MeOD-d4)[b]	δ_H (MeOD)	COSY	δ_H (MeOD)[a]
1							
2							
3							
4	6.47, *d* (1.9)	6	6.43, *brs*	6.44, *d* (2.0)	6.16, *brs*	6	6.07, *d* (2.5)
5							
6	6.08, *d* (1.9)	4	6.04, *d* (1.2)	6.05, *d* (2.0)	6.49, *brs*	4	6.40, *d* (2.5)
7							
1`							
2`							
3`	6.36, *q* (1.4)	CH_3-2`	6.34, *s*	6.34, *q* (1.5)	a:3.04,*dd* (7.3,17.5)	3`b, 4`	a:3.00, *dd* (6.9,17.3)
					b:2.55, *d* (17.0)	3`a, 4`,CH_3-2`	b:2.49, *brd*(17.0)
4`					4.31,*brs*		4.38, *dd* (2.8, 6.3)
5`	4.76, *s*		4.73, *s*	4.74, *s*			
CH_3-2`	1.87, *d* (1.4)	3`	1.85, *s*	1.86, *d* (1.5)	2.02, *s*	3`b	1.99, *s*
OCH_3-5	3.82, *s*		3.79, *s*	3.80,*s*	3.84, *s*		3.78, *s*

(a) Hassan, 2007. (b) Ayer and Racok, 1990.

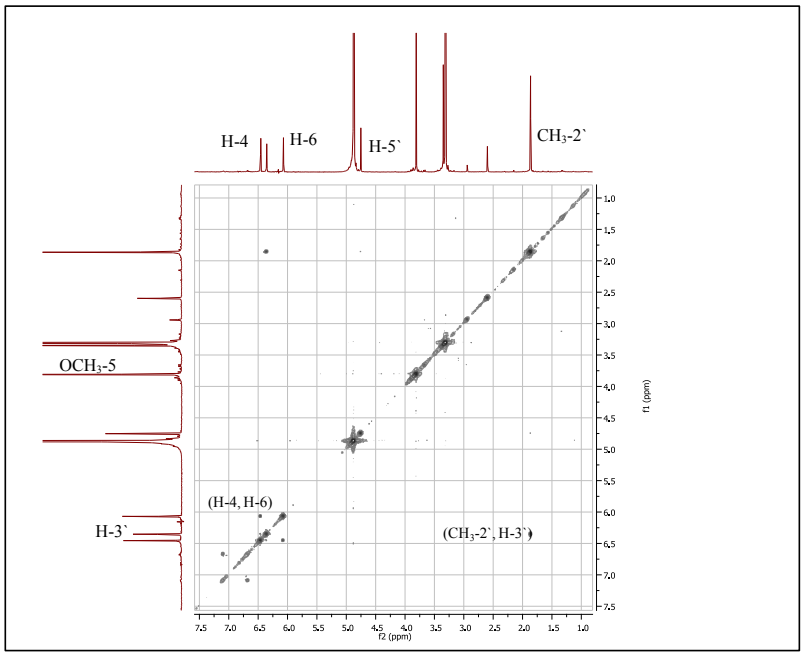


Figure 3.17: ^{1}H-^{1}HCOSY spectrum of compound (**16**)

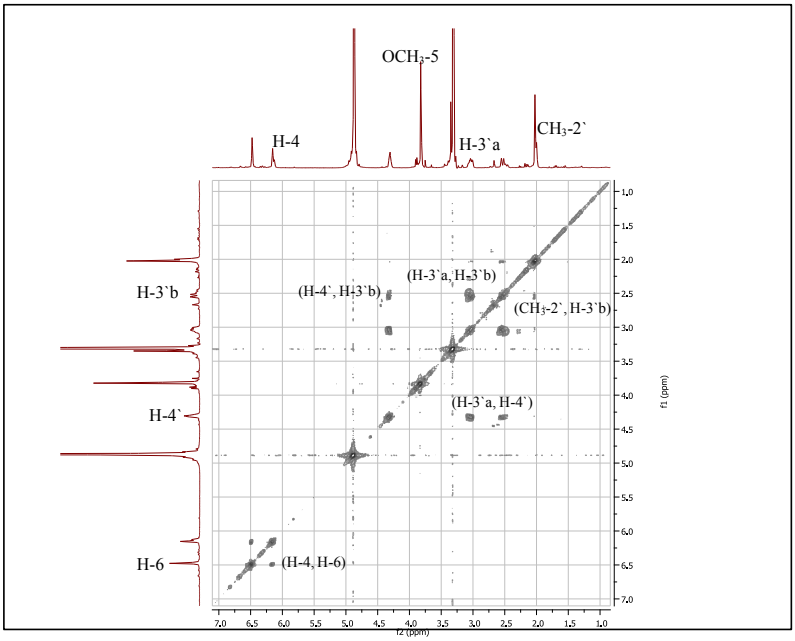
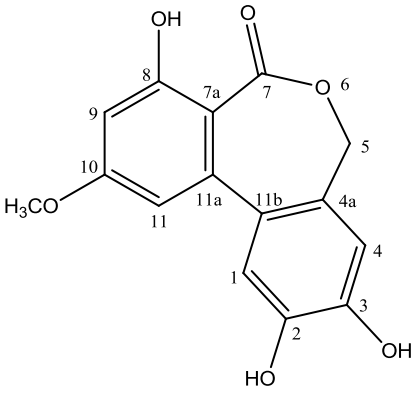
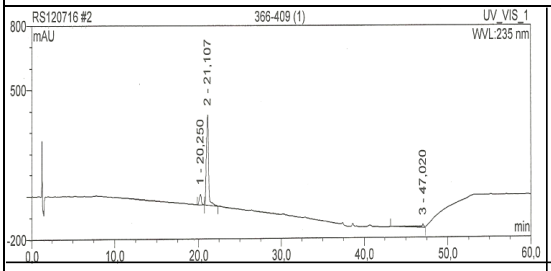


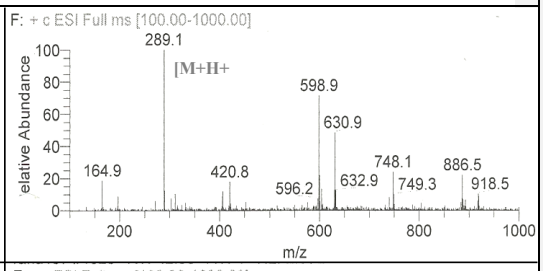
Figure 3.18: ^{1}H-^{1}HCOSY spectrum of compound (**17**)

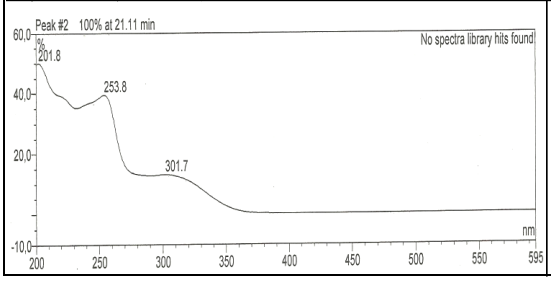
3.1.18. Alterlactone (18, known compound)

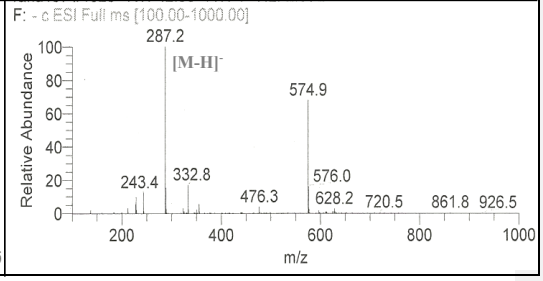
Alterlactone	
Sample code	366-409 (1)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	1.9 mg
Physical Description	white flakes
Molecular Formula	$C_{15}H_{12}O_6$
Molecular Weight	288 g/mol
Retention time HPLC	21.1 min (standard gradient)







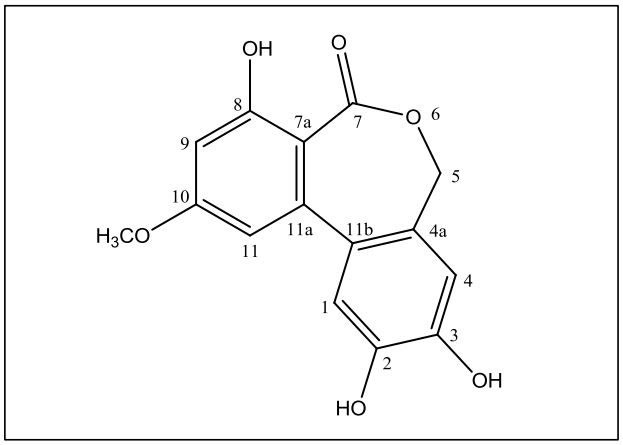




Compound (**18**) was isolated from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. The white flakes showed a UV spectrum with λ_{max} (MeOH) at 201.8, 253.8 and 301.7 nm. Its ESI-MS showed a *pseudo*-molecular ion peak at *m*/*z* 287.2 $[M-H]^-$ upon negative ionization and *m*/*z* 289.1 $[M+H]^+$ upon positive ionization indicating a molecular weight of 288 g/mol.

Structural elucidation of compound (**18**) was based on results of 1D and 2D NMR spectral analyses including ^1H NMR, ^{13}C NMR and HMBC spectra (Table 3.10). The ^1H NMR spectrum displayed four aromatic signals; two singlets at δ_H 7.03 and 6.91 ppm for H-1 and H-4 respectively, and a pair of doublet (*meta*-coupled) protons at δ_H 6.50 (*J*=2.4 Hz) and 6.46 (*J*=2.4 Hz) for H-9 and H-11, respectively. Moreover, a pair of geminally coupled protons at δ_H 4.82 [d (*J*=16.0 Hz)] and 4.85 [d (*J*=16.0 Hz)] for CH_2-5 and a singlet at δ_H 3.82 assigned for OCH_3-10 were also observed. In addition, three exchangeable protons corresponding to hydroxyl protons each appear as a broad singlet signals at δ_H 10.21, 9.45 and 9.36 ppm for 8-OH, 3-OH and 2-OH, respectively.

The planar structure of compound (**18**) is confirmed from the HMBC correlations (Figure 3.19) of the methylene protons CH_2-5 which located at the seven member lactone ring to the aromatic carbon C-4 (δ_C 115.3). Further inspection of the HMBC spectrum revealed the correlations from H-4 to C-5 (δ_C 67.7) and to C-11b (δ_C 129.9), H-9 to C-7a (δ_C 109.4), and H-11 to C-7a and C-11b. Moreover, the HMBC spectrum confirms the location of the substituents through the correlation of methoxyl protons OCH_3-10 to C-10 (δ_C 162.8) and the 8-OH group to C-8 (δ_C 159.5), C-7a (δ_C 109.4) and C-9 (δ_C 100.0). The structure was further confirmed by comparison of UV, mass spectra, ^1H NMR and ^{13}C NMR with published data for alterlactone (Hassan, 2007).



Structure for alterlactone (**18**).

Table 3.10: ^{1}HNMR, ^{13}C NMR and HMBC data for compound (**18**) at 600 (^{1}H) and 150 (^{13}C) MHz:

Position	Compound(**18**)			Compound(**18**)[a]		
	δ_H (DMSO)	HMBC	δ_C (DMSO)	δ_H (DMSO)	HMBC	δ_C (DMSO)
1	7.03, s	3,4a,11a	116.2	7.03, s	3,4a,11a,11b	115.5
2			146.6			146.6
3			145.7			145.9
4	6.91, *s*	2,5,11b	115.3	6.90, *s*	2,3,5,11b,4a	115.5
4a			139.3			140.0
5	4.82, *d* (16.0)	4	67.7	4.80, *d* (11.3)	4,7,11b	67.8
	4.85, *d* (16.0)			4.85, *d* (11.0)		
7						168.7
7a			109.4			109.5
8			159.5			159.9
9	6.50, *d* (2.4)	7a,10,11	100.0	6.45, *d* (2.2)	7a,8,10,11	100.8
10			162.8			162.2
11	6.46, *d* (2.4)	7a,9,11b	105.0	6.50, *d* (2.2)	7a,9,10,11b	105.0
11a			127.0			126.6
11b			129.9			129.8
1-OH						
2-OH	9.36, *brs*[b]	1,2		9.37, *brs*[b]		
3-OH	9.45, *brs*[b]	1,3		9.47, *brs*[b]		
8-OH	10.21, *brs*	7a,8,9		10.21, *brs*		
OCH_3-10	3.82, s	10	55.6	3.81, s	10	55.4

(a) Hassan, 2007 at at 500 (^{1}H) and 125 MHz (^{13}C)

(b) Assignements may be interchanged.

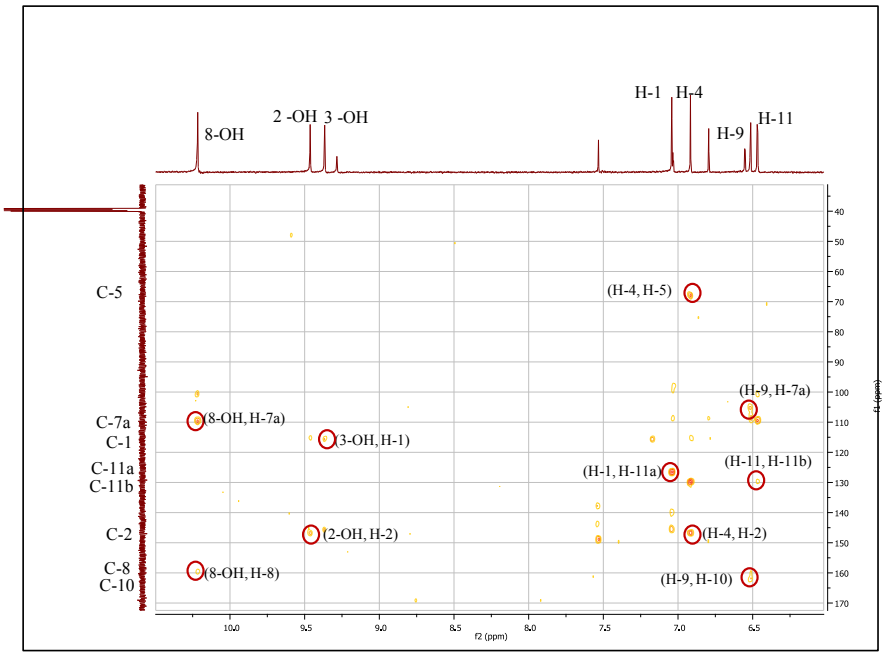
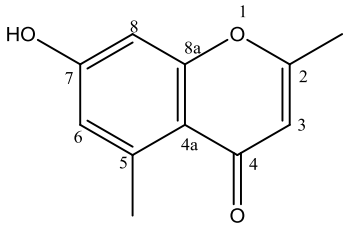
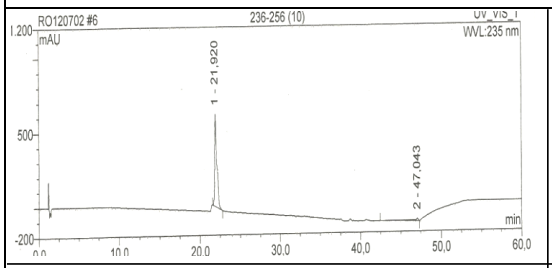


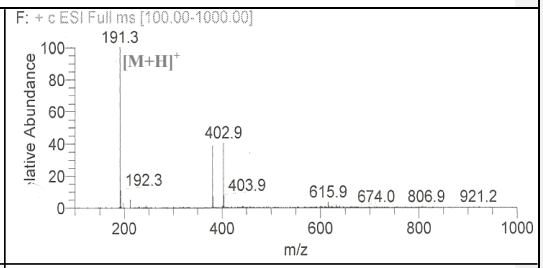
Figure 3.19: Expansion of HMBC spectrum of compound (**18**)

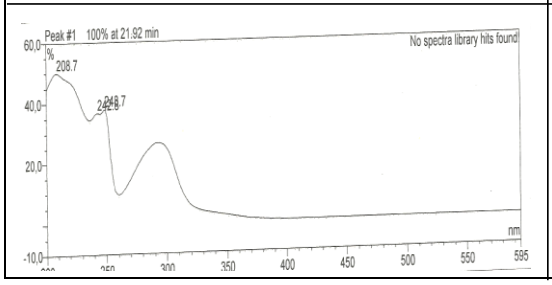
3.1.19. 2, 5-Dimethyl-7-Hydroxychromone (19, known compound)

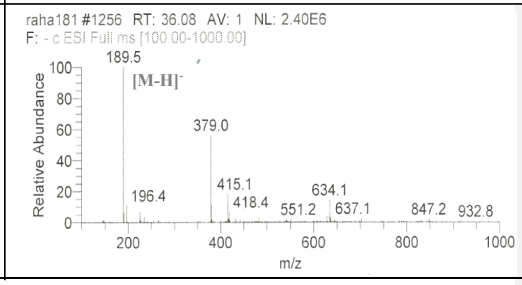
2,5-Dimethyl-7-Hydroxychromone	
Synonym(s)	7-Hydroxy-2,5-dimethyl-4H-1-benzopyran-4-one
Sample code	236-256(10)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	6.4 mg
Physical Description	Viscous yellow oil
Molecular Formula	$C_{11}H_{10}O_3$
Molecular Weight	190 g/mol
Retention time HPLC	21.9 min (standard gradient)









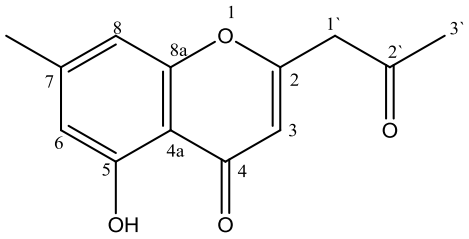


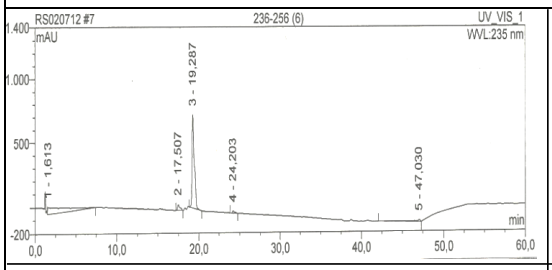
Compound (**19**) was obtained as a viscous yellow oil, isolated from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It displayed a UV spectrum with λ_{max} (MeOH) at 208.7, 242.8 and 248.7 nm. Positive and negative ESI-MS showed *pseudo*-molecular ions at m/z 191.3 $[M+H]^+$ and 189.5 $[M-H]^-$, respectively indicating a molecular weight of 190 g/mol.

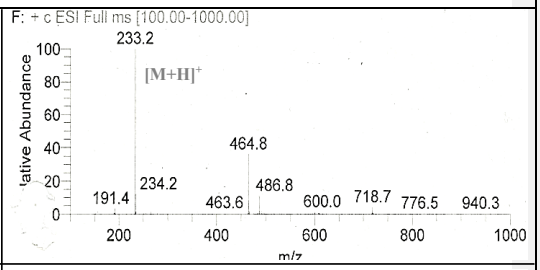
The ^{1}H NMR spectrum (Table 3.11) showed *meta*-coupled protons at δ_H 6.65 [d (*J*=2.3 Hz)] and 6.64 *(brs),* corresponding to H-8 and H-6, respectively. A vinylic singlet hydrogen H-3 at δ_H 6.02 ppm and two singlet methyl groups resonate at δ_H 2.33 and 2.72 ppm for CH_3-2 and CH_3-5, respectively. The ^{1}H-^{1}H-COSY spectrum (Table 3.11) showed two spin systems, the first one is CH_3(2)CH(3) and the second one is CH_3(5)CH(6)CH(8) indicating that the two methyl protons are substituted in two different aromatic rings. The structure was further confirmed by HMBC data (Table 3.11 and Figure 3.20) which showed correlations of the *meta*-coupled protons, H-6 to C-7 (δ_C 160.9) and H-8 to C-6 (δ_C 116.8), C-7 (δ_C 160.9) and C-8a (δ_C 159.2). Furthermore, correlations of the aromatic methyl group CH_3-2 to C-2 (δ_C 163.8) and C-3 (δ_C 110.7) and from H-3 to C-4 (δ_C 170.0) were also detected. The obtained UV, ^{1}H, ^{13}C NMR and mass spectral data were found to be identical with published data for 2,5-dimethyl-7-hydroxychromone (Ayer and Racok, 1990; Hassan, 2007), previously isolated from the roots of *Polygonum cuspidatum* (Kimura *et al*., 1983) and from rhubarb, *Rhei rhizoma* (Kashiwada *et al*., 1984).

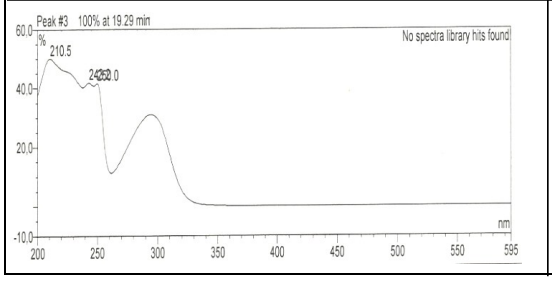
3.1.20. Altechromone B (20, known compound)

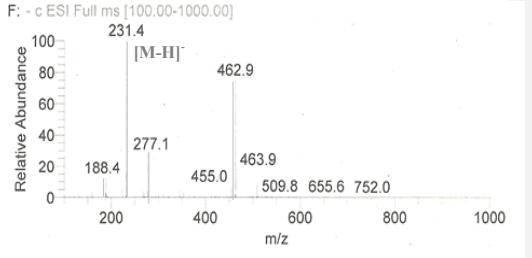
Altechromone B	
Synonym(s)	2-Acetonyl-5-hydroxy-7-methyl chromone
Sample code	236-256(6)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	3.6 mg
Physical Description	Colorless needles
Molecular Formula	$C_{13}H_{12}O_4$
Molecular Weight	232 g/mol
Retention time HPLC	19.2 min (standard gradient)







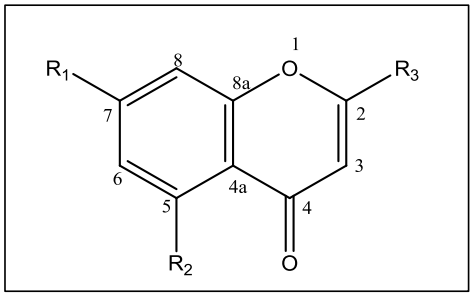




Compound (**20**) was isolated as colorless needles (3.6mg) from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. It displayed an UV spectrum with λ_{max} (MeOH) at 210.5, 243 and 250 nm, similar to the spectrum of compound (**19**). Positive and negative ESI-MS showed *pseudo*-molecular ions at m/z 233.2 $[M+H]^+$ and 231.4 $[M-H]^-$, respectively indicating a molecular weight of 232 g/mol. Both mass spectra showed ionization loss of an ethenone molecule CH_2CO- i.e. *m/z* 191.4 and 188.4 in positive and negative ESI-MS, respectively which means it differs from compound (**19**) by 42 amu.

In the ^1H NMR spectrum (Table 3.11) a clear difference in comparison to compound (**19**) was observable, whereas a singlet of a methylene protons CH_2-1` was observed at δ_H 3.64 ppm. Moreover, the ^1H NMR data of compound (**20**) (Table 3.11) indicated the presence of a pair of *meta*-coupled protons at δ_H 6.63 [d (*J*=2.4Hz)] and 6.65 (*brs*) ppm for H-6 and H-8, respectively, an olefinic singlet at δ_H 6.09 ppm for H-3 and two singlets resonating at δ_H 2.73 and 2.26 ppm for CH_3-7 and CH_3-3`, respectively. The ^1H-^1H-COSY spectrum (Table 3.11) showed a spin system, CH(6)CH_3(7)CH(8). The attachment of the aromatic methyl group at C-7 was further established by the HMBC via a strong correlation from the CH_3-7 to C-6 at (δ_C 117.8) and C-8 at (δ_C 102.8). Furthermore, the HMBC spectrum (Figure 3.21 and Table 3.11) showed a correlation from the deshielded methyl group CH_3-3` to the carbonyl group C-2` at (δ_C 204.9) confirming the attachment of an acetyl group to 1`and clarifying the molecular weight difference between (**19**) and (**20**).

From the above observation, and by comparison of UV, ^1H, ^{13}C NMR and mass spectral data with the literature, compound (**20**) was identified as altechromone B (Yasuo *et al*., 1992).



Nr.	Compound	R_1	R_2	R_3
19	2, 5-Dimethyl-7-hydroxychromone	OH	CH_3	CH_3
20	Altechromone B	CH_3	OH	CH_2COCH_3

Table 3.11: ^{1}H, ^{13}C NMR, COSY and HMBC data of compound (**19**) and (**20**) at 600 (^{1}H) and 150 (^{13}C) MHz:

	Compound (**19**)							Compound (**20**)					
#	δ_H (MeOD)	COSY	δ_C (MeOD)[c]	HMBC	δ_H (DMSO -d_6)[a]	δ_C (DMSO -$d6$)[a]	HMBC[a]	δ_H (MeOD)	δ_C (MeOD)[c]	COSY	HMBC	δ_H (DMSO -d_6)[b]	δ_C (DMSO -d_6)[b]
2			163.8			163.8			163.3				162.7
3	6.02,*s*	2-CH_3	110.7	4	5.96, *s*	110.7	2,4a,9	6.09,*s*	113.7		2	6.02,*s*	113.9
4			170			178.2							179.1
4a			118			114.2							
5			141			141.5			143.5				142.8
6	6.64,*bs*	5CH_3, 8	116.8	7	6.59, *d* (2.2)	116.4	4a,7,8, 10	6.63,*d* (2.4)	117.8	7-CH_3 ,8		6.59,*br. d* (2.0)	117.8
7			160.9			160.9							160.4
8	6.65,*d* (2.3)	6	101.8	6,7,8a	6.62, *d* (2.2)	100.5	4a,6,7, 8a	6.65, *br.s*	102.8	6	6	6.63,*br. d* (2.0)	101.6
8a			159.2			159.1							160.4
1`								3.64,*s*				3.84,*s*	48.2
2`									204.9				201.9
CH_3-2	2.33,*s*	3		2,3	2.25, *s*	19.3	2,3						
CH_3-5	2.72,*s*	6		4a,5,6	2.63, *s*	22.4	4a,5,6						
CH_3-7								2.73, *s*		6	6,8	2.65, *br.s*	24.5
CH_3-3`								2.26,*s*			2`	2.22,*s*	29.6
7-OH					10.58, *brs*								

(a) Hassan, 2007 at 500 (^{1}H) and 125 MHz (^{13}C) (C) Confirmed from HMBC

(b) Yasuo *et al*., 1992 at 500 (^{1}H) and 125 MHz (^{13}C)

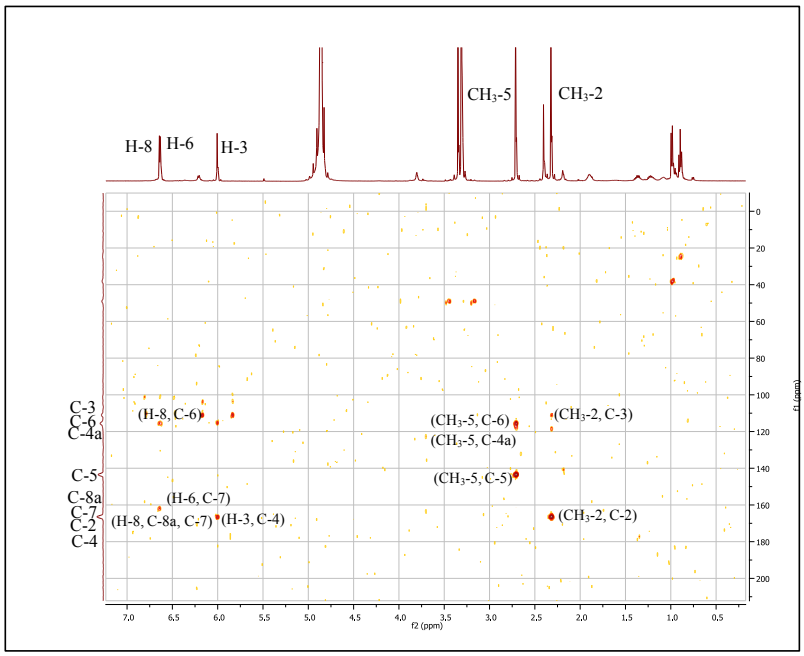


Figure 3.20: HMBC spectrum of compound (**19**)

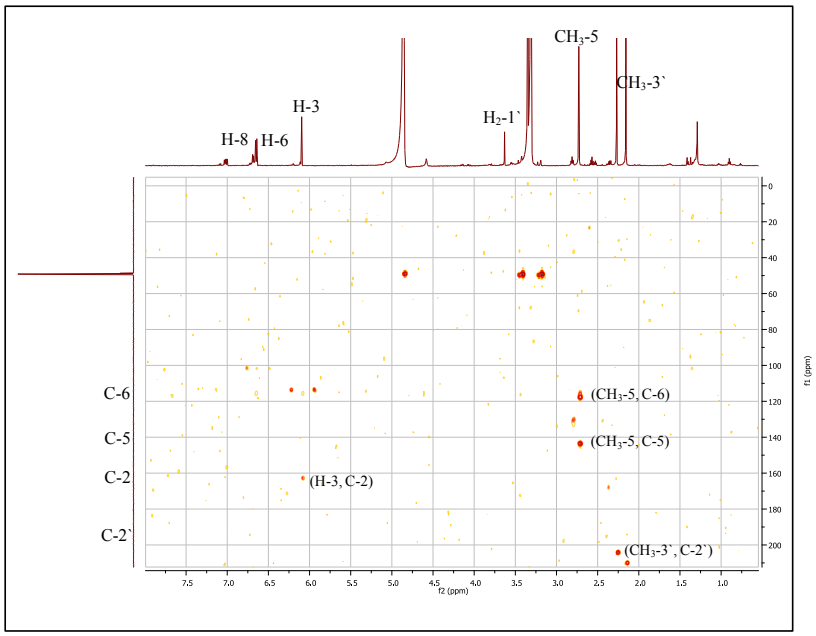
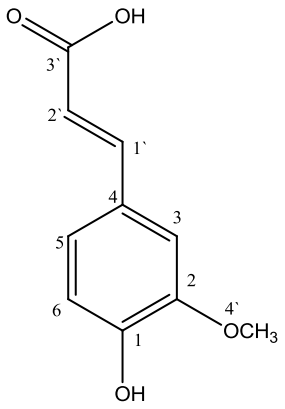
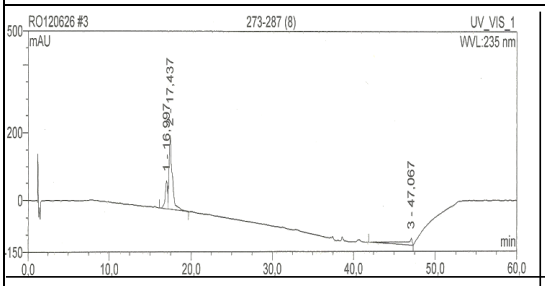


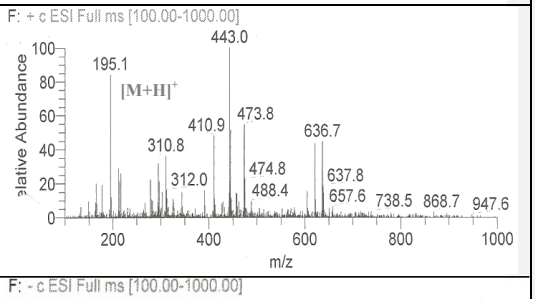
Figure 3.21: HMBC spectrum of compound (**20**)

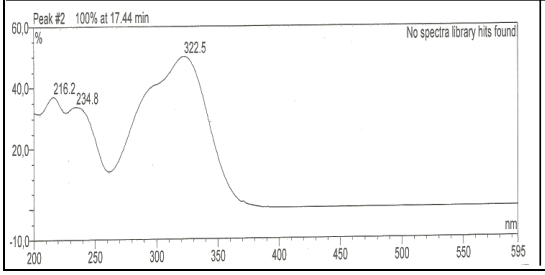
3.1.21. Ferulic Acid (21, known compound)

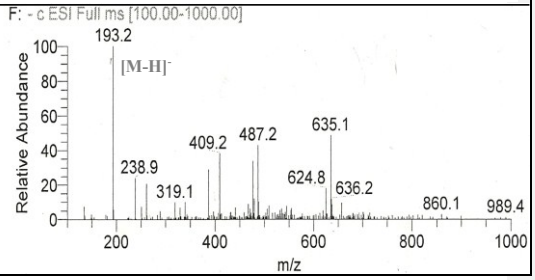
Ferulic Acid	
Synonym(s)	Coniferic acid
Sample code	273-287(8)
Biological source	*Alternaria* sp. (from *Ziziphus jujube*)
Sample amount	2 mg
Physical Description	Reddish brown solid
Molecular Formula	$C_{10}H_{10}O_4$
Molecular Weight	194 g/mol
Retention time HPLC	17.4 min (standard gradient)







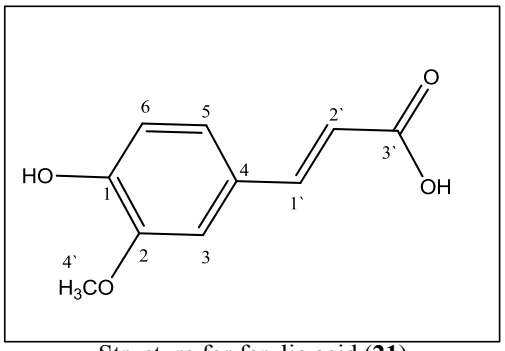




Compound (**21**) was isolated as a reddish brown solid from the EtOAC extract of the large scale rice cultures of *Alternaria* sp. The UV spectrum showed absorbances at λ_{max} (MeOH) 216.2, 234.8 and 322.5 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 195.0 $[M+H]^+$ (base peak) and *m/z*193.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 194g/mol.

The ^{1}H NMR spectrum (Table 3.12) displayed the characteristic signal for a methoxy group at δ_H 3.90 ppm assigned for OCH_3-4`. In addition, three aromatic protons at $\delta_H$ 6.82 [d (*J*=8.2 Hz)], 7.07 [dd (*J*=8.2 1.9 Hz)] and 7.19 [d (*J*=1.9 Hz)] for H-6, H-5 and H-3, respectively were also detected and two *trans*-coupled olefinic protons with coupling constant (*J*=15.9 Hz) at $\delta_H$ 6.32 and 7.61 ppm for H-2` and H-1`, respectively were also observed.

The ^{1}H-^{1}H-COSY spectrum (Figure 3.22 and Table 3.12) showed two spin systems, the first one is CH(1`)CH(2`) and the second one is CH(3)CH(5)CH(6). From the aforementioned data, compound (**21**) is in agreement with the structure of the known phenolic compound, ferulic acid (Sajjadi *et al*., 2012). Ferulic acid is the most abundant hydroxycinnamic acid in the plant kingdom and occurs mainly in the cell wall of cereal plants (Macadam and Grabber, 2002).



Structure for ferulic acid (**21**)

Table 3.12: ^{1}H NMR and COSY data of compound (**21**) at 500 (^{1}H) and 125 (^{13}C) MHz:

Position	Compound (**21**)		
	δ_H (MeOD)	COSY	δ_H ($CDCl_3$)[a]
3	7.19,*d* (1.9)	5	7.09,*d* (2)
5	7.07,*dd* (8.2,1.9)	6,3	7.14,*dd* (8.0,2.0)
6	6.82,*d* (8.2)	5	6.97,*d* (9)
1`	7.61,*d* (15.9)	2`	7.75,*d* (15)
2`	6.32,*d* (15.9)	1`	6.34,*d* (15)
OCH_3-4`	3.90,*s*		3.98,*s*

(a) Sajjadi *et al*., 2012.

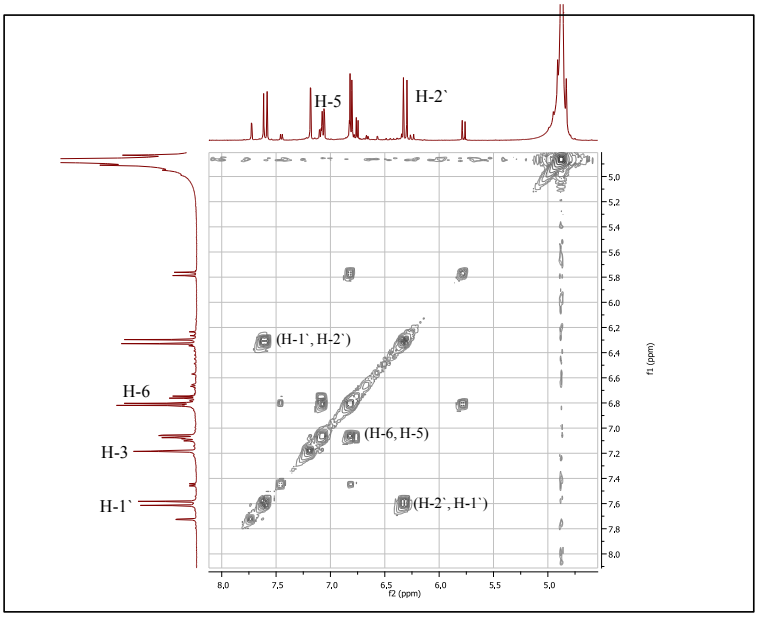


Figure 3.22: Expansion of ^{1} H-^{1}H COSY spectrum of compound (**21**)

3.1.22. Bioactivity data for compounds isolated from the endophytic fungus *Alternaria* sp.

The isolated compounds from the ethyl acetate extract of the small and large scale of *Alternaria* species were subjected to cytotoxicity (MTT) assay against mouse lymphoma (L5178Y) cells whose results (Table 3.13).

Table 3.13: Cytotoxicity assay results for the compounds isolated from *Alternaria* sp. liquid and rice extracts

Nr.	Sample tested	L5178Y growth in % (@ 10 μg/mL)	EC50* (µg/mL)	EC50 (µmol/L)
1	Citreoisocoumarinol	112.8		
2	6-Methyl-citreoisocoumarin	122.9		
3	Citreoisocoumarin	114.1		
4	Orthosporin	104.7		
5	Diaportinol	119.0		
6	Alternariol	-0.9	1.7	6.6
7	Alternariol-5-*O*-methyl ether	-4.3	7.8	28.7
8	Altenusin	8.8	6.8	23.4
9	Tenuazonic acid	134.1		
10	Stemphytriol	74.4		
11	Altertoxin II	0.3	6.2	22.5
12	Altenuene	88.2		
13	4`-Epialtenuene	75.9		
14	Isoochracinic acid	94.8		
15	7-Methoxyphthalide-3-acetic acid	95.1		
16	Talaroflavone	101.4		
17	Alteric acid	103.6		
18	Alterlactone	23.1		
19	2,5-Dimethyl-7-hydroxychromone	92.2		
20	Altechromone B	93.3		
21	Ferulic acid	98.1		

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

The cytotoxic results interestingly revealed that all of the alternariol derivatives in addition to the alterlactone (**18**) and altertoxin II (**11**) showed a significant cytotoxic activity against L5178Y cell line. The isocoumarins showed weak cytotoxic activity. In contrast, citreoisocoumarin (**3**) proved to be active against Gram positive bacteria multi-resistant

Staphylococcus aureus (MRSA) with MIC values of 0.49μg/mL. Regarding the alternariol derivatives, only alternariol (**6**) and altenusin (**8**) possess antibacterial activity against (MRSA) with MIC values of 0.49 and 31.25μg/mL as well as against *Streptococcus pneumonia* with MIC values of 0.98 and 15.63 μg/mL, respectively. Moreover, compounds (**10**), (**11**) and (**21**) showed significant activities against *Staphylococcus aureus* ATCC 29213 at a concentration of 64 µg/ml.

3.2. Compounds isolated from the fungus *Penicillium* sp.

The fungal strain *Penicillium* sp. was isolated from the sediment of Wadi El-Natron lake, a hyper saline lake located in Egypt. The pure fungal strain was identified and then cultivated on solid rice medium. HPLC chromatogram of the EtOAc extract of the fungus grown on solid rice medium showed that 6-hydroxy-8-methoxy-3,5-dimethylisochroman (**23**) is the main component (see Figure 3.23). Antibacterial, antifungal and cytotoxicity testing-results showed that the rice cultures extract of the fungus is promising. Based on these preliminary biological screenings, the extract of the fungus WN-11-1-3-1-2 had been subjected to further chromatographic procedures leading to the isolation of;

6-Hydroxy-8-methoxy-3,5-dimethylisochromanol(**22**),6-Hydroxy-8-methoxy-3,5-dimethylisochroman (**23**), arohynapene D (**24**), 1,6-Dihydroxy-3,5-dimethyl-8 methoxy-isochroman (**25**), 6-Hydroxy-8-methoxy-3,5-dimethyl-3,4-dihydro- isocoumarin (**26**), 6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin (**27**), 6-Methylcurvulinic acid (**28**), 2-Methyl-penicinoline (**29**), pretichodermamide C (**30**), N-Methylpretichodermamide B (**31**), and methyltriacetic lactone (**32**).

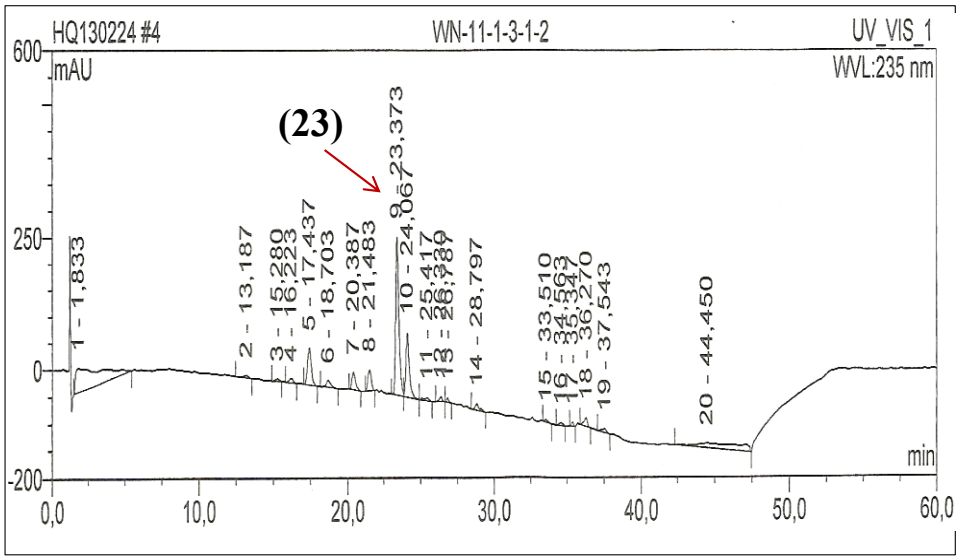
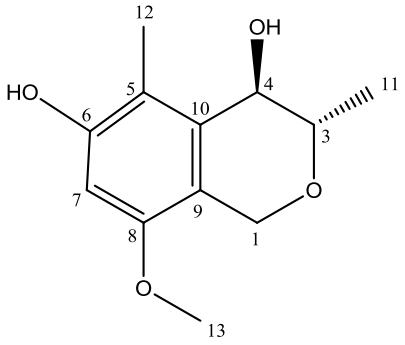
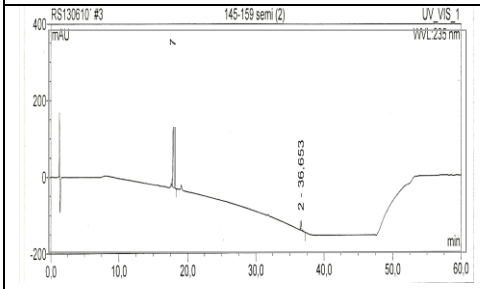


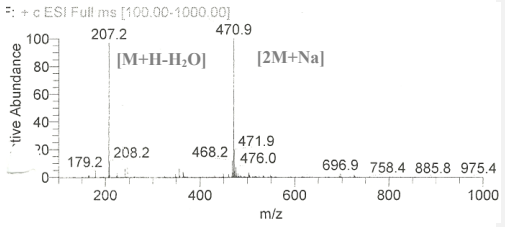
Figure 3.23: HPLC chromatogram of EtOAc extract of large scale of *Penicillium* sp. on rice medium. (**23):** 6-Hydroxy-8-methoxy-3, 5-dimethylisochroman.

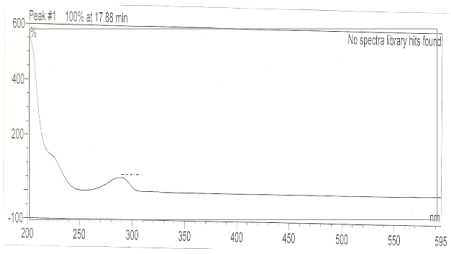
3.2.1. (3S, 4R)-6-Hydroxy-8-methoxy-3, 5-dimethylisochromanol (22, new compound)

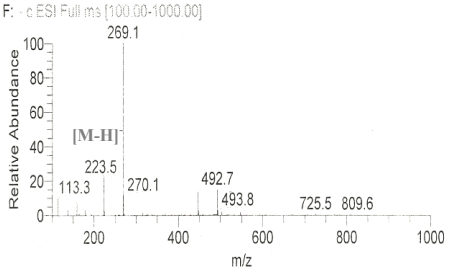
(3*S,* 4*R*)-6-Hydroxy-8-methoxy-3,5-dimethylisochromanol	
Synonym(s)	(3*S,* 4*R*)-6-Hydroxy-8-methoxy-3,5dimethylisochromanol
Sample code	145-159 (2)
Biological source	*Penicillium* sp.
Sample amount	6.9 mg
Physical Description	Brown solid
Molecular Formula	$C_{12}H_{16}O_4$
Molecular Weight	224.10 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 60 (*c* 0.3, MeOH)
Retention time HPLC	17.8 min (standard gradient)







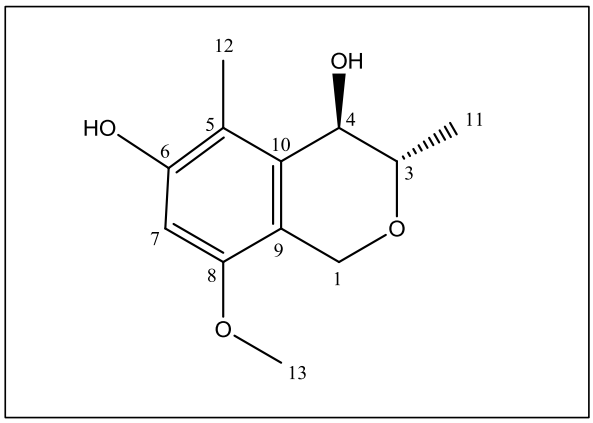




Compound (**22**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. as a brown solid (6.9 mg). It showed UV absorbance maxima at λ_{max} (MeOH) 288.6 nm. Negative ESI-MS showed molecular ion peak at m/z 223.5 $[M-H]^-$ while the positive ESI-MS showed a strong peak at m/z 207.2 $[M-H_2O+H]^+$. The molecular formula $C_{12}H_{16}O_4$ was obtained from HRESI-MS which exhibited a strong peak at *m*/*z* 247.0940 $[M+Na]^+$ (calculated 247.0941, *Δ* 0.0001).

Structural elucidation of compound (**22**) was based on results of 1D and 2D NMR spectral analysis including ^1H NMR, ^1H-^1H COSY, HMBC and ROESY spectra (Table 3.14). The ^1H NMR spectrum showed two methyl groups (one appears as a doublet at δ_H 1.27 ppm [d, (*J*= 6.5 Hz)] assigned for CH_3-11 while the other one appears as a singlet at δ_H 2.17 ppm for CH_3-12), a singlet at δ_H 3.73 ppm for OCH_3-13, a quartet doublet at δ_H 3.81 ppm [qd,(*J*=5.2 and 1.2 Hz)] for H-3, a doublet at δ_H 4.35 ppm [d,(*J*=5.2 Hz)] for H-4, two doublets at δ_H 4.55 and 4.64 ppm, [d,(*J*= 15.2 Hz)] for the desheilded geminally coupled protons CH_2-1 and an aromatic singlet at δ_H 6.38 ppm for H-7. The ^1H-^1H COSY correlations showed two spin systems, CH_2(1) and CH_3(11)CH(3)CH(4). The HMBC spectrum (Figure 3.24) showed correlations from CH_3-12 to C-5 (δ_C 116.8), C-10 (δ_C 135.6) and C-6 (δ_C 155.8). Further inspection of the HMBC spectrum revealed correlations from the methylene protons CH_2-1 at δ_H (4.55 and 4.64 ppm) to C-3 (δ_C 77.4), C-8 (δ_C 154.7), C-9 (δ_C 116) and C-10 (δ_C 135.6), H-3 at δ_H 3.81 ppm to C-1 (δ_C 62.3), C-4 (δ_C 69.2), C-10 (δ_C 135.6) and CH_3-11(δ_C 17.7). Moreover, the aromatic proton H-7 showed ^2J-^3J and ^4J HMBC correlations, to C-6(^2J), C-9(^3J), and C-1 (ω) which secures its position at C-7. Moreover, the aromatic methoxy OCH_3-13 is confirmed through the HMBC correlation of its protons to C-8. From the previous data, compound (**22**) was identified as the 6-hydroxy-8-methoxy-3, 5-dimethylisochromanol hinting a new natural product.

The ROESY experiment showed that the aromatic proton H-7 showed a strong ROESY correlation (Figure 3.25) with the OCH_3-13 group at δ_H 3.73 ppm confirming their *ortho*-location to each other. The *trans*-configuration between H-3 and H-4 in (**22**) was also confirmed from ROESY experiment (Figure 2b). Moreover, irradiation of an oxymethine proton at δ_H 4.35 (H-4) affected the signal intensity of a methyl proton at δ_H 1.27. The absolute configurations at C-3, biogenetically deduced to be *S* configuration and the absolute configuration at C-4 was deduced from the relative configuration. Thus (**22**) was elucidated as a new chromone (3*S**, 4*R**)-6-hydroxy-8-methoxy-3,5-dimethyl isochromanol.



Structure for 6-hydroxy-8-methoxy-3,5-dimethylisochromanol (**22**).

Table 3.14: ^{1}H, ^{13}C NMR, COSY, HMBC and ROESY data of compound **(22)** at 600 (^{1}H) and 150 (^{13}C) MHz:

Compound **(22)**					
Position	δ_H(MeOD)	COSY	ROESY	HMBC	δ_C(MeOD)
1a	4.64, *d* (15.2)	1b, CH_3-11	1b, CH_3-11	3,8,9,10	62.3
b	4.55, *d* (15.2)	1a,4	1a	3,8,9,10	
2					
3	3.81, *qd*(5.2, 1.2)	4, CH_3-11	1a,1b,4, CH_3-11	CH_3-11,1,4,10	77.4
4	4.35, *d* (5.2)	1b,3	1a,1b,3, CH_3-11, CH_3-12	CH_3-11,1,9,10	69.2
5					116.8
6					155.8
7	6.38, *s*		OCH_3-13	1,6,9	98.3
8					154.7
9					116
10					135.6
CH_3-11	1.27, *d* (6.5)	1a,3	1a,1b,3,4	1,3	17.7
CH_3-12	2.17, *s*		1a,1b,4	5,6,7,10	11.2
OCH_3-13	3.73, *s*		7	6,8	56

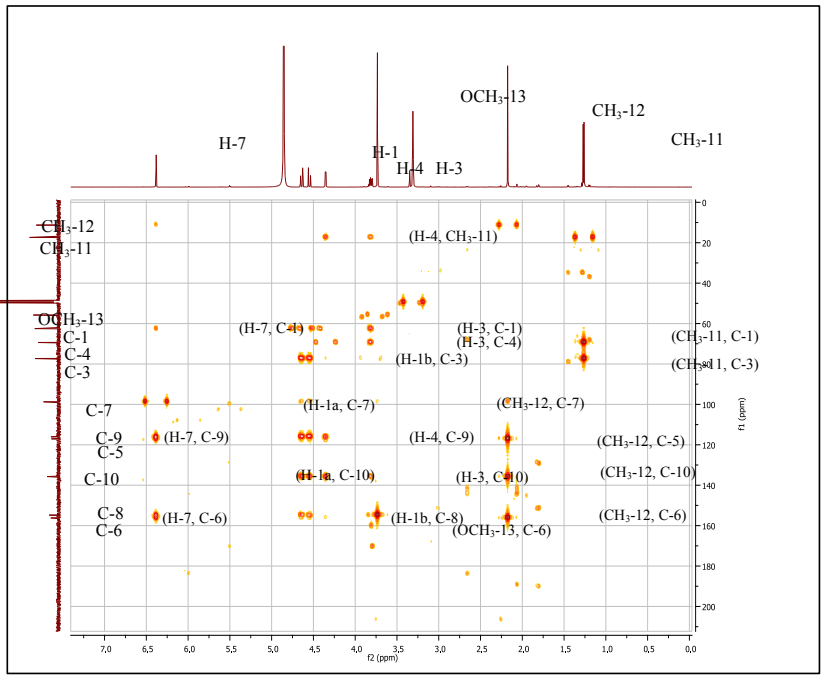


Figure 3.24: HMBC spectrum of compound (**22**)

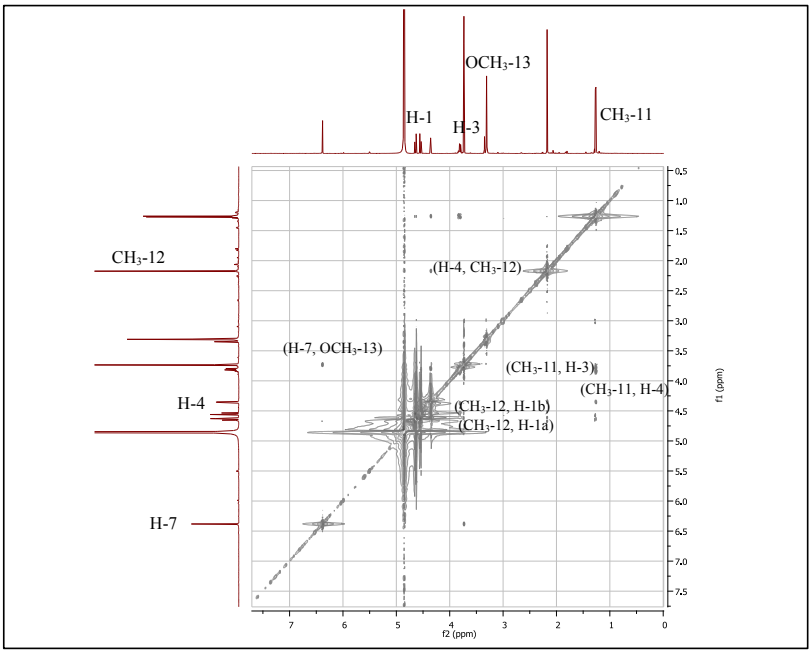
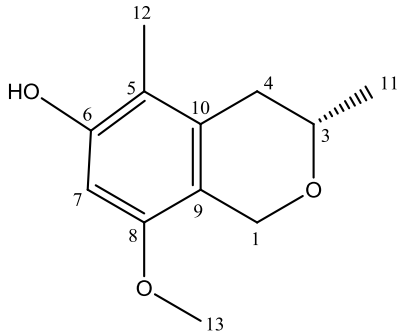
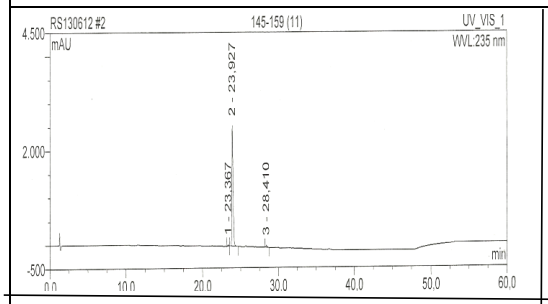


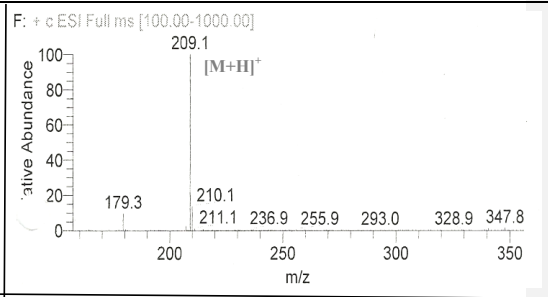
Figure 3.25: ROESY spectrum of compound (**22**)

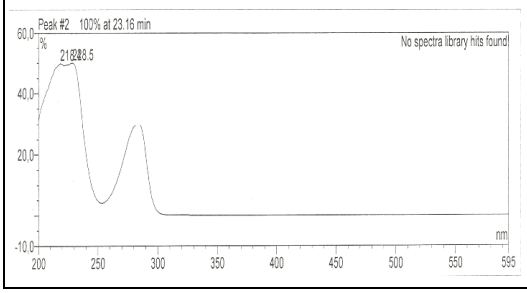
3.2.2. (3S)-6-Hydroxy-8-methoxy-3, 5-dimethylisochroman (23, known compound)

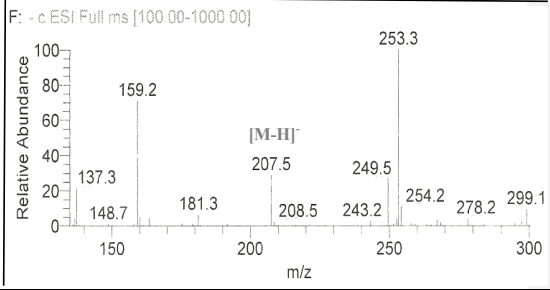
(3*S*)-6-Hydroxy-8-methoxy-3, 5-dimethylisochroman	
Synonym(s)	(3*S*)-6-Hydroxy-8-methoxy-3,5-dimethylisochroman
Sample code	145-159 (11)
Biological source	*Penicillium* sp.
Sample amount	39.7 mg
Physical Description	White solid
Molecular Formula	$C_{12}H_{16}O_3$
Molecular Weight	208.11 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 90 (*c* 0.1, MeOH)
Retention time HPLC	23.9 min (standard gradient)









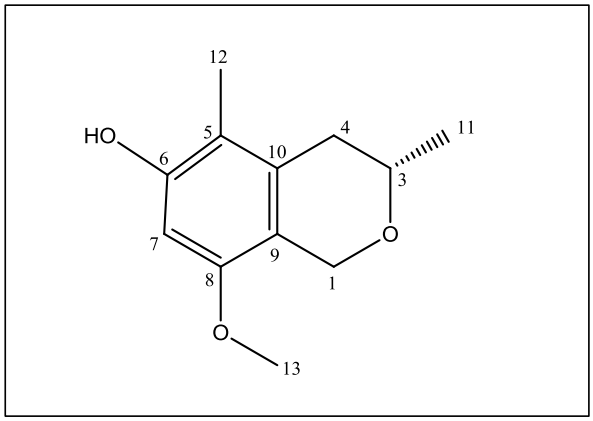


Compound (**23**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. as a white solid (39.7 mg). It showed UV absorbance maxima at λ_{max} (MeOH) 218.4, 228.5 and 283.6 nm. Positive and negative ESI-MS showed molecular ion peaks at m/z 209.1 $[M+H]^+$ (base peak) and m/z 207.4 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 208 g/mol. The difference in the molecular weight between compound (**23**) and the previous compound (**22**) is 16 amu which indicates the less of a hydroxyl group in compound (**23**).

The ^{1}H NMR spectrum (Table 3.15) for compound (**23**) is closely related to the previous compound (**22**). It displayed also characteristic signals attributable to two methyl protons CH_3-11 and CH_3-12, appearing at δ_H 1.32 [d (*J*=6.2 Hz)] and 1.99, *s*, respectively, upfield geminally-coupled methylene protons at [δ_H 2.33 [dd (*J*=16.5, 10.7 Hz)] and 2.63 [dd (*J*=16.5, 3.0 Hz)] for CH_2-4, deshielded methylene protons at [δ_H 4.50 and 4.79 [d, (*J*=14.9 Hz)] for CH_2-1, a methine signal at δ_H 3.69 for H-3, a singlet at δ_H 3.68 ppm for OCH_3-13, and an aromatic singlet at δ_H 6.28, *s* attributed for H-7. The presence of an extra upfielded methylene signal at δ_H 2.33 and 2.63 ppm in (**23**) and the lack of the oxygenated methine signal CH(4) which is present in (**22**) confirm the removal of the 4-OH group in (**23**).

The ^{1}H-^{1}H-COSY (Table 3.15) showed two spin systems. The first one is CH_2(1) and the second one is CH_3(11)CH(3)CH_2(4). The HMBC spectrum (Figure 3.26) showed strong correlations from the CH_3-12 protons at δ_H 1.99,(*s*) to C-5 at δ_C (114.7), C-6 at δ_C (155.3), and C-10 at δ_C (135.0) securing its position at C-5. Furthermore, the aromatic proton H-7 at δ_H 6.28 ppm showed correlations to carbons at δ_C 10.4, 114.7, 115,155.3 and 155.8 assigned to CH_3-12, C-5, C-9, C-6 and C-8, respectively which supported the assignment of the planar structure of (**23**).

The $[\alpha]_D$ is in full agreement with the literature (Guochun *et al.*, 2004). Thus compound (**23**) was identified as (3*S*)-6-hydroxy-8-methoxy-3,5-dimethylisochroman, the confirmation of the aforementioned data based on the comparison with the reported literature (Masuma *et al*., 1994; Guochun *et al.*, 2004; El-Neketi *et al*., 2013), in which (**23**) has been previously isolated from different *Penicillium* species.



Structure for (3*S*)-6-hydroxy-8-methoxy-3, 5-dimethylisochroman (**23**)

Table 3.15: ^{1}H, ^{13}C NMR, COSY, ROESY and HMBC data of compound (**23**) at 600 (^{1}H) and 150 (^{13}C) MHz:

#	Compound (**23**)						
	δ_H(MeOD)	COSY	ROESY	HMBC	δ_C(MeOD)	δ_H($CDCl_3$)[a]	δ_C($CDCl_3$)[a]
1a	4.50, *d* (14.9)	1b	1b, 3	3, 8,9,10	65.8	4.57, *d* (15.2)	64.6
b	4.79, *d* (14.9)	1a	1a	3, 8,9,10		4.89, *d* (15.2)	
2							
3	3.69, *m*	4a, 4b, CH_3-11	4a, 4b, CH_3-11		72.1	3.74, *m*	70.6
4a	2.33, *dd* (16.5, 10.7)	3, 4b	1a, 4b, CH_3-11, CH_3-12	3, 5,9,10, CH_3-11	34.9	2.42, *dd* (16.5, 10.6)	34
b	2.63, *dd* (16.5, 3.0)	3, 4a	1a, 3, 4a, 11-CH_3, CH_3-12	3,5,9,10		2.61, *dd* (16.5, 2.3)	
5					114.7		112.9
6					155.3		152.7
7	6.28, *s*		OCH_3-13	5, 6, 8, 9, CH_3-12	97	6.23, *s*	96.2
8					155.8		153.9
9					115		115.4
10					135		134.1
CH_3-11	1.32, *d* (6.2)	3	3, 4a, 4b	3, 4, 10	22.7	1.38, *d* (6.3)	21.7
CH_3-12	1.99, *s*		4a, 4b	5, 6,7,10	10.4	2.04, *s*	10
OCH_3-13	3.68, *s*		7	7, 8	55.5		

a) Masuma *et al*., 1994.

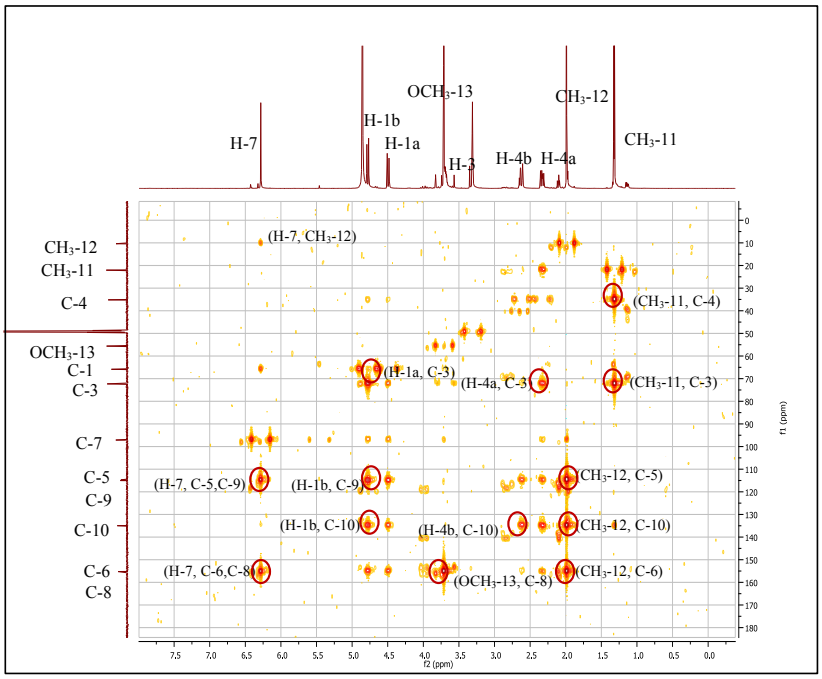


Figure 3.26: HMBC spectrum of compound (**23**)

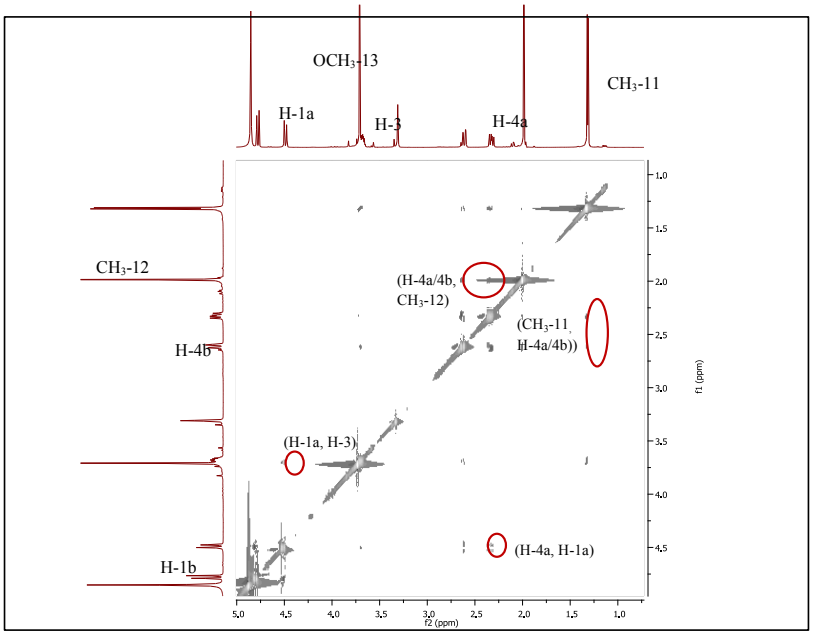
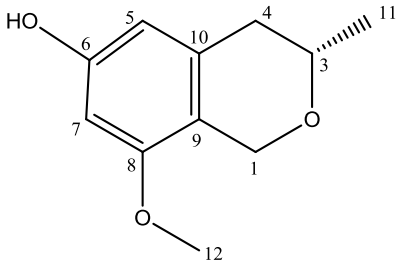
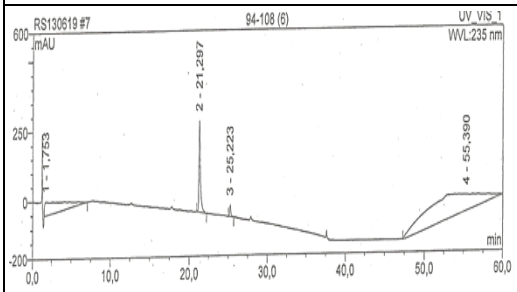


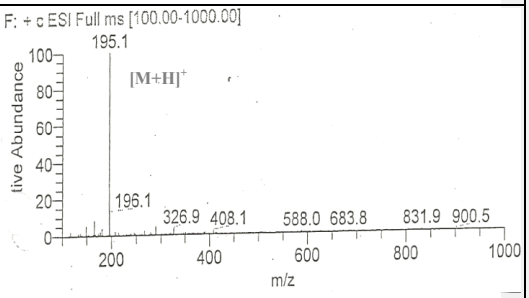
Figure 3.27: Expansion of ROESY spectrum of compound (**23**)

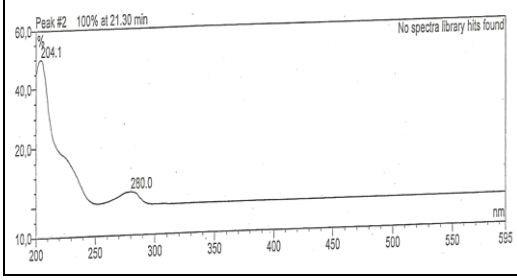
3.2.3. (+)-Arohynapene D (24, new compound)

(+)-Arohynapene D	
Synonym(s)	(3*S*)-6-Hydroxy-8-methoxy-3-methylisochroman
Sample code	94-108 (6)
Biological source	*Penicillium* sp.
Sample amount	3.4 mg
Physical Description	Brown solid
Molecular Formula	$C_{11}H_{14}O_3$
Molecular Weight	194.1 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 45 (*c* 0.1, MeOH)
Retention time HPLC	21.29 min (standard gradient)









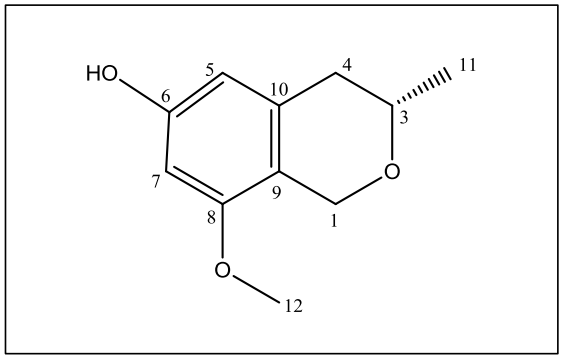
(-)-ESI-MS: No ionization

Compound (**24**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. in the form of a brown solid (3.4mg). It exhibited UV absorbances at λ_{max} (MeOH) 204.1 and 280 nm. The HRESI-MS exhibited a strong peak at *m/z* 195.1017$[M+H]^+$ indicating a molecular formula of $C_{11}H_{14}O_3$ (calculated 195.1016, Δ 0.0001). The molecular weight of (**24**) differes from that of (**23**) by 14 amu indicating the removal of a methyl group in (**24**).

Similar to (**23**) the ^{1}H NMR spectrum (Table 16) showed two geminally coupled methylene protons at [δ_H 2.43 [dd, (*J*=16.3, 5.5 Hz)] and 2.55 [dd, (*J*=16.3, 2.5 Hz)] for CH_2-4, and a deshielded one at [δ_H 4.40 and 4.62 ppm [each, d, (*J*=15.0 Hz)] for CH_2-1, a methine signal at δ_H 3.65 ppm for H-3, an exchangeable proton at δ_H 9.19 ppm appearing as a singlet for 6-OH, a singlet at δ_H 3.67 ppm for OCH_3-12, and only one methyl group at δ_H 1.20 [d, (*J*=6.12 Hz)] for CH_3-11. The *meta*- coupled aromatic protons at δ_H 6.10 and 6.20 ppm (each doublet, *J*=1.9 Hz) for H-5 and H-7, respectively confirmed the demethylation of (**23**). The ^{1}H-^{1}H COSY spectrum (Table 16) of (**24**) showed three spin systems. The first one is CH_2(1), the second one is CH_3(11)CH (3)CH_2(4) and the third one is CH(5)CH(7).

The HMBC spectrum (Figure 3.28) showed a strong correlation from OCH_3-12 at δ_H 3.67 to C-8 (δ_C 155.9) securing its position at C-8. Further inspection of the HMBC spectrum revealed correlations from CH_2-4 to C-5 (δ_C106.6), from CH_2-1 to C-8 (δ_C 155.9) and from H-5 to C-4 (δ_C 35.5). The ROESY experiment (Figure 3.29) showed that the CH_3-11 showed a cross peak with the CH_2-4 at δ_H 2.43 and 2.55 ppm and H-3 at δ_H 3.65 ppm, OCH_3-12 correlates with H-7 at δ_H 6.20 and 6-OH with H-7 and H-5 at δ_H 6.10 in the same spectrum confirming the location of the substituents.

The obtained UV, ^{1}H, ^{13}C NMR and mass spectral data were found to be identical with the published data for 6-hydroxy-8-methoxy-3-methylisochroman (arohynapene D) previously reported from *Penicillium* sp. FO-2295 (Noriko *et al.*, 1994) except the difference in the configuration as approved from the comparison of the $[\alpha]_D$ values of both compounds. The absolute configuration of C-3 were proposed to be *S*, by comparison $[\alpha]_D$ value of (**24**) with the literature which was + 116.4 (MeOH) (Guochun *et al.*, 2004). This led to the conclusion that (**24**) is the enantiomer of (-)-arohynapene D and it was thus assigned as the previously unreported (3*S*)-6-hydroxy-8-methoxy-3-methylisochroman.



Structure of (+)-arohynapene D (**24**)

Table 16: ^{1}H, ^{13}C NMR, HMBC and ROESY data of compound (**24**) at 600 (^{1}H) and 150 (^{13}C) MHz:

	Compound (**24**)					Compound (**24**)[a]		
#	δ_H(DMSO)	COSY	ROESY	HMBC	δ_C(DMSO)	δ_H($CDCl_3$)	HMBC	δ_C($CDCl_3$)
1a	4.40, *d* (15.0)	1b	1b,3	3,8,9,10	67.8	4.57,*dt* (15.0, 2.0)	3,8,9	64.4
1b	4.62, *d* (15.0)	1a	1a	3,8,9,10		4.85,*d* (15.0)		
2								
3	3.65,*m*	4a,CH_3-11	1a,4a,4b, CH_3-11		70.0	3.73,*m*	1	70.2
4a	2.43,*dd* (16.3 ,5.5)	3,4b	1a,1b.3,4b,5	3,10, CH_3-11	35.5	2.60,*m*	3,5,9, 10,CH_3-11	35.8
4b	2.55,*dd* (16.3 ,2.5)	4a	3,4a,5	5,9,10				
5	6.10,*d* (1.9)		4a,4b,6-OH	4,6,7,9	106.6	6.10,*d* (2.0)	4,6,7,9	106.5
6					156.6			154.8
7	6.20,*d* (1.9)		6-OH, OCH_3-12	5,6,9,	96.6	6.23,*d* (2.0)	5,6,8,9	96.2
8					155.9			156.5
9					113.1			115.6
10					135.2			135.7
CH_3-11	1.20,*d* (6.12)	3	3,4a,4b	3,4	21.2	1.33,*d* (6.0)	3,4	21.4
OCH_3-12	3.67,*s*		7	8	56.2	3.75,*s*	8	55.1
6-OH	9.19,*s*		5,7	5,6,7				

a) Noriko *et al.*, 1994.

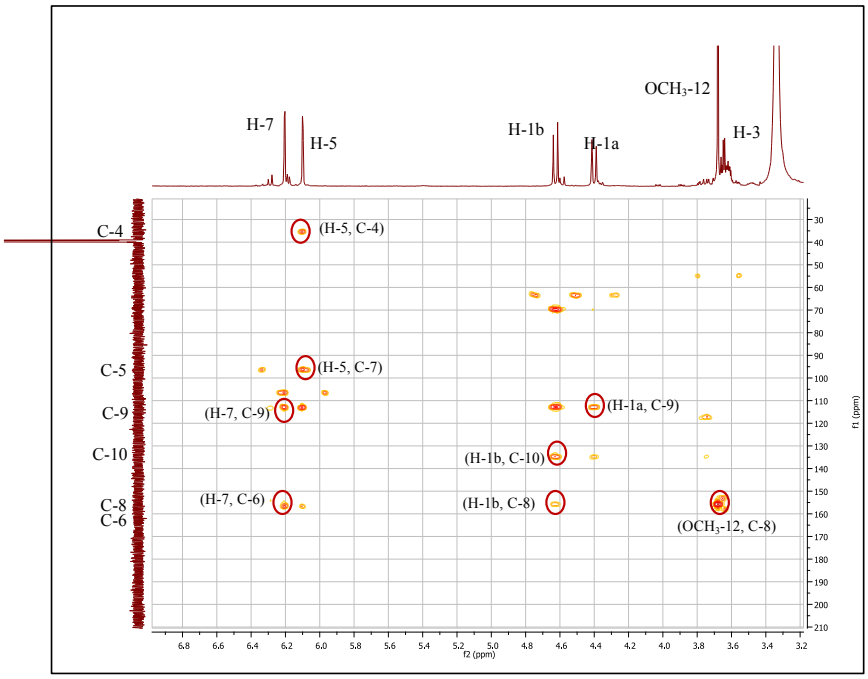


Figure 3.28: Expansion of HMBC spectrum of compound (**24**)

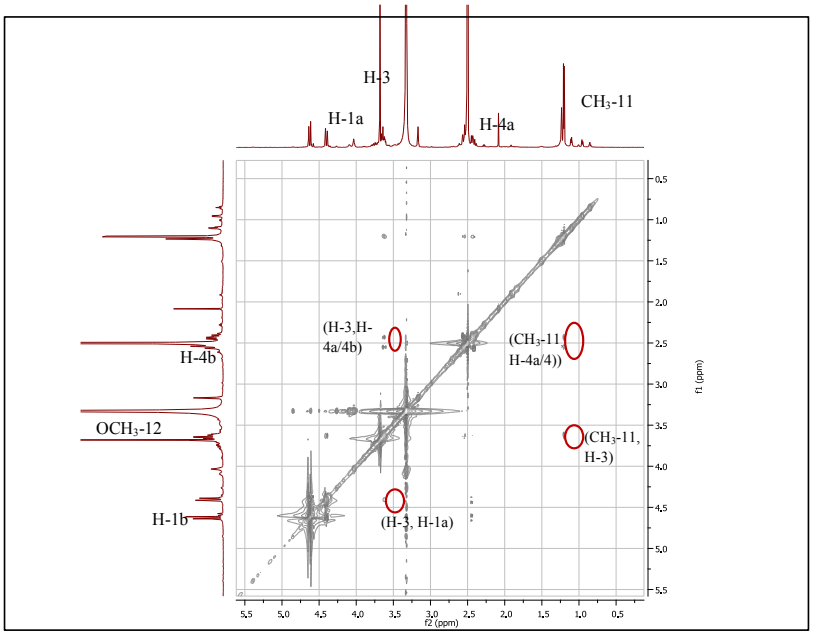
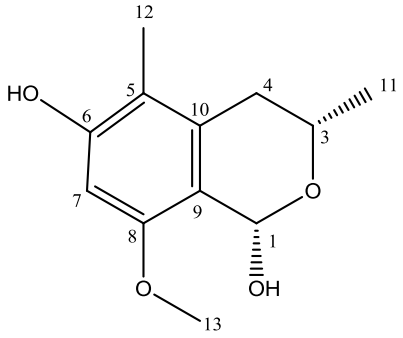
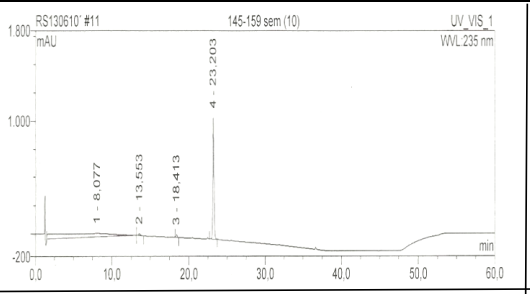


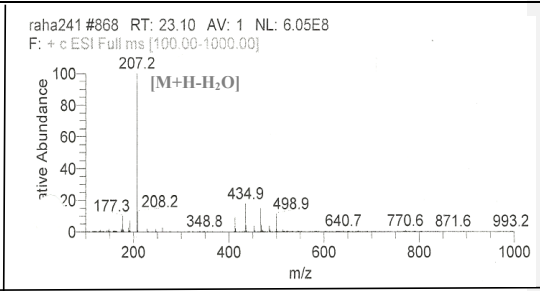
Figure 3.29: Expansion of ROESY spectrum of compound (**24**)

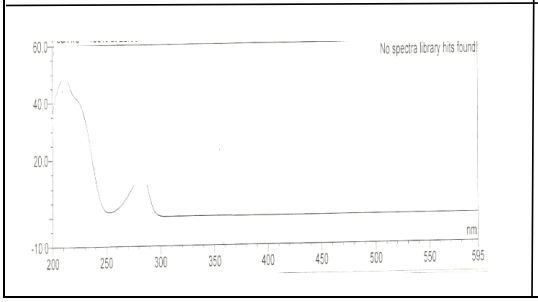
3.2.4. (1*S*, 3*S*)-1, 6-Dihydroxy-3, 5-dimethyl -8-methoxyisochroman (25, known compound)

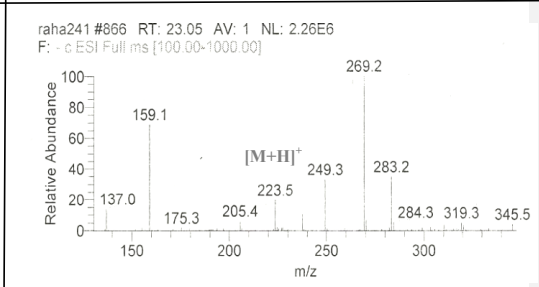
(1*S*, 3*S*)-1, 6-Dihydroxy-3, 5-dimethyl -8-methoxyisochroman	
Synonym(s)	1, 6-Dihydroxy-3, 5-dimethyl -8-methoxyisochroman
Sample code	145-159 (10)
Biological source	*Penicillium* sp.
Sample amount	5.5 mg
Physical Description	Orange brown solid
Molecular Formula	$C_{12}H_{16}O_4$
Molecular Weight	224.2 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 24.6 (*c* 0.1, MeOH)
Retention time HPLC	23.2 min (standard gradient)









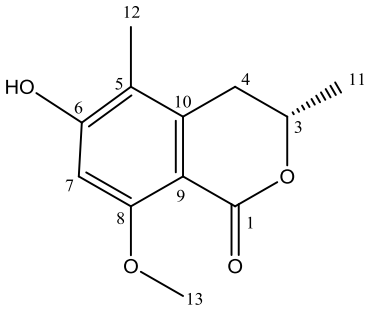


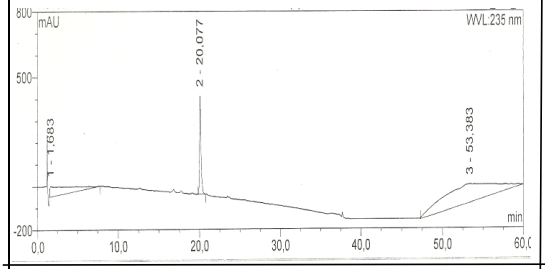
Compound (**25**) was isolated from the EtOAc extract of the solid rice cultures of *Penicillium* sp. as an orange brown solid (5.5 mg). It displayed UV absorbance maxima at λ_{max} (MeOH) 211.4 and 283.1 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 207.2 for $[M\text{-}H_2O+H]^+$ (base peak) and 223.5 $[M\text{-}H]^-$ (base peak) respectively, revealing a molecular weight of 224.2 g/mol.

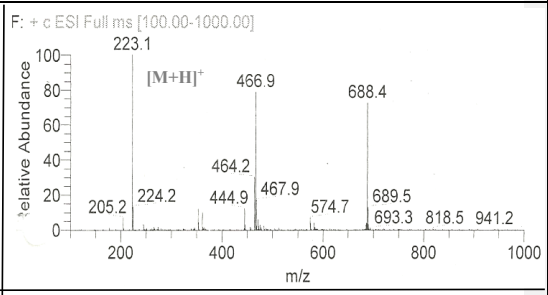
Analysis of ^{1}H NMR spectrum (Table 3.17) revealed the presence of two methyl groups at δ_H 1.34 [d (*J*=6.2 Hz)] and 1.99 ppm assigned for CH_3-11 and CH_3-12, respectively, a methylene signal at [δ_H 2.27 [dd (*J*=16.8, 11.5 Hz)] and 2.64 [dd (*J*=16.8, 3.4 Hz)]] for CH_2-4, a methoxy group at δ_H 3.75 ppm for OCH_3-13, two oxygenated methine signals at δ_H 5.47 ppm for H-1, and at δ_H 4.20 ppm for H-3 and one aromatic signal at δ_H 6.33 ppm for H-7. The ^{1}H-^{1}H COSY spectrum of compound (**25**) (Figure 3.31) showed one spin system, which is CH_3(11)CH(3)CH_2(4). The HMBC spectrum (Figure 3.30) confirmed the structure by strong correlation from the methoxy proton OCH_3-13 at δ_H 3.75 to C-8 (δ_C 156.3) confirming its position at C-8. Further inspection of the HMBC spectrum revealed correlations from H-1 at δ_H 5.47 to OCH_3-13 (δ_C 56.3), C-3 (δ_C 64), C-9 (δ_C 114.8), C-10 (δ_C 135.8) and C-8 (δ_C 156.3) and from the methyl proton CH_3-12 at δ_H 1.99 ppm to C-5 (δ_C 113.5), C-9 (δ_C 114.8), C-10 (δ_C 135.8) and C-6 (δ_C 155) which supported the assignment of the planar structure of (**25**). Thus, compound (**25**) was identified as the known 1,6-dihydroxy-3,5-dimethyl -8-methoxyisochroman, by comparing its UV, ^{1}H NMR, $[\alpha]_D$ and mass spectral data with the published data (Guochun *et al.*, 2004). This compound was originally isolated from *Penicillium expansum* (Guochun *et al.*, 2004).

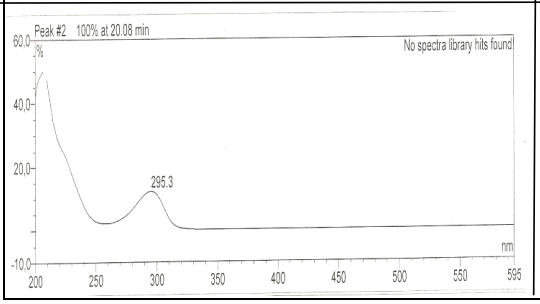
3.2.5. (3S)-6-Hydroxy-8-methoxy-3, 5-dimethyl-3,4-dihydroisocoumarin (26, known compound)

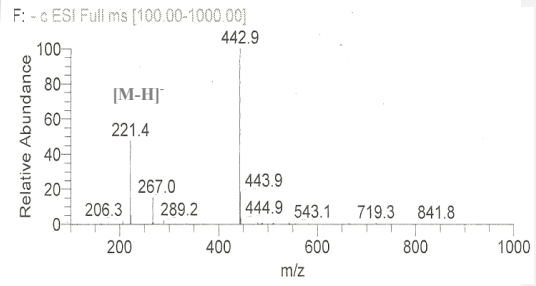
(3*S*)-6-Hydroxy-8-methoxy-3,5-dimethyl-3,4-dihydroisocoumarin	
Synonym(s)	(3*S*)-6-Hydroxy-8-methoxy-3,5-dimethyl-3,4-dihydroisocoumarin
Sample code	145-159 (6)
Biological source	*Penicillium* sp.
Sample amount	4.3 mg
Physical Description	brown solid
Molecular Formula	$C_{12}H_{14}O_4$
Molecular Weight	222.09 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 102 (*c* 0.118, MeOH)
Retention time HPLC	20.07 min (standard gradient)











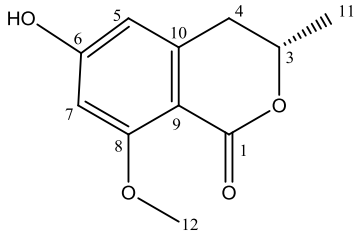
Compound (**26**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. in the form of brown solid (4.3 mg). Its UV spectrum showed λ_{max} (MeOH) at 206.9 and 295.3 nm. Positive and negative ESIMS showed molecular ion peaks at *m/z* 223.1 $[M+H]^+$ (base peak) and *m/z* 221.4 $[M-H]^-$ (base peak), respectively indicating a molecular weight of 222 g/mol which is less by 2 amu than (**25**).

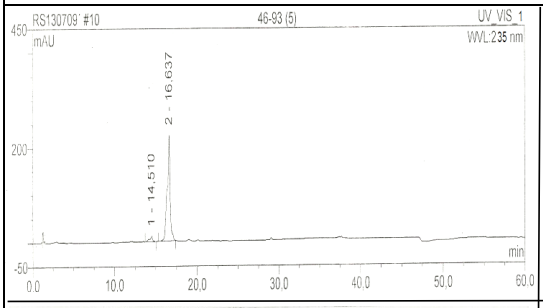
The ^{1}H NMR spectrum (Table 3.17) for compound (**26**) is closely related to the previous compound (**25**). It displayed also characteristic signals attributable to two methyl protons CH_3-11 and CH_3-12, resonating at δ_H 1.42 [d (*J*=6.2 Hz)] and δ_H 2.02 ppm (*s*), respectively, a geminally-coupled methylene protons at δ_H 2.68 [dd (*J*=7.6, 15.3 Hz)] and 3.21 [dd (*J*=8.6, 15.3 Hz)] for CH_2-4, a multiplet for a methine signal at δ_H 4.61 ppm for H-3, a methoxy signal at δ_H 3.75 ppm for OCH_3-13 and an aromatic singlet at δ_H 6.29(*s*) for H-7. Comparison of the ^{13}C-NMR of (**26**) (Table 3.18) with that of (**25**) exhibited an extra carbonyl signal resonating at δ_C 170.6 in (**26**) suggesting the possible oxidation of the secondary alcoholic group (CH(1)OH) in (**25**) to this carbonyl group in (**26**) and confirming the difference in the molecular weight between (**25**) and (**26**). The ^{1}H-^{1}H- COSY spectrum of compound (**26**) (Figure 3.33) showed a spin system CH_3(11)CH(3)CH_2(4).

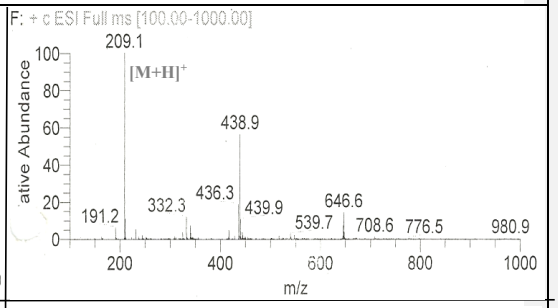
The HMBC spectrum (Figure 3.32), showed correlations from the methoxy group to C-8 at (δ_C 143.2) which secures its position at C-8. In addition, the CH_3-12 correlates to C-5 at (δ_C 113.7), C-6 at (δ_C 150) and C-10 at (δ_C 129.3) confirming its position at C-5. Comparison of ^{1}H, ^{13}C NMR, HMBC and COSY data (Table 3.17 and 3.18) with the published data (Sheng *et al*., 1990) revealed that compound (**26**) is identified as (3*S*)-6-hydroxy-8-methoxy-3,5-dimethyl-3,4-dihydroisocoumarin, wherein the absolute configuration at C-3 position was determined by comparing its optical rotation $[\alpha]_D^{20}$ + 102 (MeOH) with that of the same compound $[\alpha]_D^{20}$ + 106 (MeOH) which was isolated from the *Penicillium citreo-viride B* (Sheng *et al*., 1990).

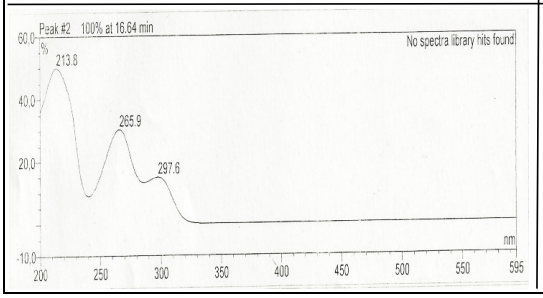
3.2.6. (3S)-6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin (27, new compound)

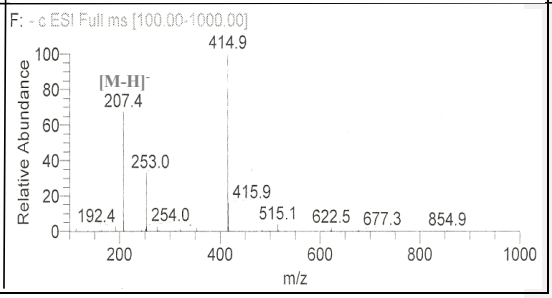
(3*S*)-6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin	
Synonym(s)	6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin
Sample code	46-93 (5)
Biological source	*Penicillium* sp.
Sample amount	1.7 mg
Physical Description	Orange solid
Molecular Formula	$C_{11}H_{12}O_4$
Molecular Weight	208.07 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 51 (*c* 0.32, $CHCl_3$)
Retention time HPLC	16.63 min (standard gradient)









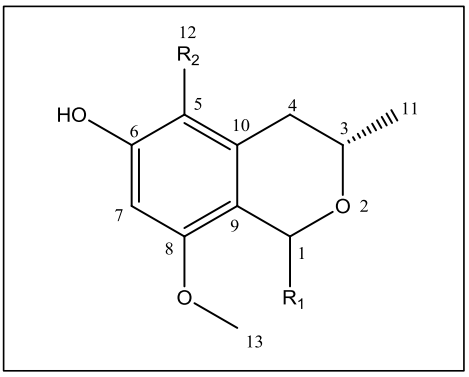


Compound (**27**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. It was isolated as an orange solid (1.7 mg). The UV absorption data λ_{max} (MeOH) at 213.8, 265.9 and 297.6 indicated that it is isocoumarin congener (**26**). The ESI-MS showed molecular ion peaks at *m/z* 209.1 $[M+H]^+$ (base peak) and *m/z* 207.4 $[M-H]^-$ (base peak), respectively indicating a molecular weight of 208.07g/mol. The molecular weight of (**27**) is less than (**26**) by 14 amu indicating a lack of one methyl group in (**27**). The HRESI-MS exhibited a prominent peak at *m/z* 209.0808 $[M+H]^+$ indicating a molecular formula of $C_{11}H_{12}O_4$ (calculated 209.0808, Δ 0.0000).

The ^{1}H- NMR spectrum (Table 3.17) exhibited signals similar to compound (**26**), except for the absence of CH_3-12 in (**26**) which is replaced by an aromatic proton at δ_H 6.29 ppm (H-5) (*meta*-coupled with H-7 at δ_H 6.41 ppm). In addition, a methoxy signal at δ_H 3.83 ppm assigned for OCH_3-12 was observed. Similar to (**26**), the aliphatic moiety showed a methyl group at δ_H 1.41 ppm [d (*J*=6.2 Hz)] for CH_3-11, methylene protons at δ_H 2.79 [d (*J*=16.0 Hz)] and 2.85 [dd (*J*=16.0, 3.06 Hz)] for CH_2-4 and a multiplet assigned for methine group at δ_H 4.51 ppm for H-3.

The HMBC spectrum (Figure 3.34) showed a strong correlation from the OCH_3-12 at δ_H 3.83 to C-8 (δ_C 164.6) securing its position at C-8. Further inspection of the HMBC spectrum revealed the methylene protons CH_2-4 to C-10 (δ_C 146.2), the methyl group CH_3-11 to C-3 (δ_C 75.1) and C-4 (δ_C 36.5) and the aromatic proton H-7 to C-5 (δC 105.2) and C-8 at (δC 164.6) which supported the assignment of the planar structure as shown.

The relative configuration of (**27**) was obtained from the ROESY spectrum (Figure 3.35). The ROESY experiment showed cross peaks from CH_3-11 to CH_2-4 at δ_H 2.79 and 2.85 ppm and H-3 at δ_H 4.51 ppm and from OCH_3-12 to H-7 at δ_H 6.41 ppm confirming the location of these substituents. By comparison of the previous data including optical rotation value of (**27**) it was found that the $[\alpha]_D^{20}$ +51 (*c* 0.32, $CHCl_3$) while that in the literature $[\alpha]_D^{20}$ –46.5 (*c* 0.043, $CHCl_3$) (Dethoup *et al.*, 2007). Thus, compound (**27**) was identified as an enantiomer of (-)-6-hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin. Hence, (**27**) was assigned as the new (3*S*)-6-hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin.



#	Compound	R_1	R_2
25	1, 6-Dihydroxy-3, 5-dimethyl -8-methoxyisochroman	OH	CH_3
26	6-Hydroxy-8-methoxy-3, 5-dimethyl-3,4-dihydroisocoumarin	=O	CH_3
27	6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin	=O	-H

Table3.17: ^{1}H NMR & HMBC of compound **(25)**,compound (**26**) & compound (**27**) at 600 MHz:

#	Compound (**25**)			Compound (**26**)			Compound (**27**)			
	δ_H (MeOH)	HMBC	δ_H ($CDCl_3$)[a]	δ_H (MeOH)	HMBC	δ_H ($CDCl_3$)[b]	δ_H(MeOH)	ROESY	HMBC	δ_H ($CDCl_3$)[c]
1	5.47,*s*	3,8,9,10, OCH_3-13	5.41,*s*							
2										
3	4.20,*m*		4.15,*m*	4.61,*m*		4.47,*m*	4.51,*m*	4a,4b,, CH_3-11		4.41,*m*
4a	2.64,*dd* (16.8,3.4)	5,10,11	2.57,*dd* (17,3.5)	3.21,*dd* (8.6.,15.3)	10	2.91,*dd* (2.7,16.4)	2.85,*dd* (16.0,3.06)	3,4b	10	2.86,*dd* (16,3.1)
b	2.27,*dd* (16.8,11.5)	3,5,6,10, 11	2.20,*dd* (16.7,11.6)	2.68,*dd* (7.6,15.3)	10, CH_3-11	2.66,*dd* (11.5,16.4)	2.79,*d* (16.0)	3,4a,5		2.27,*dd* (16,10.9)
5							6.29,*s*	4b		6.26,*s*
6										
7	6.33,*s*	5,8,9	6.26,*s*	6.29,*s*	8,9	6.43,*s*	6.41,*s*	OCH_3-12	5,8	6.37,*s*
8										
9										
10										
CH_3-11	1.34,*d*(6.2)	3,4,10	1.27,*d* (6.1)	1.42,*d* (6.2)	3,4	1.49,*d* (6.3)	1.41,*d* (6.2)	3,4a,4b	3,4	1.32,*d* (6.2)
CH_3-12	1.99,*s*	1,5,6,7,9	1.93,*s*	2.02,*s*	5,6,10	2.10,*s*				
OCH_3-12							3.83,*s*	7	8	3.74,*s*
OCH_3-13	3.75,*s*	8	3.68,*s*	3.75,*s*	1,8	3.82,*s*				
6-OH										10.52,*brs*

a) Guochun *et al.*, 2004. b) Sheng *et al*., 1990. C) Dethoup *et al.*, 2007.

Table 3.18: ^{13}C NMR of compound (**25**), compound (**26**) and compound (**27**) at 150 MHz:

#	Compound (**25**)		Compound (**26**)	Compound (**27**)	
	δ_C (MeOH)[b]	δ_C ($CDCl_3$)[a]	δ_C (MeOH)[b]	δ_C (MeOH)[b]	δ_C ($CDCl_3$)[c]
1	95.6	88.5	170.6	165.1	162.8
2					
3	64	62.6	80.9	75.1	72.9
4a	33.7	30.9	38.2	36.5	35.5
b					
5	113.5	112.8	113.7	105.2	106.2
6	155	154.2	150		161.3
7	96.9	96.9	100.5		98.4
8	156.3	155	143.2	164.6	162.9
9	114.8	114.5	114.4		104.6
10	135.8	135.2	129.3	146.2	144
CH_3-11	21.4	21.6	22		20.4
CH_3-12	10.8	10.1	10.1		
OCH_3-12				55.6	
OCH_3-13	56.3	54.8	56.4		

a) Guochun *et al.*, 2004.
b) Derived from HMBC spectrum
c) Dethoup *et al.*, 2007.

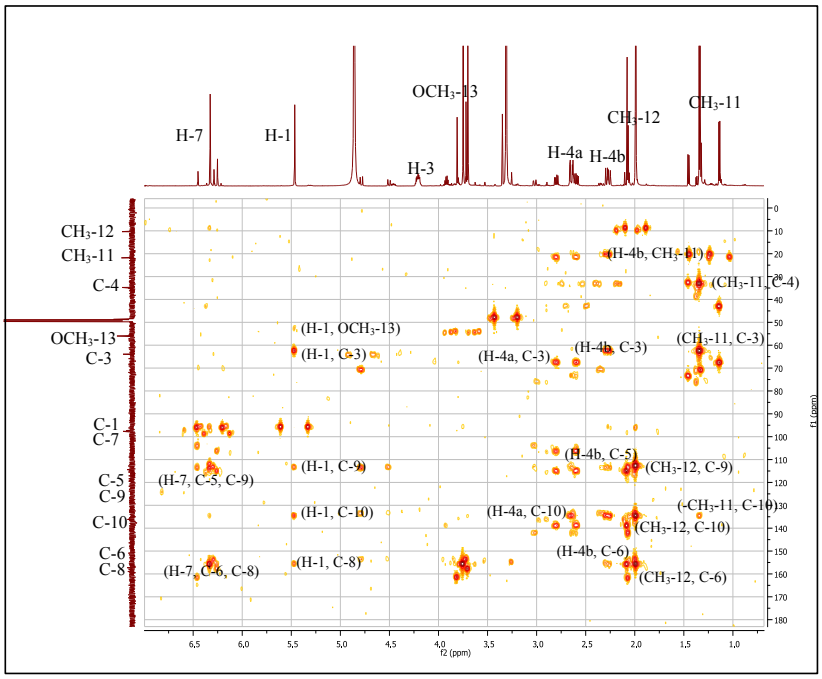


Figure 3.30: HMBC spectrum of compound (**25**)

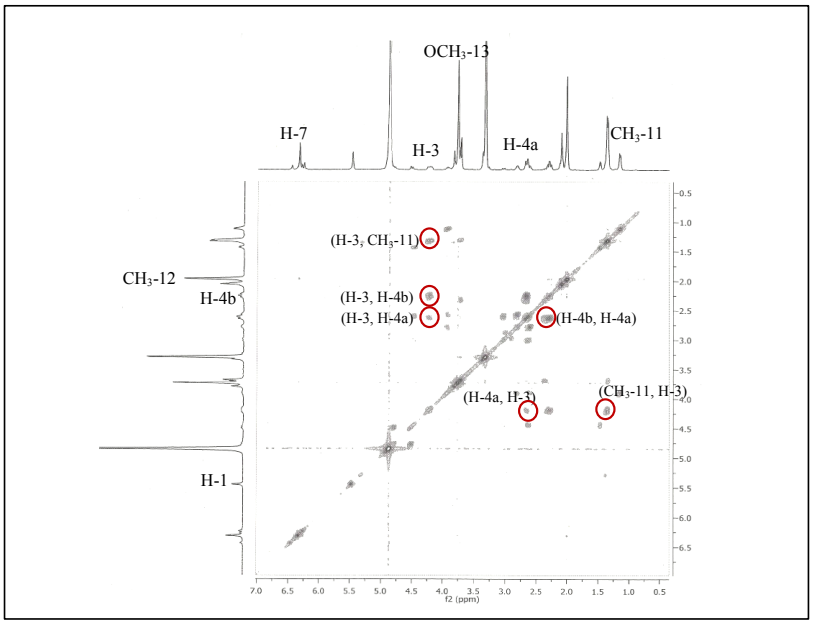


Figure 3.31: ^{1}H-^{1}H-COSY spectrum of compound (**25**)

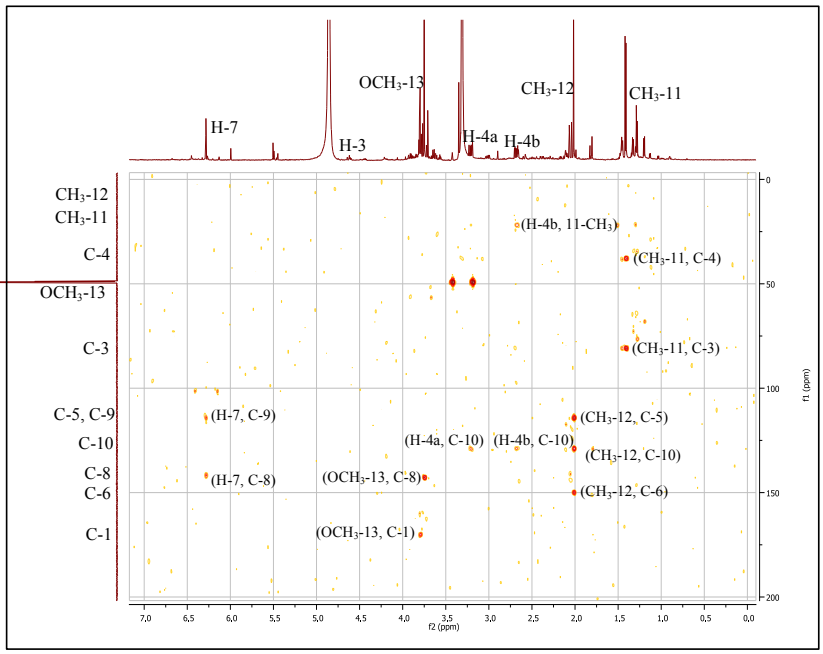


Figure 3.32: HMBC spectrum of compound (**26**)

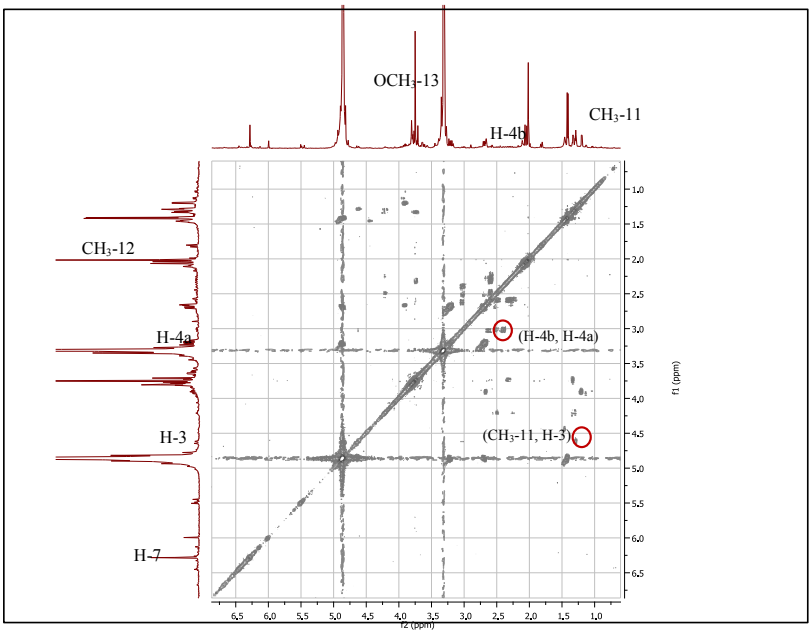


Figure 3.33: ^{1}H-^{1}H-COSY spectrum of compound (**26**)

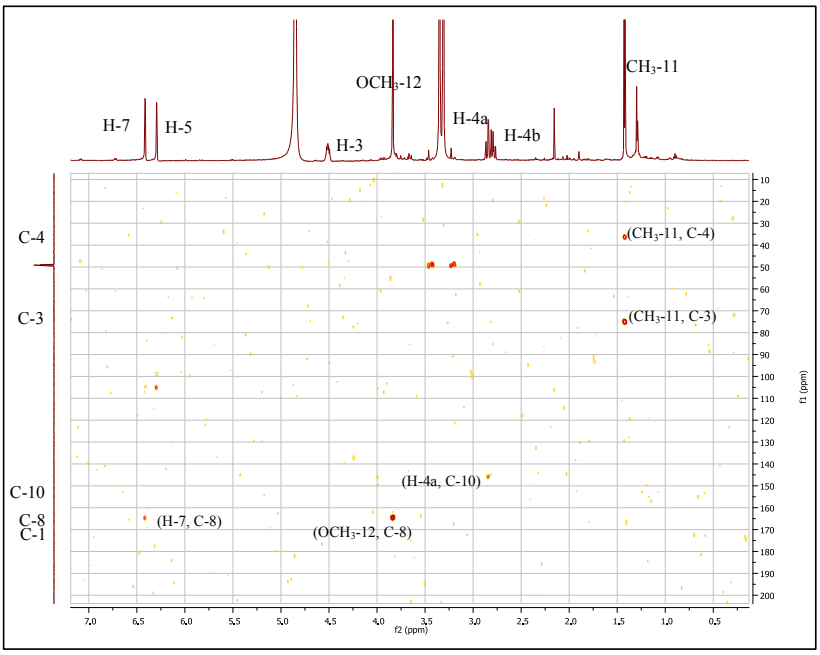


Figure 3.34: HMBC spectrum of compound (**27**)

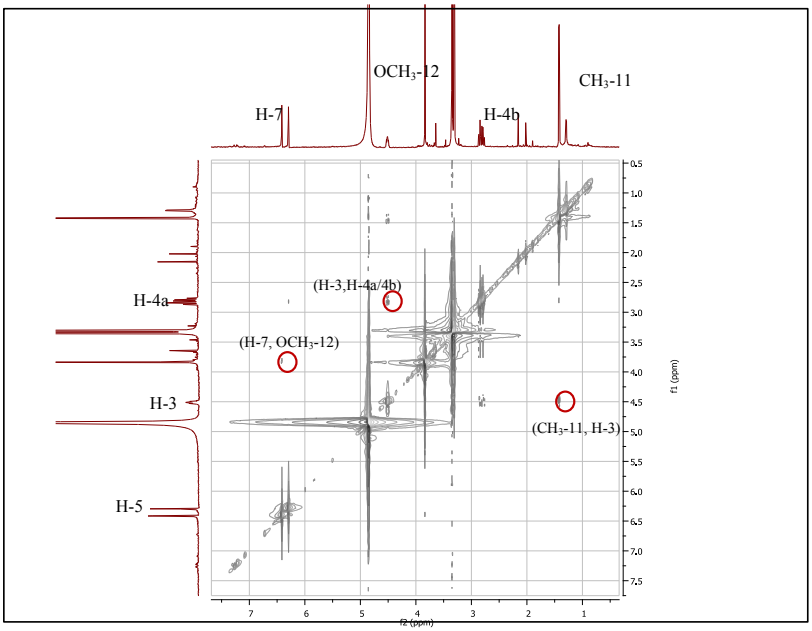
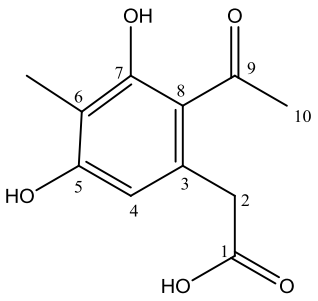
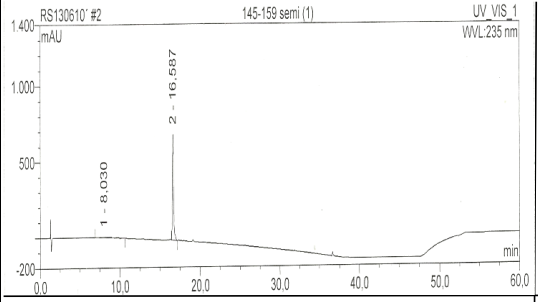


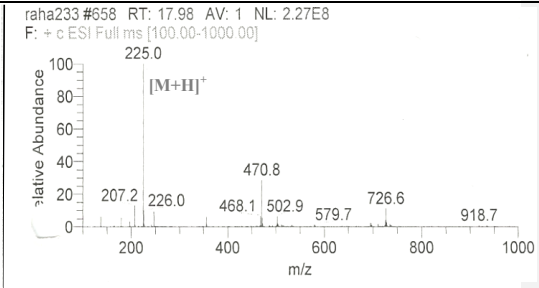
Figure 3.35: ROESY spectrum of compound (**27**)

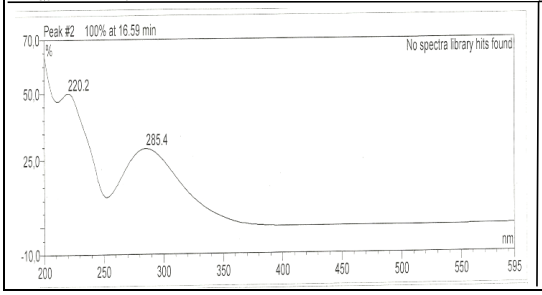
3.2.7. 6-Methylcurvulinic acid (28, known compound)

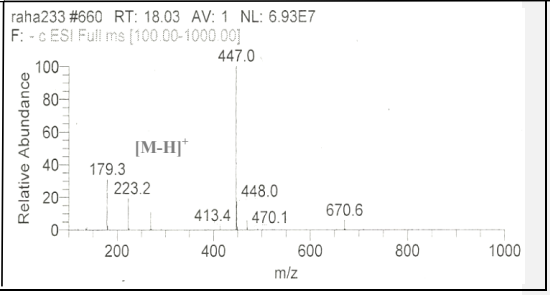
6-Methylcurvulinic acid	
Synonym(s)	6-Methylcurvulinic acid
Sample code	145-159 (1)
Biological source	*Penicillium* sp.
Sample amount	6.5 mg
Physical Description	White amorphous solid
Molecular Formula	$C_{11}H_{12}O_5$
Molecular Weight	224.07 g/mol
Retention time HPLC	16.58 min (standard gradient)







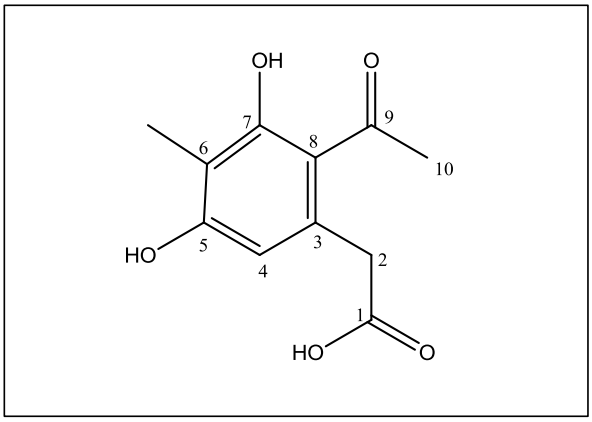




Compound (**28**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. in the form of a white amorphous solid (6.5 mg). It exhibited UV absorbances at λ_{max} (MeOH) 220.2 and 285.4 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 225 $[M+H]^+$ (base peak) and *m/z* 223.2 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 224 g/mol.

The ^1H NMR spectrum (Table 3.19) indicated the presence of two methyl groups each appearing as a singlet; at δ_H 2.05 and 2.57 ppm for CH_3-6 and CH_3-10, respectively, methylene protons appearing as singlets at δ_H 3.77 ppm for CH_2-2, and an aromatic singlet at δ_H 6.29 ppm for H-4. The ^{13}C NMR spectrum displayed 11 carbon atoms, *viz*, two methyl carbons (C-10 and C-6), a methylene carbon (C-2), six aromatic carbons and two carbonyl carbons (C-1 and C-9), respectively.

The HMBC spectrum (Table 3.19 and Figure 3.36) showed a strong correlation from CH_3-6 at δ_H 2.05 to C-6 (δ_C 111.6), C-5 (δ_C 160.3), and C-7 (δ_C 161) securing its position at C-6. Further inspection of the HMBC spectrum showed correlations from CH_3-10 to C-9 (δ_C 206.1), and C-8 (δ_C 118.5) confirming that an acetyl group is attached to C-8. Furthermore, the methylene protons CH_2-2 at δ_H 3.77 displayed correlations to C-4 (δ_C 112.8), C-8 (δ_C 118.5), C-3 (δ_C 135.1) and C-1 (δ_C 175.2). Thus, compound (**28**) was identified as the known 6-methylcurvulinic acid by comparison of UV, ^1H, ^{13}C NMR and mass spectral data with the published data (El-Neketi *et al*., 2013).



Structure of 6-methylcurvulinic acid (**28**)

Table 3.19: ^{1}H, ^{13}C NMR and HMBC data of compound (**28**) at at 600 (^{1}H) and 150 (^{13}C) MHz:

	Compound (**28**)			Compound (**28**)[a]		
#	δ_H(MeOD)	δ_C(MeOD)[b]	HMBC	δ_H(CD_3OD)	δ_C(CD_3OD)	HMBC
1		175.2			175.2	
2	3.77,*s*	41.8	4,8,3,1	3.76,*s*	41.4	4,8,3,1
3		135.1			134.9	
4	6.29,*s*	112.8	6-CH_3,2,6,8,5	6.29,*s*	112.3	2,6,8,5
5		160.3			160.8	
6		111.6			111.8	
7		161			161.3	
8		118.5			118.5	
9		206.1			206.2	
CH_3-10	2.57,*s*	32.3	8,9	2.57,*s*	32	8,9
CH_3-6	2.05, *s*	8	6, 5,7	2.04,*s*	8	6 ,5,7

a) El-Neketi *et al*., 2013.
b) Derived from HMBC spectrum

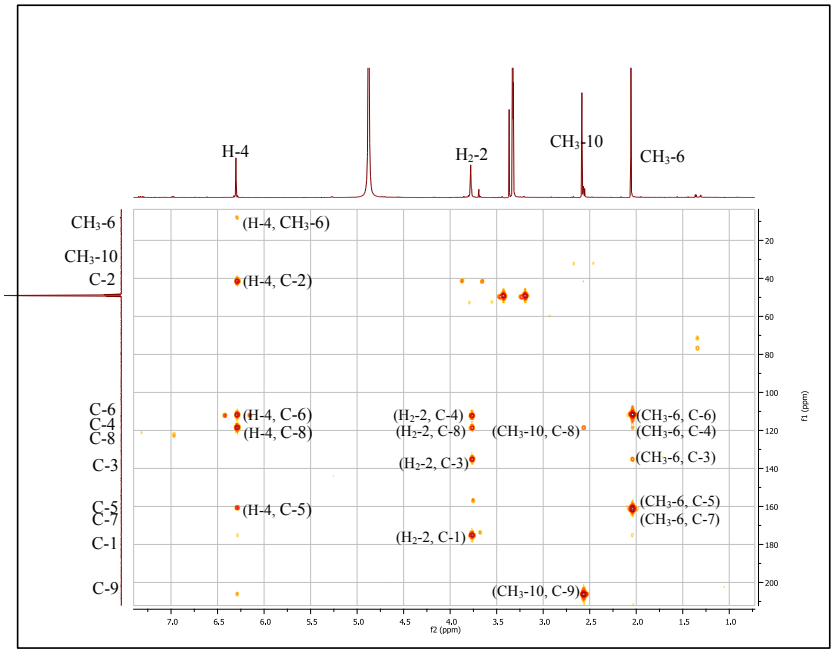
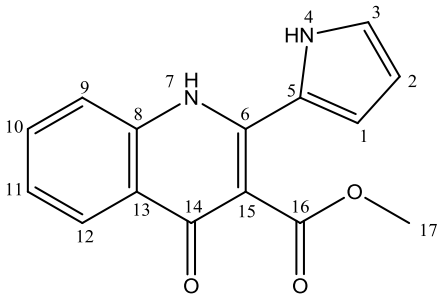
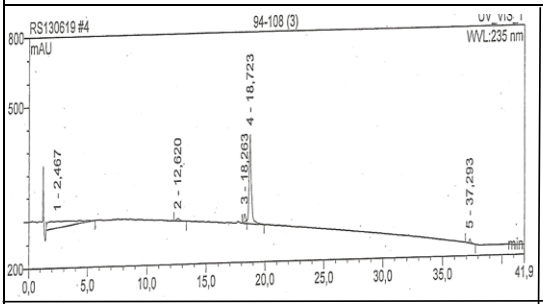


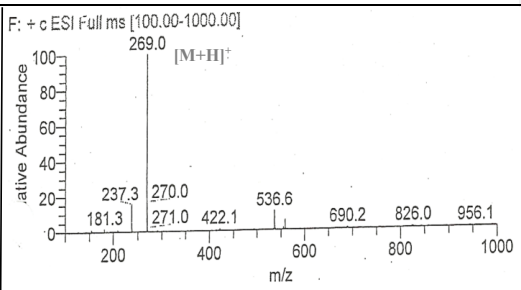
Figure 3.36: HMBC spectrum of compound (**28**)

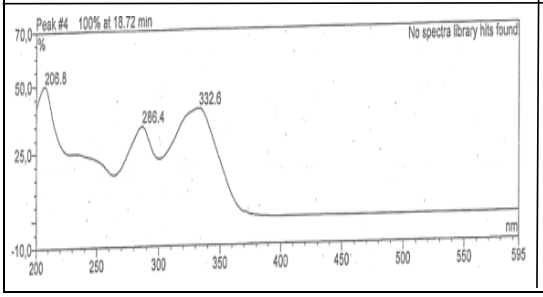
3.2.8. 2-Methyl-penicinoline (29, known compound)

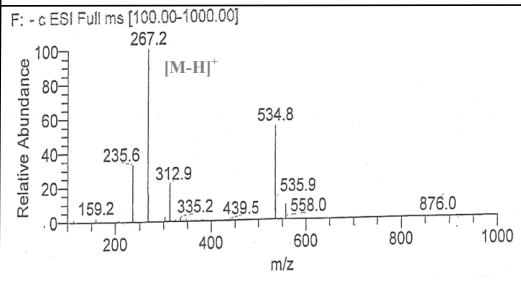
2-Methyl-penicinoline	
Synonym(s)	3-Quinolinecarboxylic acid, 1,4-dihydro-4-oxo-2-(1*H*-pyrrol-2-yl)-, methyl ester
Sample code	94-108 (3)
Biological source	*Penicillium* sp.
Sample amount	3.2mg
Physical Description	Brown solid
Molecular Formula	$C_{15}H_{12}N_2O_3$
Molecular Weight	268 g/mol
Retention time HPLC	18.72min (standard gradient)









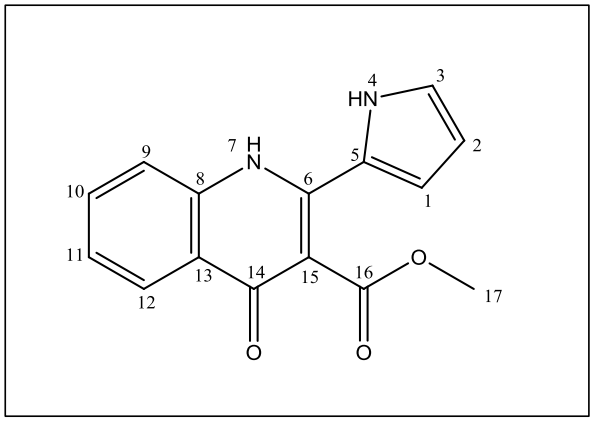


Compound (**29**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. in the form of a brown solid (3.2 mg). It exhibited UV absorbances at λ_{max} (MeOH) 206.8, 286.4 and 332.6 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 269 $[M+H]^+$ (base peak) and *m/z* 267.2 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 268.2 g/mol.

Structural elucidation of compound (**28**) was based on the results of 1D and 2D NMR spectral analysis including ^{1}H- and ^{13}C-NMR, ^{1}H-^{1}H COSY, and HMBC spectra (Table 3.20). The ^{1}H NMR spectrum showed a methyl ester singlet at δ_H 3.66 ppm for OCH_3-17, seven aromatic protons; resonating at δ_H 6.46 (brs), 6.25 [dd (*J*= 2.5, 5.3 Hz)], 7.13 (brs), δ_H 8.07 [d (*J*=7.8 Hz)], 7.35 [brt (*J*=7.8 Hz)], 7.69 [t, (J=8.1 Hz)] and 7.65 [d (*J*=8.1 Hz)] for H-1, H-2, H-3, H-12, H-11, H-10, and H-9, respectively. In addition, the ^{1}H NMR spectrum displayed two extra signals at δ_H 11.59 and 11.64, for NH-4 in the pyrrol ring and NH-7 in the quinolinone moiety, respectively. The ^{1}H-^{1}H COSY correlations (Table 3.20) showed two spin systems, the first one is CH(9) CH(10) CH(11) CH(12) and the second one is CH(1)CH(2)CH(3).

The HMBC spectrum (Figure 3.37) showed correlations from H-12 to the conjugated carbonyl group C-14 (δ_C 173.7) confirming the planar structure of the quinolone moiety. The attachment of the pyrrol ring to C-6 was secured through correlations from H-1(δ_H 6.46ppm) to C-6 (δ_C 140.9) and correlations from H-1 and H-3 to C-5 (δ_C 123.0).

Based on these data and by comparing them with these published references (Elsebai *et al*., 2011; Gao *et al*., 2012), compound (**29**) was identified as the known 2-methyl-penicinoline. Compound (**29**) has been previously isolated from the fungus *Auxarthron reticulatum* (Elsebai *et al*., 2011) but for the first time from a *Penicillium* species.



Structure of 2-methyl-penicinoline (**29**)

Table 3.20: ^{1}H, ^{13}C NMR, HMBC and ROESY data of compound (**29**) at at 600 (^{1}H) and 150 (^{13}C) MHz:

	Compound (**29**)					Compound (**29**)[a]			
#	δ_H(DMSO-d_6)	COSY	ROESY	HMBC	δ_C(DMSO-d_6)	δ_H(DMSO-d_6)	δ_C(DMSO-d_6)	NOESY	HMBC
1	6.46,*brs*	2,3	2, OCH_3-17	2,3,5,6	112.0	6.47,*dd* (1.5, 3.7)	112.1	2	2,3,5
2	6.25,*dd* (2.5, 5.3)	1,3	1,3	1,3	109.9	6.25,*dd* (2.6, 3.7)	110.1	1,3	1,3,5
3	7.13,*brs*	1,2	2	1,2,5	122.6	7.13,*dd* (1.5, 2.6)	122.8	2	1,2,5
NH-4	11.59,*s*	1,2,3				11.6,*s*		3	
5					123.0		123.0		
6					140.9		140.9		
NH-7	11.64,*s*			5,6,9,13,15		11.7,*s*		1,9	5,6,9,13, 15
8					139.6		139.7		
9	7.65,*d* (8.1)	10	10,11	11,13	118.7	7.66,*d* (7.7)	118.7	10, NH-7	11,13
10	7.69,*t* (8.1)	9,11	9,11	8,12	132.4	7.70,*t* (7.7)	132.6	9,11	8,12
11	7.35,*brt* (7.8)	10,12	9,10,12	8,9,10,13	123.6	7.36,*brt* (7.7)	123.9	9,10	9,13
12	8.07,*d* (7.8)	11	11	8,10,14	124.9	8.07,*d* (7.7)	124.9	11	8,10,14
13					124.3		124.3		
14					173.7		173.8		
15					113.6		113.6		
16					167.6		167.8		
OCH_3 -17	3.66, *s*		1	16	51.8	3.67, *s*	52.1	1	15,16

a) Elsebai *et al*., 2011.

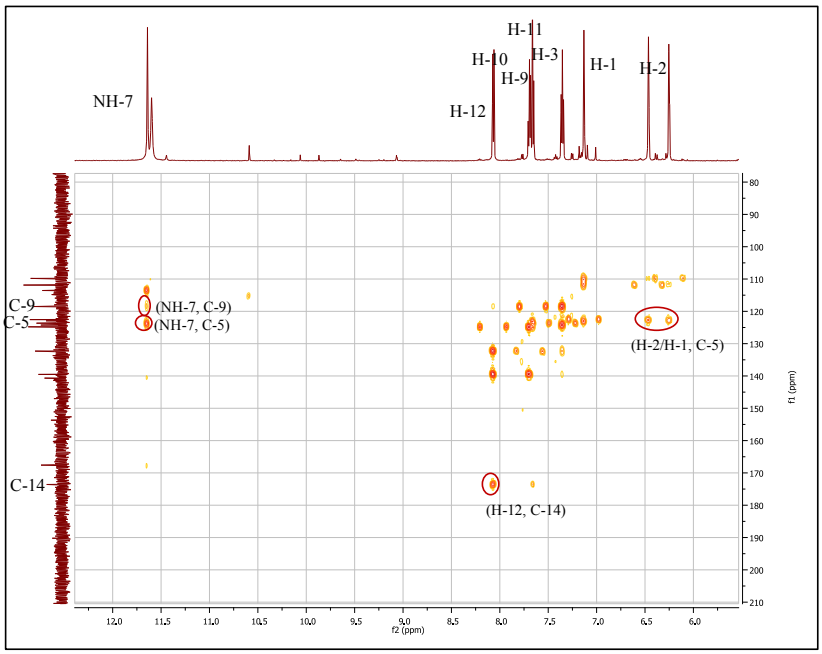


Figure 3.37: Expansion of HMBC spectrum of compound (**29**)

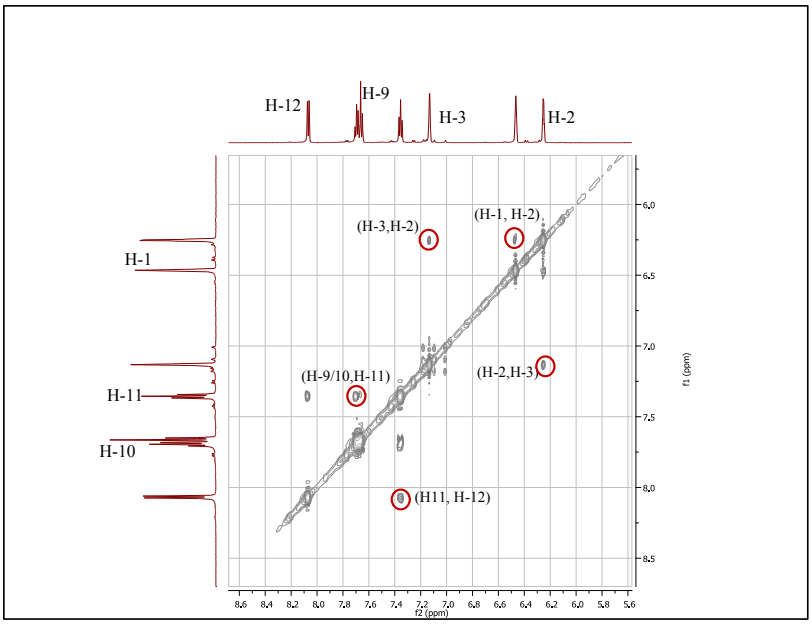
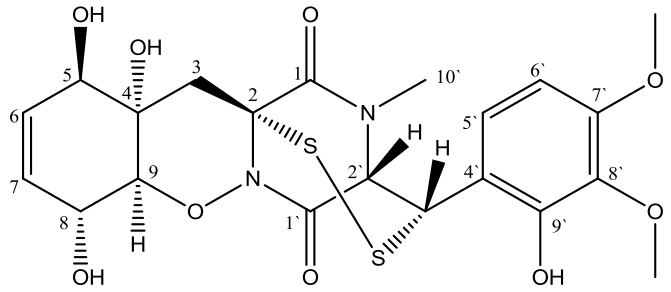
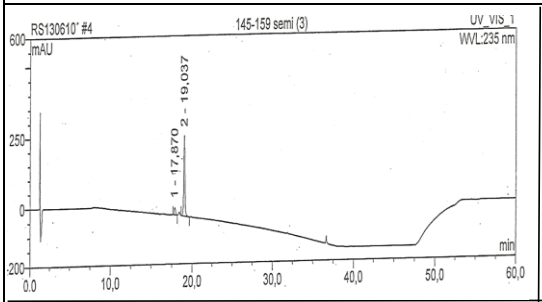


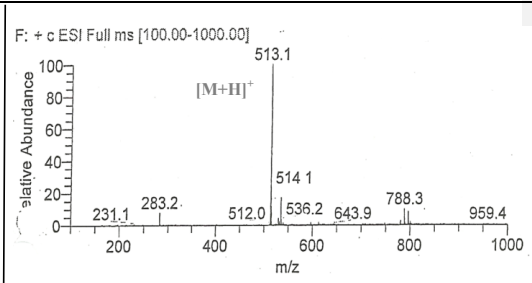
Figure 3.38: Expansion of ROESY spectrum of compound (**29**)

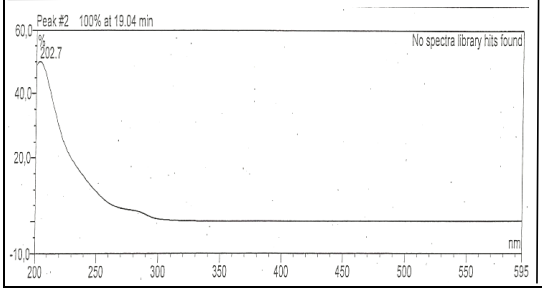
3.2.9. Pretichodermamide C, (30, new compound)

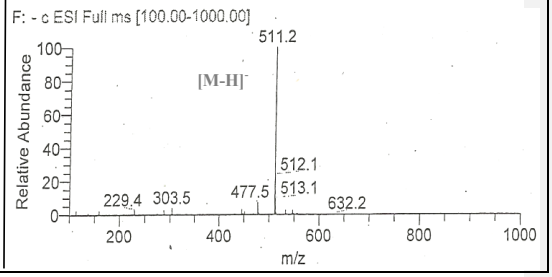
Pretichodermamide C	
Synonym(s)	Pretichodermamide C
Sample code	145-159 (3)
Biological source	*Penicillium* sp.
Sample amount	4.2 mg
Physical Description	Brownish orange solid
Molecular Formula	$C_{21}H_{24}N_2O_9S_2$
Molecular Weight	512.09 g/mol
Optical Rotation $[\alpha]_D^{25}$	-167 (c.0.12, MeOH)
Retention time HPLC	19.03 min (standard gradient)











Compound (**30**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp., as a brownish orange solid, it displayed UV absorbance at λ_{max} (MeOH) 202.7 nm. The HRESI-MS exhibited a strong peak at m/z 535.0815 $[M+Na]^+$ indicating a molecular formula of $C_{21}H_{24}N_2O_9S_2$ (calculated 535.0815, Δ 0.000), indicating ten elements of unsaturation.

Inspection of the ^{1}H NMR spectrum of compound (**30**) (Table 3.21) revealed the presence of signals of two aromatic methoxy groups at δ_H 3.78 and 3.68 ppm (7`-$OCH_3$ and 8`-OCH_3, respectively), two *ortho*-coupled aromatic protons at δ_H 7.34 ppm[d, (*J* = 8.8 Hz, H-5`)] and 6.55 ppm [d (*J* = 8.8 Hz, H-6`)], and one phenolic hydroxy proton at δ_H 9.45 (OH-9`), suggested the presence of a 2,3 dimethoxy phenol ring unit. This assumption was supported by HMBC cross-peaks (Figure 3.41) detected from H-5` to C-7`(δc 152.8) and C-9`(δc 148.0), from H-6` to C-4`(δc 116.6) and C-8`(δc 152.8), as well as from the two methoxy groups 7`-OCH_3 and 8`-$OCH_3$ to their respective carbons C-7` and C-8`, respectively.

In addition, the ^{1}H NMR spectrum showed resonances of one methylene group at δ_H 2.17 and 1.96 ppm for (CH_2-3), two vinylic protons at δ_H 5.41 (H-6) and 5.47 ppm (H-7), three oxygenated aliphatic methines, resonating downfield at δ_H 4.16, 4.21, and 3.92 ppm (H-5, H-8, and H-9, respectively), and three hydroxy protons at δ_H 5.15, 5.16, and 5.27 ppm (4-OH, 8-OH, and 5-OH, respectively). These signals were indicative of an oxazine-cyclohexene moiety, as confirmed by the ^{1}H-^{1}H-COSY correlations (Figure 3.39a) comprising the five methine protons from H-5 to H-9, and by the HMBC correlations from H-6 to C-4 (δc 71.3) and C-8 (δc 64.3); H-7 to C-5(δc 74.1) and C-9 (δc 85.5); H-9 to C-4 and C-8; and H-3 to C-4 and C-9.

Finally, the remaining signals of a nitrogen-bearing methyl group at δ_H 2.98 ppm (δc 33.1) (10`-$CH_3$) and an AB system of overlapping methines at $\delta_H$ 4.59 ppm (δc 66.0 and 41.5) (H-2` and H-3`, respectively), in addition to the signals of two carbonyl groups with chemical shift characteristic of amides at δc 165.4 (C-1) and 165.7 ppm (C-1`), and the presence of two sulfur atoms in the molecule, as indicated by the HRESIMS, indicated a N-methylated 2, 5-epidithio-3, 6-diketo group (Kirby and Robins, 1980), hence accounting for the remaining four degrees of unsaturation.

HMBC correlations of H-3 to C-1 and C-2 (δc 69.4), and of H-3` to C-1`, C-2`, C-4`, C-5', and C-9`, established the connection of the identified substructures as shown in (Figure 3.39

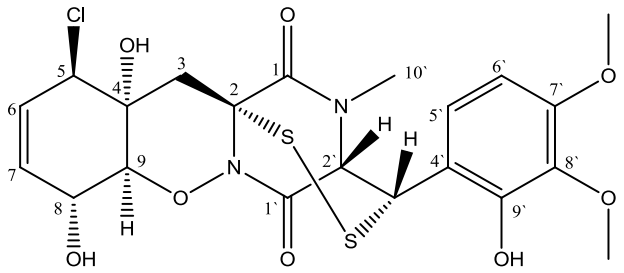
a). It is worth to mention that compound (**30**) represents an analogue of petrichodermamide A (Seephonkai *et al*., 2006), differing in the presence of a methyl group at N-10`, which accounts for the molecular weight difference of 14 amu observed between both compounds. This was further corroborated by the HMBC correlations of 10`-CH_3 to C-1 and C-2`, and by the shielding and deshielding effects for C-3` (δc 41.5) and C-2`(δc 66.0), respectively, as a result of the steric interaction between the N-methyl group and the C-3` methine proton (Yokose *et al*., 1984).

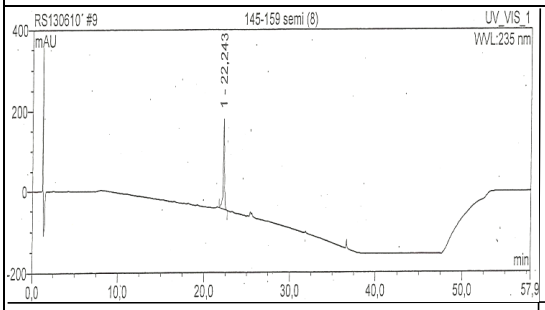
The relative configuration of (**30)** was determined by analysis of the coupling constants and the ROESY spectrum (Figure 3.42). Key correlations were observed for both H-5 and 4-OH with H-9, as well as between H-8 and 5-OH, thus indicating a cis-orientation of H-9, H-5, 4-OH, and 8-OH in the benzene ring fused to the oxazine portion (Figure 3.39b). In addition, the small vicinal coupling between H-2` and H-3` (J < 1 Hz) suggested their *gauche* relationship, hence indicating the same relative stereochemistry as observed for petrichodermamide A. On the basis of the above data, (**30)** was identified as a new natural product, for which the name pretichodermamide C is proposed.

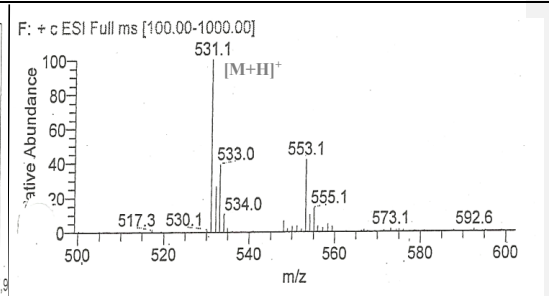
The absolute configuration of pretichodermamide C (**30)** is assumed to be identical to that of pretichodermamide A based on the $[\alpha]_D$ values of (**30**): $[\alpha]_D^{25}$-167 (c.0.12, MeOH) which are both in the negative side as observed for pretichodermamide A: $[\alpha]_D^{25}$-206 (c.0.10, MeOH), implying that both compounds share the 2*S*, 4*S*, 5*R*, 8*R*, 9*S*, 2`*S* , 3`*S* configuration.

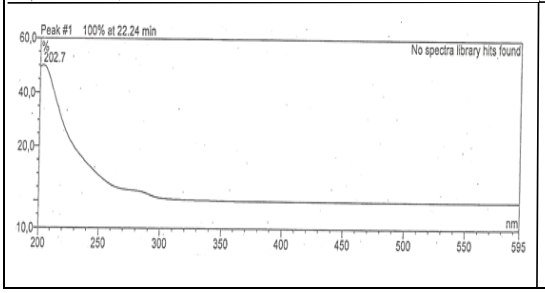
3.2.10. N-methylpretrichodermamide B, (31, new compound)

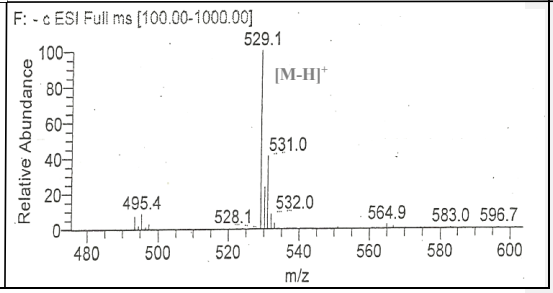
N-methylpretrichodermamide B	
Synonym(s)	N-methylpretrichodermamide B
Sample code	145-159 (8)
Biological source	*Penicillium* sp.
Sample amount	3.1 mg
Physical Description	Brownish orange solid
Molecular Formula	$C_{21}H_{23}N_2O_8S_2Cl$
Molecular Weight	530.06 g/mol
Optical Rotation $[\alpha]_D^{25}$	-102 (c.0.07, MeOH)
Retention time HPLC	22.24 min (standard gradient)











Compound (**31**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp., as a brownish orange solid, It showed UV absorbance at λ_{max} (MeOH) 202.7 nm. The HRESIMS spectrum, showed the characteristic chlorine isotope pattern corresponding to the *pseudo*-molelecular ion $[M+Na]^+$ (m/z 553.0479) for the formula $C_{21}H_{23}N_2O_8S_2Cl$ (calculated 553.0477, Δ 0.0002), thus corresponding to ten elements of unsaturation

The ^{1}H NMR spectrum of (**31**) (Table 3.21) was similar to that of (**30**), except for the marked downfield shifts observed for 4-OH and H-5 resonating at δ_H 5.71 and 4.86 ppm, respectively, as well as for the absence of the hydroxy group at C-5. The above spectral differences suggested that (**31**) features the same molecular skeleton as (**30**), bearing a chlorine substituent at C-5 instead of a hydroxyl group as found in (**31**), which accounts for the 18 amu molecular weight difference between both compounds. This assumption was further supported by inspection of the respective COSY and HMBC correlations (Figure 3.40 a). The HMBC spectrum (Figure 3.41) displayed correlations from H-5 to C-4 (δ_C 70) and C-6 (δ_C 126.6); H-6 to C-4 (δ_C 70) and C-8 (δ_C 65.1); H-7 to C-9 (δ_C 85.1); 8-OH to C-7 (δ_C 132.1), C-8 (δ_C 65.1) and C-9 (δ_C 85.1); H-9 to C-4(δ_C 70) and C-8 (δ_C 65.1); H-2`and H-3`to C-1` ($\delta_C$ 164.4), and C-4` (δ_C 116.8); CH_3-10` to C-1`(δ_C 164.4), C-2` ($\delta_C$ 65.8), and C-3` (δ_C 41.7); H-5`to C-3`(δ_C 41.7), C-7`($\delta_C$ 153.2) and C-9`(δ_C 148); H-6`to C-4`(δ_C 116.8) and C-8`($\delta_C$ 136.1); $OCH_3$-7`to C-7`($\delta_C$ 153.2), and $OCH_3$-8`(δ_C 60.8); OCH_3-8` to C-8`(δ_C 136.1), and OCH_3-7`($\delta_C$ 55.9); and 9`-OH to C-4`($\delta_C$ 116.8), and C-9`(δ_C 148).

The relative configuration of (**31**) was deduced to be the same as that of (**30**), according to the ROESY experiment as well as the coupling patterns of the relevant protons (Figure 3.40 b and 3.44). Therefore, (**31**) was identified as a new natural product and was given the name N-methylpretichodermamide B with the same absolute conftiguration of (**30**). To the best of our knowledge, this is only the second report of a chloro-epidithiodiketopiperazine compound in nature. A previous report includes the isolation the N-demethyl analogue of (**31**), named DC1149B, from *Thrichoderma longibrachiatum* (Hirofumi *et al.*, 1990).

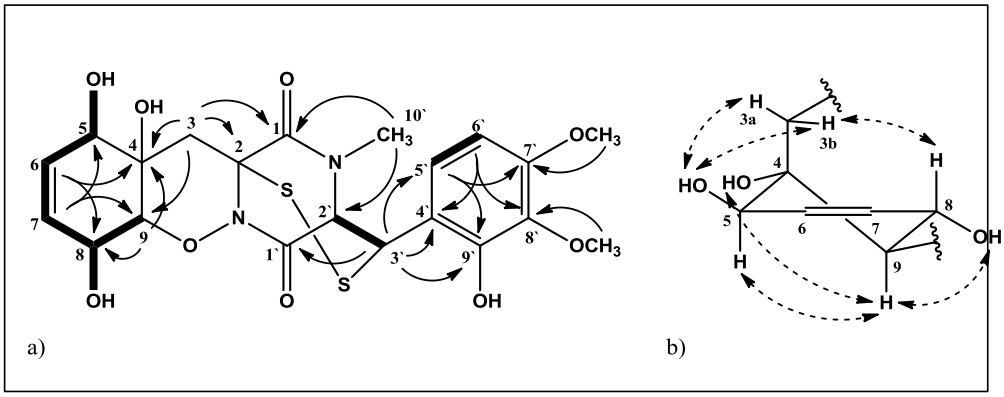


Figure 3.39: a) ^{1}H-^{1}H COSY (**bold**) and selected HMBC (H → C) correlations of (**30**), b) selected ROESY correlations of the oxazine-cyclohexene moiety of (**30**).

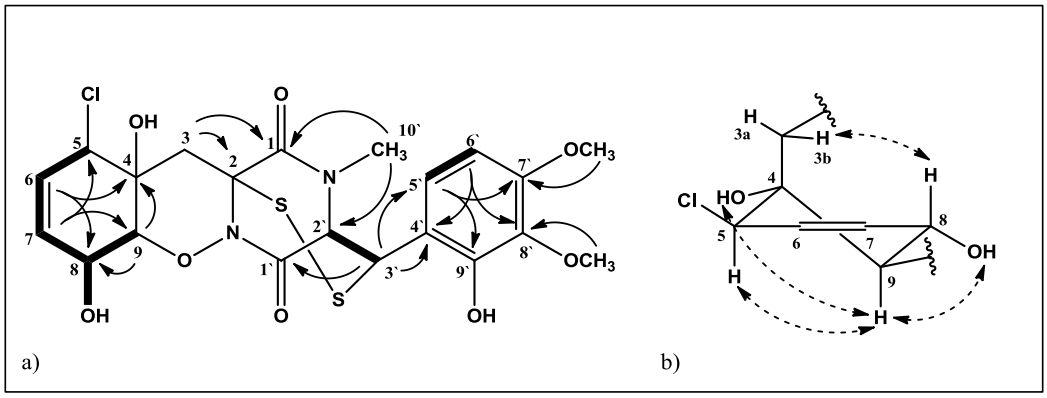


Figure 3.40: a) ^{1}H-^{1}H COSY (**bold**) and selected HMBC (plain) correlations of (**31**), b) selected ROESY correlations of the oxazine-cyclohexene moiety of (**31**).

Table 3.21: ^{1}H, ^{13}C NMR, COSY, ROESY and HMBC data of compounds (**30**) and (**31**) at 600 (^{1}H) and 150 (^{13}C) MHz:

#	Compound (**30**)					Compound (**31**)				
	δ_H(DMSO-d_6)	COSY	ROESY	HMBC	δ_C(DMSO-d_6)[a]	δ_H(DMSO-d_6)	COSY	ROESY	HMBC	δ_C(DMSO-d_6)[a]
1					165.4					165.4
2					69.4					69.5
3a	2.17, d(15.9)	3b, 9	3b,5,5-OH	4, 9	32.1	2.25, d (17.0)	3b,9	3b		33.8
3b	1.96, d (15.9)	3a	3a,5 6,7, 8,9	1, 2		2.08, d (17.0)	3a, 4-OH	3a, 8	1, 2	
4					71.3					70
5	4.16, brs	6, 5-OH	3a,3b,6,7, 9, 4-OH,5-OH, 8-OH	4,6	74.1	4.86, brs	6,8	6, 9, 4-OH	4, 6	67.9
6	5.41, d (10.3)	5, 5-OH	3b, 5, 7	4, 8	129.8	5.57, d (10.3)	5,4-OH	5,7	4,8	126.6
7	5.47, d (10.3)	8	3b,5,6, 8	5, 9	128.6	5.62, d (10.3)	5,8-OH	6,8,8-OH	9	132.1
8	4.21, brs	7,9, 8-OH	3b, 7, 9, 5-OH,8-OH	9	64.3	4.34, brs	5,9, 8-OH	3b,7,9,8-OH		65.1
9	3.92, d (6.9)	3a, 8	3b,5, 7,8, 4-OH	3, 4, 8	85.5	4.04, d (7.5)	3a, 8	5, 8-OH, 4-OH	4, 8	85.1
1`					165.7					164.4
2`	4.59, brs		5`, $CH_3$-10`, 9`-OH	1`,3`, 4`, $CH_3$-10`	66	4.62,d (4.74)		5`, $CH_3$-10`, 9`-OH	1`, 4`	65.8
3`	4.59, brs		5`, $CH_3$-10`, 9`-OH	1`, 2`, 4` ,5`, CH_3-10`, 9`	41.5	4.62,d (4.74)		5`, $CH_3$-10`, 9`-OH	1`, 4`	41.7
4`					116.6					116.8
5`	7.34, d (8.8)	6`	2`,3`, 6`	3`,7`,8`, 9`	123.1	7.33, d (8.8)	6`	2`, 3`, 6`	3`, 7`, 9`	123.5
6`	6.55, d (8.8)	5`	5`, $OCH_3$-7`	4`, 7`, 8`, 9`	103.1	6.55, d (8.8)	5`	5`, $OCH_3$-7`	4`, 8`	103.9
7`					152.8					153.2
8`					136.2					136.1
9`					148					148
OCH_3-7`	3.78, s	OCH_3-8`	2`,3`, 6`, $OCH_3$-8`	7`, $OCH_3$-8`	56	3.78, s	OCH_3-8`	6`, $OCH_3$-8`	7`,$OCH_3$-8`	55.9
OCH_3-8`	3.68, s	OCH_3-7`	2`,3`,CH_3-10`,9`-OH	7`, 8`, OCH_3-7`	60.8	3.68, s	OCH_3-7`	9`-OH, $OCH_3$-7`	8`, $OCH_3$-7`	60.8
9`-OH	9.45, S		2`,3`, OCH_3-8	4`, 8`, 9`		9.46,S		2`, 3`, OCH_3-8`	4`, 9`	
CH_3-10`	2.98, s		2`,3`	1`, 2`	33.1	2.99, s		2`, 3`	1`, 2`,3`	32.9
5-OH	5.27, d (5,8)	5,6	3a,5,8	6						
8-OH	5.16, d (5.8)	8	5,7,8,9	9		5.35, d (6.9)	7, 8	8,9	7, 8, 9	
4-OH	5.15, d (5,8)	5	5, 9	9		5.71, brs	3b,6	5, 9	6	

[a] Derived from HMQC and HMB

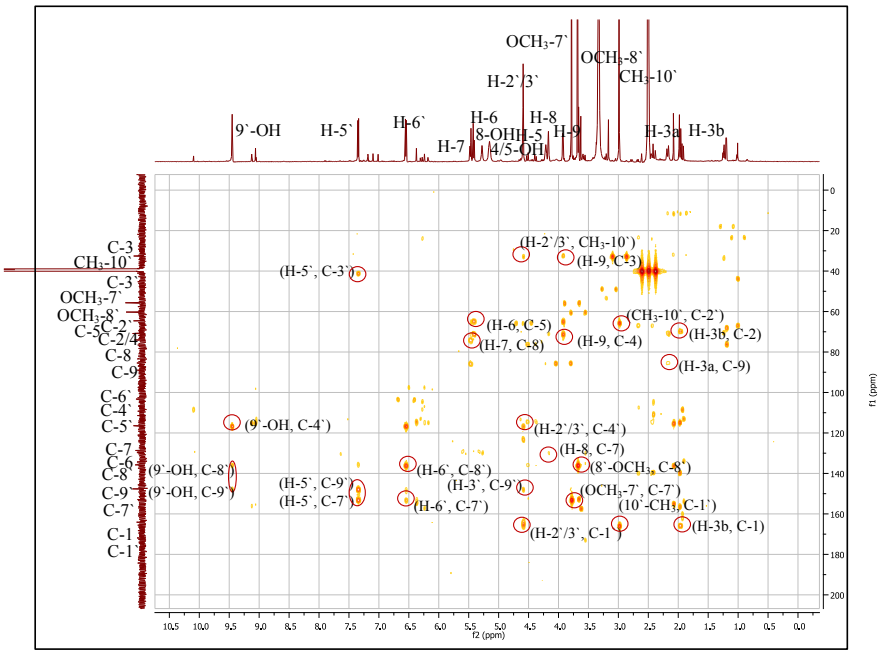


Figure 3.41: HMBC spectrum of compound (**30**)

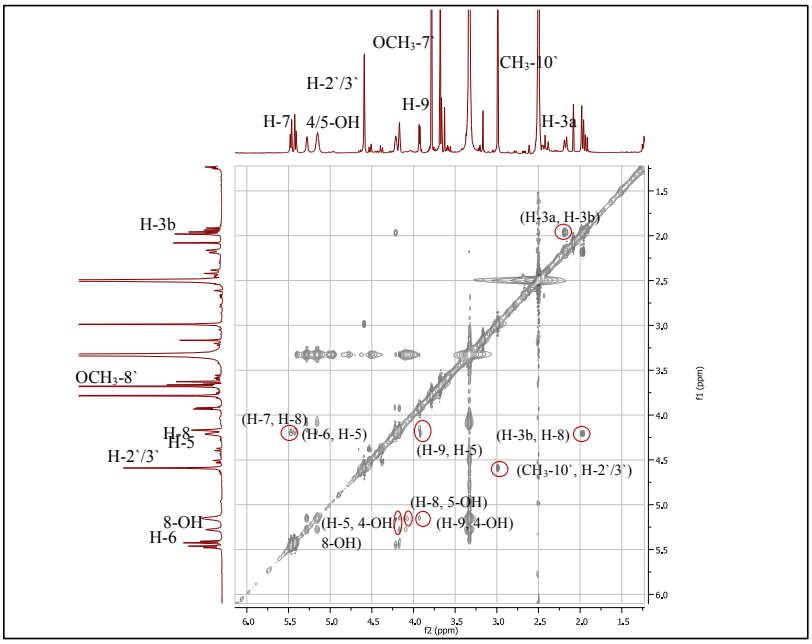


Figure 3.42: Expansion of ROESY spectrum of compound (**30**)

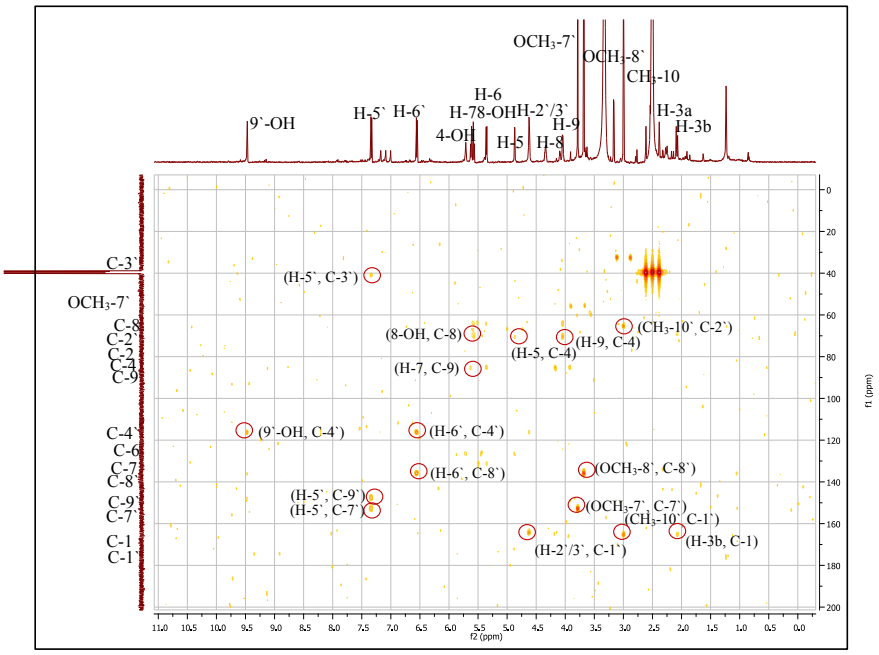


Figure 3.43: HMBC spectrum of compound (**31**)

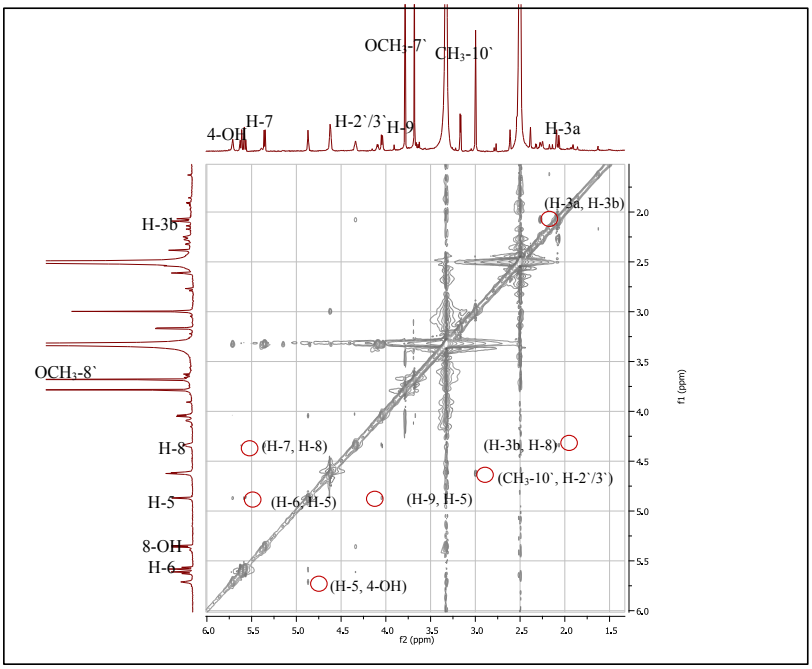
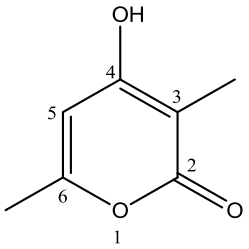
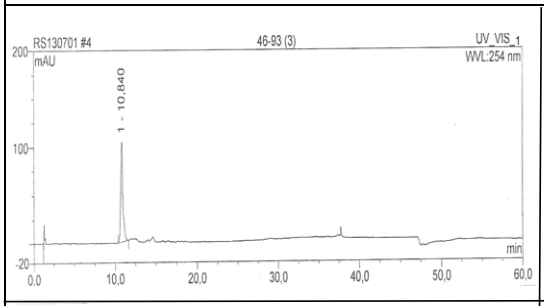


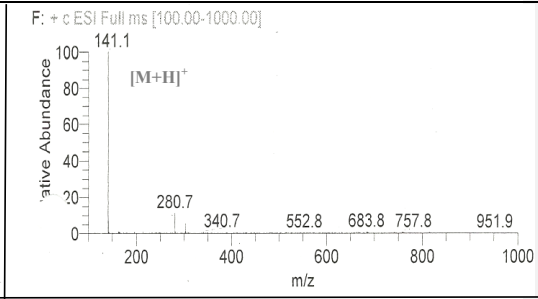
Figure 3.44: Expansion ROESY spectrum of compound (**31**)

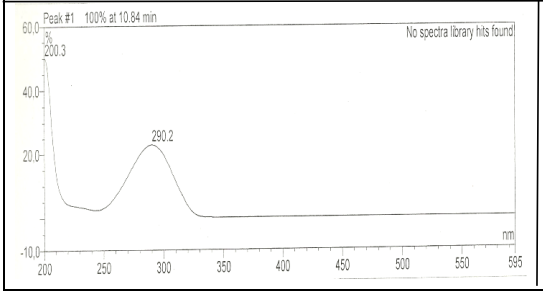
3.2.11. Methyltriacetic lactone, (32, known compound)

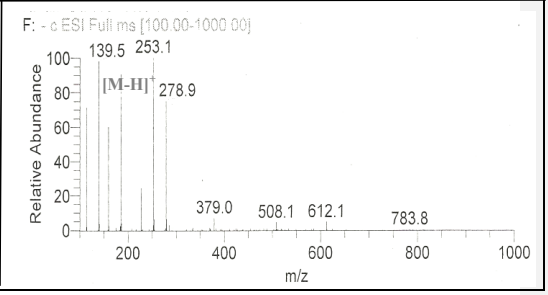
Methyltriacetic lactone	
Synonym(s)	4-Hydroxy-3,6-dimethyl-2H-pyran-2-one
Sample code	46-93 (3)
Biological source	*Penicillium* sp.
Sample amount	1.1 mg
Physical Description	Brown crystals
Molecular Formula	$C_7H_8O_3$
Molecular Weight	140.04 g/mol
Retention time HPLC	10.84 min (standard gradient)







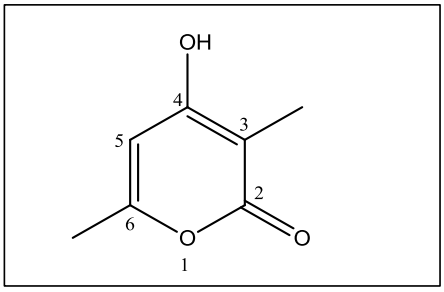




Compound (**32**) was isolated from the EtOAc extract of solid rice cultures of *Penicillium* sp. in the form of brown crystals (1.1 mg). It exhibited UV absorbances at λ_{max} (MeOH) 200.3 and 290.2 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 141.1 $[M+H]^+$ (base peak) and *m/z* 139.5 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 140.04 g/mol.

The ^{1}H NMR spectrum of compound (**32**) recorded in DMSO-*d6* (Table 3.22) exhibited two methyl singlets at δ_H 1.73 and 2.14 for CH_3-6 and CH_3-3, an aromatic singlet at δ_H 5.98 for H-5. Moreover, a hydroxyl aromatic singlet at δ_H 11.09 for 4-OH was observed.

The ^{1}H-^{1}H-COSY spectrum (Figure 3.45 and Table 3.22) showed the following spin system; CH_3(6)CH(5). Comparison of the UV, MS and ^{1}H NMR data with reported data (Mathias *et al*., 1999) established that compound (**32**) is the known methyltriacetic lactone. Compound (**32**) was previously isolated from *Penicillium* sp. (Savard *et al*., 1994) and *Ampelomyces* species (Hassan, 2007).



Structure of methyltriacetic lactone (**32**)

Table 3.22: ^{1}H, ^{13}C NMR and COSY data of compound (**32**) at 500 (^{1}H) and 125 (^{13}C) MHz:

#	Compound (**32**) δ_H(DMSO-d_6)	COSY	δ_H(DMSO-d_6)[a]	δ_H($CDCl_3$)[b]
2				
3				
4				
5	5.98,*s*	CH_3-3, CH_3-6	5.98,*s*	5.8,*s*
6				
CH_3-3	2.14,*s*	CH_3-6,5	2.14,*s*	2.53,*s*
CH_3-6	1.73,*s*	CH_3-3,5	1.74,*s*	1.92,*s*
4-OH	11.09,*s*	CH_3-3	11.08,*brs*	

a) Mathias *et al*., 1999. b) Savard *et al*., 1994.

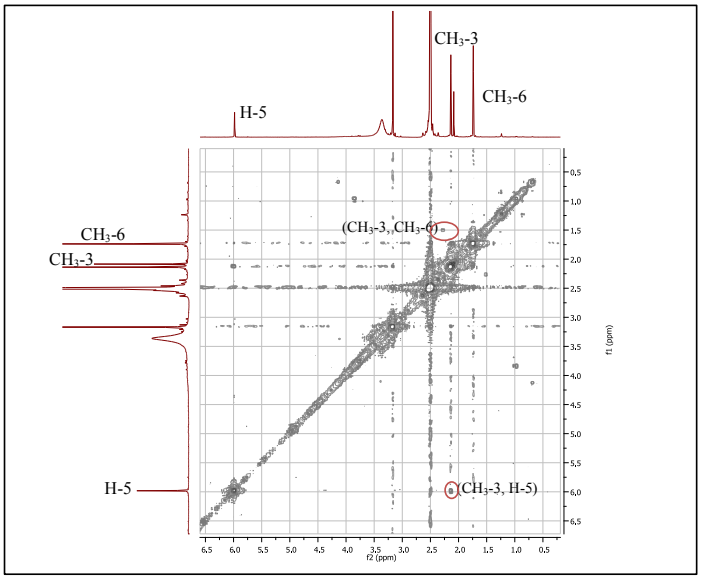


Figure 3.45: Expansion of ^{1}H-^{1}H-COSY spectrum of compound (**32**)

3.2.12. Bioactivity test results for compounds isolated from the fungus *Penicillium* sp.

The isolated compounds from the ethyl acetate extract of the rice cultures of *Penicillium* sp. were subjected to cytotoxicity (MTT) assay against mouse lymphoma (L5178Y) cells whose results are shown below (Table 3.23).

Table 3.23: Cytotoxicity assay results for the compounds isolated from *Penicillium* sp. rice extracts

Nr.	Sample tested	L5178Y growth in % (@ 10 μg/mL)	EC50* (μg/mL)	EC50 (μmol/L)
22	6-Hydroxy-8-methoxy-3,5dimethylisochromanol	73.8		
23	6-Hydroxy-8-methoxy-3, 5-dimethylisochroman	103.4		
24	Arohynapene D	83.9		
25	1, 6-Dihydroxy-3, 5-dimethyl -8-methoxyisochroman	59.6		
26	6-Hydroxy-8-methoxy-3,5-dimethyl-3,4-dihydro- isocoumarin	40.5		
27	6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin	86.3		
28	6-Methylcurvulinic acid	87.8		
29	2-Methyl-Penicinoline	78.5		
30	Pretichodermamide C	85.6		
31	N-methylpretichodermamide B	3.5	2.0	3.8
32	Methyltriacetic lactone	90.2		

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

The N-methylpretichodermamide B (**31**) proved to be highly effective against the L5178Y cancer cell line. Furthermore, the other isolated compounds proved to be moderate to weak active against the same cell line. On the other hand, all pure isolated compounds (**22**-**32**) were evaluated for their antimicrobial activities against different Gram + and Gram – bacteria including, *Staphylococcus aureus* ATCC, *Streptococcus pneumonia* ATCC 49619, and *Escherichia coli* ATCC 25922; unfortunately, none exhibited any activity.

4. Discussion:

4.1. Selection of culture media

Growth media and incubation conditions have a very strong effect on the secondary metabolite production. The variation in the environment and nutrition have been shown to have an essential effect on the quantity and diversity of secondary metabolite production (Fiedurek *et al*., 1996; Mohanty and Prakash, 2009).

Manipulation of culture conditions such as temperature, shaker speeds, media with at least two different pH levels, salinity, carbon and nitrogen sources at different concentrations, high- or low-phosphate content, adding trace minerals, *etc.* have varying effects on the production of fungal secondary metabolites ((Knight *et al.*, 2003; Bode *et al*., 2002; Miao *et al*., 2006).

The variation of culture conditions is used to optimize the yield of a specific compound, this including cultivation on solid and liquid media. Cultivation of fungi using liquid culture techniques had played an important role in industrial applications (Smith and Berry, 1975). However, liquid media are far from the natural environment of fungi. Actually, they decrease the importance of specific phenomena such as osmotic resistance or cytoplasmic transfer.

Thus, the argue of choosing appropriate culture media is still around. We should focus on a limited set of parameters in order to produce novel and bioactive metabolites. So, the investigated fungal strains were cultured on different media resulting to have various secondary metabolites.

In the present study and from literature (Hassan, 2007), it is observed that the solid media produce yields with larger masses than the liquid media of the same fungus.

4.2. Methodologies for metabolite profiling

Natural product research involves mainly isolation and purification of pure compounds from crude extracts or fractions of different natural product sources and this is followed by structure elucidation of the isolated compounds. The investigation of these metabolites in crude natural product extracts or fractions requires high degree of sensitivity and selectivity. Therefore, it is necessary to generate advanced analytical and spectroscopic methods like the hyphenated techniques. Hyphenated techniques combine both chromatographic and spectral methods in order to obtain the advantages of both. Such techniques have the ability to solve complex analytical

problems (Kalpesh *et al*., 2010). The power of combining hyphenated techniques to HPLC can give a good idea about the different substructures and/or functional groups of the structure. Such combinations have been applied in this study.

4.2.1. HPLC/UV

Nowadays, advanced HPLC columns joined with fast UV diode array detectors facilitate the identity of every single component present in the extract. The different chromophore of the components absorb in different ultraviolet regions. This feature makes the UV spectrum an important source of information related to the structure of natural products (Cannall, 1998).

In the present study, the isolated compounds that share similar chromophoric functions were examined by the HPLC/UV-photodiode array detection (LC/UVDAD) (*e.g.* citreoisocoumarin derivatives (**1**-**5**), alternariol derivatives (**6**-**7**), stemphytriol and altertoxin II (**10**-**11**), altenuene and 4`-epialtenuene (**12**-**13**), isochroman derivatives (**22**-**25**).

4.2.2. HPLC/ESI-MS

High Performance Liquid Chromatography–Electrospray Mass Spectrometry (HPLC/ESI-MS) is the most popular MS technique. It combines the separation ability of HPLC along with the sensitivity and specificity of detection from MS. One of the advantages of HPLC-MS is that it allows samples to be rapidly desalted online, accordingly, this rising (HPLC/ESI-MS) to be a highly versatile tool for studies of natural products (Smedsgaard and Frisvad, 1996).

On the other hand, modern Liquid Chromatography-Mass Spectrometry (LC/MS) is now the most useful technique with the development of electrospray ionisation (ESI). So, it can be applied to a wide range of biological molecules. Fast scanning speeds allow measuring a large number of secondary metabolites in a single analytical run in the form of positive and negative ionization peaks (James, 2009). In addition, this method is also helpful in establishing the relation between closely related natural products. In the study, this is proved to be extremely valuable in detecting the characteristic 3:1 ratio in the peak heights of the positive and negative ion peaks of N-methylpretichodermamide B (**31**) which indicates the presence of a chlorine atom.

4.3. Isolation of natural products

By using different chromatographic techniques, pure compounds have been isolated from different fungal strains originated from different sources. The endophytic fungus *Alternaria* sp. has been isolated from the seeds of *Ziziphus jujube* which yielded a number of compounds possessing bioactivity. Furthermore, the extract of *Penicillium* sp. which was isolated from the sediment of Wadi El-Natrun lake yielded plenty of secondary metabolites.

4.4. Compounds isolated from purified fungal strains

4.4.1. Compounds isolated from the endophytic fungus *Alternaria* sp.

Alternaria sp. has been isolated from the healthy seeds of *Ziziphus jujube* growing in Uzbekistan. The seeds of *Ziziphus jujube* are known to cure eye diseases and are also useful in leucorrhoea (Mahajan and Chopda, 2009). In addition, they have been used in traditional medicine as a sedative. They are known to depress activity of the central nervous system which reduces anxiety and induces sleep (Wen-Huang *et al*., 2000).

Extracts of the small scale liquid cultures afforded citreoisocoumarinol (**1**), 6-methyl-citreoisocoumarin (**2**)**,** citreoisocoumarin **(3),** orthosporin (**4**) and diaportinol (**5**). While, the extract of the large scale solid rice cultures gave: alternariol (**6**), alternariol-5-*O*-methyl ether (**7**), altenusin (**8**), tenuazonic acid (**9**), stemphytriol (**10**), altertoxin II (**11**), altenuene (**12**), 4`-epialtenuene (**13**), isoochracinic acid (**14**), 7-methoxyphthalide-3-aacetic acid (**15**), talaroflavone (**16**), alteric acid (**17**), alterlactone (**18**), 2,5-dimethyl-7-hydroxychromone (**19**), altechromone B (**20**), ferulic acid (**21**). In this part, the discussion of the biosynthesis and the bioactivity of the metabolites produced by small and large scales of the *Alternaria sp.* are presented.

4.4.1.1. Isocoumarin derivatives

4.4.1.1.1. Biosynthesis of isocoumarin and phthalide derivatives

The biosynthesis of isocoumarins has been the subject of interesting work on many fungi. These isocoumarines are made from heptaketide intermediate with or without reduction in their side chains. The production of these compounds by biosynthetic feeding studies has been investigated citreoisocoumarin using sodium [$^{13}C_2$] acetate as a labeled isotope. This revealed some insight into the cyclization and aromatization of the first ring of the heptaketide chain, the

second ring cyclization by claisen type condensation is prohibited and exclusively gives lactone ring formation (Figure 4.2) (Barton and Meth, 1999).

Additionly, from a biogenetic point of view, *β*-polyketo carboxylic acids are expected to be converted into the corresponding phenolic compounds including different types of isocoumarins (**1**-**5**) and phthalides (**14** and **15**) according to the mode of enzymatic cyclization as shown in (Figure 4.1) (Lai *et al*., 1991).

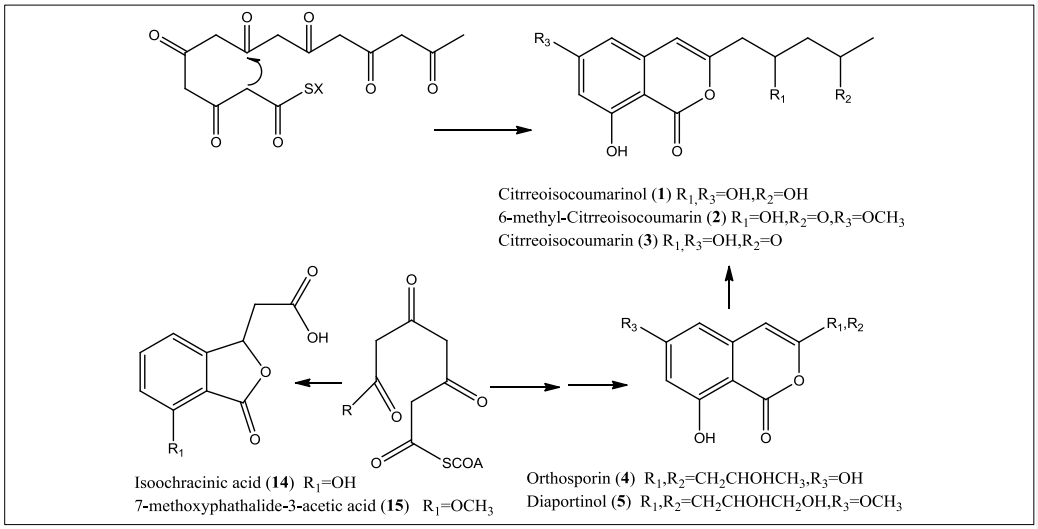


Figure 4.1: Biosynthetic pathways of isocoumarins and phthalides.

4.4.1.1.2. Bioactivity and phytotoxicity of isocoumarin derivatives

Isocoumarin derivatives were subjected to antibacterial testing. Citreoisocoumarin (**3**) proved to be active against the gram-positive bacteria, multi-resistant *Staphylococcus aureus* (MRSA) with MIC values of 0.49*μ*g/mL. In contrast, the cytotoxicity testing for isocoumarin compounds against L5178Y (mouse lymphoma) cell line (see Table 3.13) showed weak cytotoxic activity.

Orthosporin (**4**) which had been isolated from the fungus *Drechslera siccans* was reported to cause irregular brown spots in leaves of oats and both perennial and Italian ryegrass. Orthosporin (**4**) also caused necrosis when applied in solution to leaves of maize, crabgrass, soya bean, barnyard grass and spiny amaranth but not to leaves of perennial ryegrass (Hallock *et al*.,

1988). Furthermore, a study showed that orthosporin (**4**) has toxic activity against coffee tree leaves (Ichihara *et al*., 1989).

4.4.1.2. Alternariol derivatives

4.4.1.2.1. Biosynthesis of alternariol and biphenic acid derivatives

Alternariol is thought to be formed by the polyketide route of biosynthesis which is a common pathway for the formation of many fungal secondary metabolites (Crawford and Townsend, 2010). Fungal polyketide synthases (PKSs) are crucial for the first steps of the biosynthesis of alternariol and its derivatives (Gulder *et al.*, 2012; Crawford and Townsend, 2010).

Biosynthetic routes for alternariol were first extensively studied by Thomas (Thomas, 1961) who suggested that this metabolite might be synthesized by head-to-tail condensations of acetate units. Later, Gatenbeck and Hermodsson (Gatenbeck and Harmodsson, 1965) determined that malonate formed by carboxylation of acetate was the polycondensing molecule.

Based on the previous studies, alternariol can be established to be derived from a single C_{14} polyketide chain first folded then cyclized. The precise sequence of reactions involved is still unknown. The essential features in alternariol biosynthesis are the condensations of two aldol followed by enolization in both rings. This would give a biphenyl system and lactonization would then lead to alternariol (**6**). The oxygenation pattern in alternariol shows alternate oxygens on both aromatic rings and an acetate origin is readily surmised even though some oxygens have been used in ring formation processes. The lone methyl 'start-of-chain' is also usually very obvious in acetate-derived compounds though the carboxyl 'end-of-chain' can often react with convenient hydroxyl functions, which may have arisen through enolization followed by lactone formation (Dewick, 2006).

On the other hand, alternariol biosynthetic reaction may proceed through an intermediate ^{14}C-labeled norlichexanthone (NLX) as shown in (Figure 4.2) through bond cleavage and rearrangement of (NLX). The point of attachment between the enzyme and the polyketide is shown as the active end of the polyketide chain although the attachment could have migrated or could involve several points of contact. The polyketide chain is assembled on the surface of the enzyme in a configuration that facilitated the formation of NLX. After rupture of the aromatic

phloroglucinol ring, limited rotation of the aryl fragment and ring closure would produce the coupling pattern observed in alternariol (**6**). After enolization, the resulting solution also possessed O-methyltransferase activity capable of forming alternariol-5-*O*-methyl ether (**7**) and altenusin (**8**) from alternariol (**6**) by a transmethylation reaction involving S-adenosyl methionine (SAM) enzyme (see Figure 4.3) (Stinson *et al.*, 1986).

Alterlactone (1**8**) is most probably biosynthesized through the same pathway from altenusin (**8**) by oxidation of the aromatic methyl group, rotation and closure of the lactone ring at a different site (Hassan, 2007). Altenuene (**12**) and 4`-epialtenuene (**13**) have been also biosynthesized from altenusin (**8**) by oxidative cyclization.

The biphenyl intermediate which could also derived from altenusin (**8**) is the precursor to the structurally and biosynthetically biphenic acids metabolites, talaroflavone (**16**) and alteric acid (**17**). The scheme (figure 4.3) illustrating the biosynthetic pathway for alternariol and biphenic acids derivatives depended on previous studies which used labelling methods to examine the incorporation of [1-^{13}C] labelled acetate into the biosynthetically targeted metabolites including, α-dibenzopyrones and biphenic acids compounds (Ayer and Racock, 1990; Hassan, 2007).

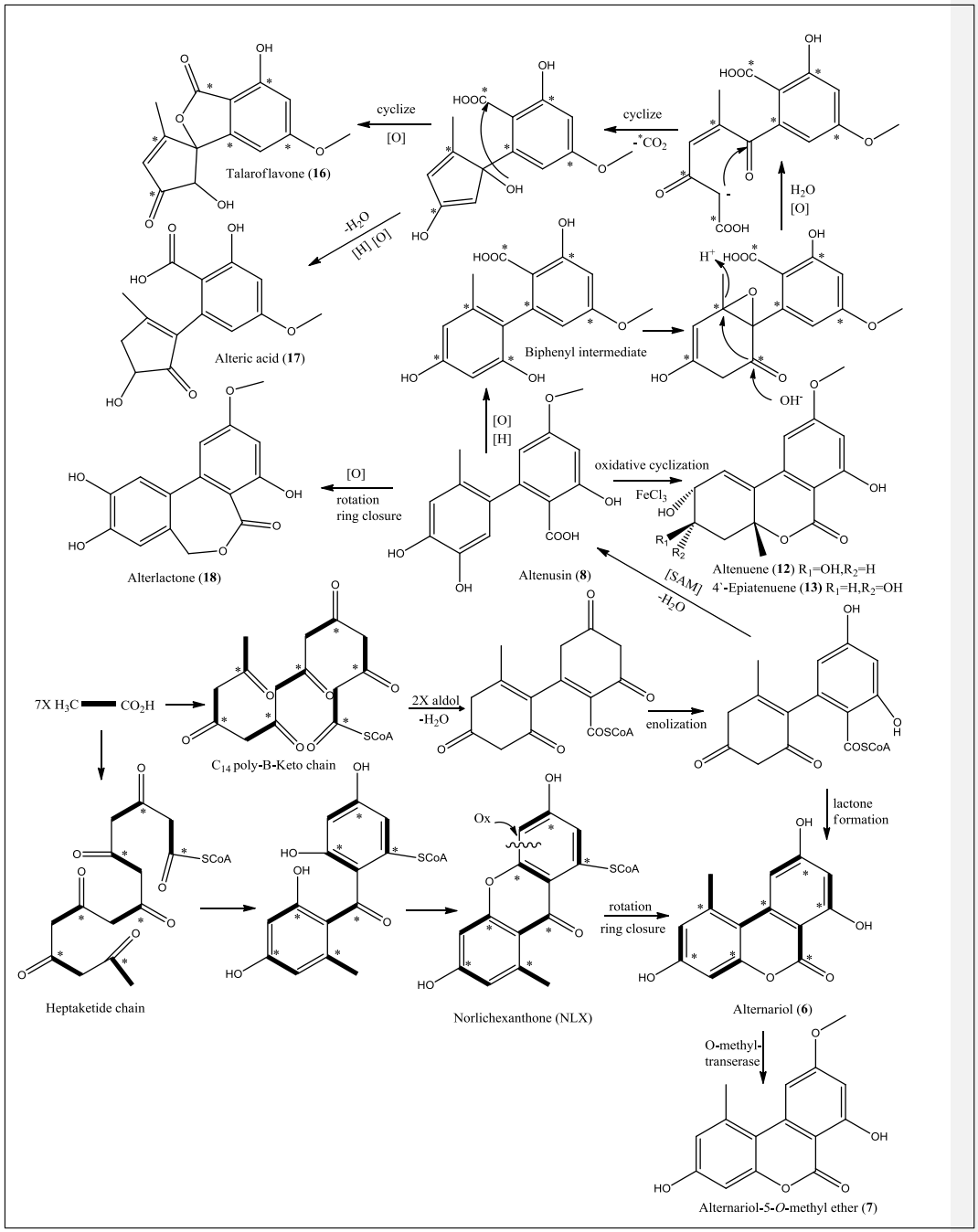


Figure 4.2: Postulated biosynthetic pathways of alternariol and biphenic acid derivatives.

4.4.1.2.2. Bioactivity and toxicity of dibenzo-α-pyrones and tenuazonic acid

Dibenzo-α-pyrones including, alternariol (**6**), alternariol-5-*O*- methyl ether (**7**) in addition to altenusin (**8**) have been reported in our group studies to be a highly cytotoxic compounds against mouse lymphoma (L5178Y) cells (Hassan, 2007). Less inhibition was shown for alterlactone (**18**), while altenuene (**12**), 4`-epialtenuene (**13**) and the biphenic acids (**16**) and (**17**) are not active.

The antibacterial activity for alternariol (**6**) and altenusin (**8**) which was reported in this study against different types of bacteria in addition to the previous studies confirmed the broad spectrum activity of these compounds (Hassan, 2007). Furthermore, altenusin (**8**) isolated from the endophytic fungus *Alternaria* sp. (UFMGCB55) was screened to show inhibitory activity on trypanothione reductase (TR) which is an enzyme involved in the protection of parasitic *Trypanosoma* and *Leishmania* species against oxidative stress and this enzyme has been considered to be a validated drug target (Cota *et al.,* 2008).

Dibenzo-α-pyranones which have been isolated from *Alternaria* fungi are known to be most commonly encountered in naturally contaminated commodities (Scott, 2011). Alternariol (**6**) and alternariol methyl ether (**7**) are major toxins produced by species of the former fungal genus. Alternariol (**6**) causes weak acute toxic effects and the LD50 is higher than 400 mg/kg of body weight for mice. It is lethal to mice embroys at levels of 100 mg/kg b.w. (Pero *et al.,* 1973). There are several reports that alternariol (**6**) and alternariol methyl ether (**7**) exhibit genotoxic potential, e.g. induction of DNA strand breaks and gene mutations in cultured human and animal cells (Brugger *et al.,* 2006; Pfeiffer *et al.,* 2007). Further investigation showed that alternariol (**6**) has been identified as a topoisomerase I and II poison which might contribute to the impairment of DNA integrity in human colon carcinoma cells (Ostry, 2008). It induced cell death by activation of the mitochondrial pathway of apoptosis in human colon carcinoma cells (Bensassi *et al.,* 2012). Alternariol (**6**) induced cytochrome P450 1A1 and apoptosis in murine heptatoma cells dependent on the aryl hydrocarbon receptor (Schreck *et al.,* 2012). Recently, a study has been done in order to identify the polyketide synthase (PKSs) which is responsible for the production of alternariol and alternariol methyl ether in order to reduce the mycotoxin effect accompanied by the production of these metabolites (Saha *et al.,* 2012).

Another major mycotoxin metabolite produced by *Alternaria* sp. is tenuazonic acid (**9**). The biosynthesis of tenuazonic acid (**9**) proceeds from one molecule *l*-isoleucine and two

molecules of acetate (Stickings and Tounsend, 1961) via N-Acetoacetyl-*l*-isoleucine as intermediate (Gatenbeck and Sierankiewicz, 1973). Tenuazonic acid (**9**) exhibits manifold biological activities and has been reported to have antiviral (Miller *et al.,* 1963), antitumor, antibacterial (antitubercular) (Sonaimuthu *et al.,* 2010) and cytotoxic activities (Gitterman, 1965). Although, the inhibition of mouse lymphoma (L5178Y) cell lines in this study has been absent. Tenuazonic acid (**9**) has phytotoxic properties (Lebrun *et al.,* 1988) and was found to be acutely toxic in mammals (Smith *et al.,* 1968) and to several animal species (Griffin and Chu, 1983). Additionally, tenuazonic acid (**9**) was found to be responsible for the outbreak of "onyalai", a human hematologic disorder disease occurring in Africa (Steyn and Rabie, 1976), but further toxicological evidence is lacking. Accordingly, tenuazonic acid (**9**) is regarded as the most toxic *Alternaria* mycotoxin (Bottalico and Logrieco, 1998).

4.4.1.3. Chromone derivatives

4.4.1.3.1. Biosynthesis of 2, 5-dimethyl-7-hydroxychromone

The biosynthesis of most chromones originates from a pentaketide precursor (Dewick, 2006). In the case of the isolated compound 2, 5-dimethyl-7-hydroxychromone (**19**) the biosynthesis does not seem to arise from a pentaketide precursor but possibly originates from a hexaketide precursor as illustrated in (Figure 4.3) (Ayer and Racock, 1990).

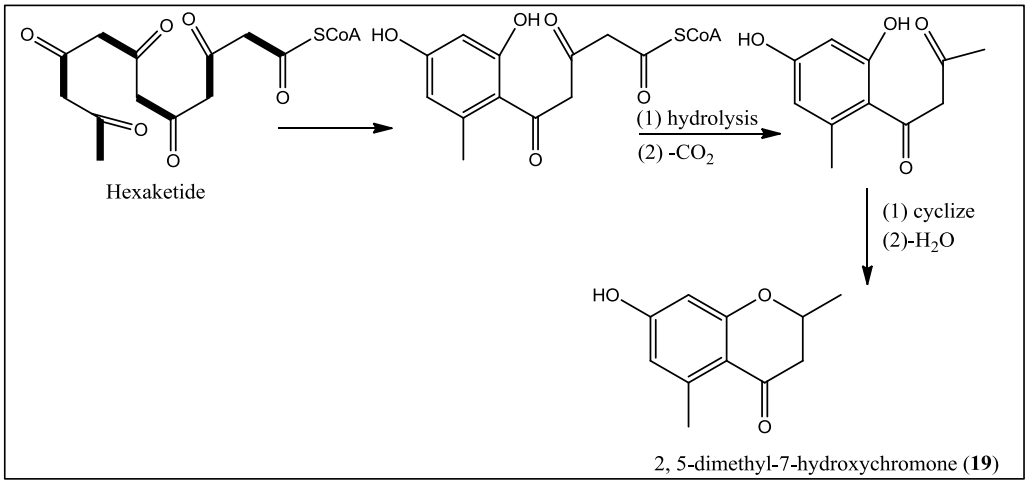


Figure 4.3: Postulated biosynthetic pathway of 2, 5-dimethyl-7-hydroxychromone (**19**).

4.4.1.3.2. Biosynthesis of altechromone B

The aromatic heptaketide is the biosynthetic precursor of altechromone B (**20**). The heptaketide precursor is followed by subsequent regiospecific C-8/C-13 aldol type cyclization of the heptaketide intermediate and the removal of a carboxyl group from the carboxyl terminal leads to formation of aloesone (2-acetonyl-7-hydroxy-5-methylchromone) (Abe *et al.,* 2006). Alterchromone B (**20**) is finally formed after methylation and oxidation processes (Figure 4.4).

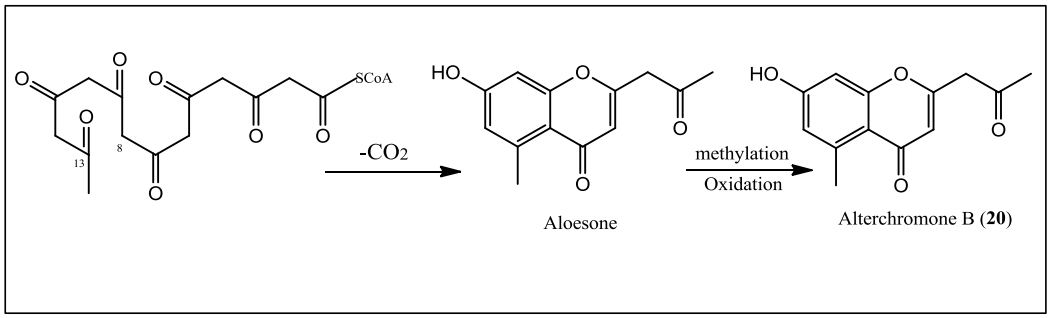


Figure 4.4: Postulated biosynthetic pathway of altechromone B (**20**).

4.4.1.4. Reduced perylenequinones

4.4.1.4.1. Biosynthesis of stemphytriol and altertoxin II

Alternaria species produce a variety of partially reduced perylenequinone derivatives which have also been isolated from the genus *Stemphylium*. Their biosynthesis so far occurs most probably via a phenol coupling of two pentaketide –derived moieties followed by reduction and hydroxylation in different positions to yield stemphytriol (**10**) and epoxidation to yield altertoxin II (**11**) (see Figure 4.5). In order to confirm this hypothesis, incorporation experiments of ^{13}C labelled sodium acetate in to these compounds were carried out (Alberto *et al.,* 1986; Okuno *et al.*1983).

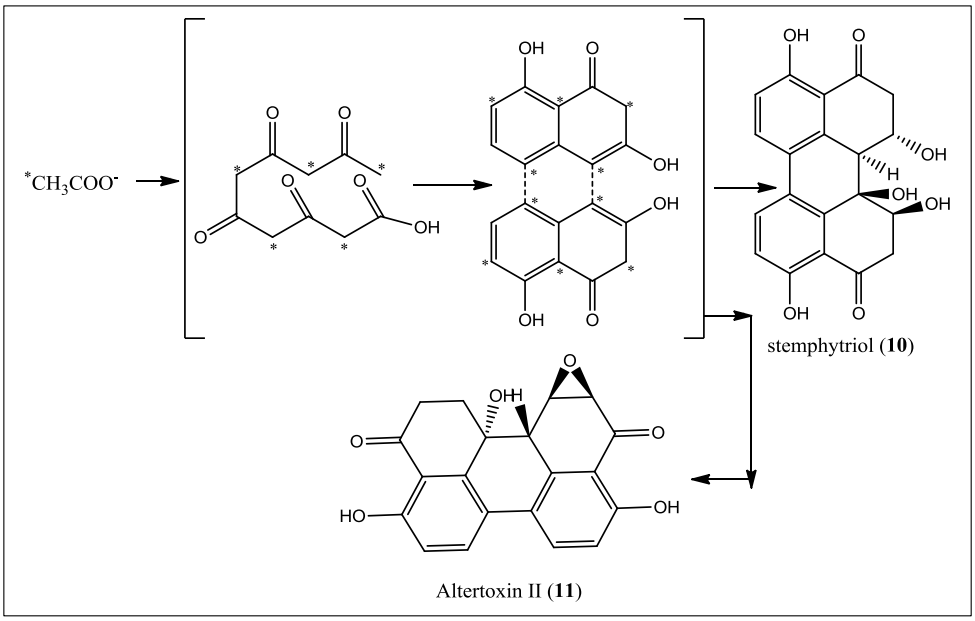


Figure 4.5: Possible biosynthetic pathway of reduced perylenequinones.

4.4.1.4.2. Bioactivity and mutagenicity of reduced perylenequinones

Altertoxin II (**11**) when subjected to MTT assay against mouse lymphoma (L5178Y) cells, showed a high cytotoxic activity. In contrast, stemphytriol (**10**) showed week cytotoxic activity against the same cell lines. Furthermore, this study showed that both compounds process significant antibacterial activity against *Staphylococcus aureus.*

In addition to dibenzo-α-pyrones, perylene quinones are also toxins generated by *Alternaria* fungi. Earlier studies have demonstrated that several congeners of this group are highly mutagenic for *Salmonella typhimurium* even in the absence of a metabolic activation system (Stack and Prival, 1986). Altertoxin II (**11**) has been reported as a very potent mutagen and DNA strand-breaking agent in Chinese hamster V79 cells, a potency exceeding that of alternariol (**6**). V79 are a mammalian cell line devoid of most xenobiotic-metabolizing enzyme activities including cytochrome P450 (Fleck *et al.,* 2012). It is hypothesized that the toxicity of altertoxin II (**11**) is due to the presence of the epoxy group (Alberto *et al.,* 1986).

4.4.2. Compounds isolated from the fungus *Penicillium* sp.

Penicillium is a large anamorphic ascomycetous fungal genus with widespread occurrence. The genus *Penicillium* produces a much diversified array of active secondary metabolites, including antibacterial (Lucas *et al.,* 2007), antifungal substances (Nicoetti, 2007), immunosuppressants, cholesterol-lowering agents (Kwon *et al.,* 2002), and also potent mycotoxins (Frisvad and Samson, 2004). Thousands of *Penicillium* isolates have probably been screened in bioprospecting programs since the discovery of penicillin and new bioactive metabolites continue to be discovered from these fungi nowadays indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industry (Petit *et al.,* 2009).

Chemical investigation of the ethyl acetate extract of the fungus grown on solid rice cultures lead to the isolation of the following pure compounds;
6-hydroxy-8-methoxy-3,5dimethylisochromanol(**22**),6-hydroxy-8-methoxy-3,5-dimethylisochroman(**23**), arohynapene D (**24**), 1,6-dihydroxy-3,5-dimethyl-8-methoxy-isochroman(**25**), 6-hydroxy-8-methoxy-3,5-dimethyl-3,4-dihydro-isocoumarin (**26**), 6-hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin (**27**), 6-methylcurvulinic acid (**28**), 2-methyl-Penicinoline (**29**), pretichodermamide C (**30**), N-methylpretichodermamide B (**31**), methyltriacetic lactone (**32**).

4.4.2.1. Isochroman derivatives

4.4.2.1.1. Biosynthesis of isochroman derivatives

Isochromans and isocoumarins (**22-27**) are derived from five acetate units (Figure 4.6). These units undergo aldol, aromatization and methylation reactions yielding mellein derivatives (**26** and **27)**. Further reduction and oxidation reactions yield the isochromans (**22-24)**. In compound (**22)** the C-4 hydroxy group is derived from molecular oxygen and is introduced in the benzylic oxidation of isochroman (**23)**. This evidence has been obtained from ^{17}O NMR spectroscopy which precludes epoxide intermediates in the biosynthesis of (**22**) and hydroxymellein derivatives (Abell *et al.,* 1987).

Kommentar [RY1]:

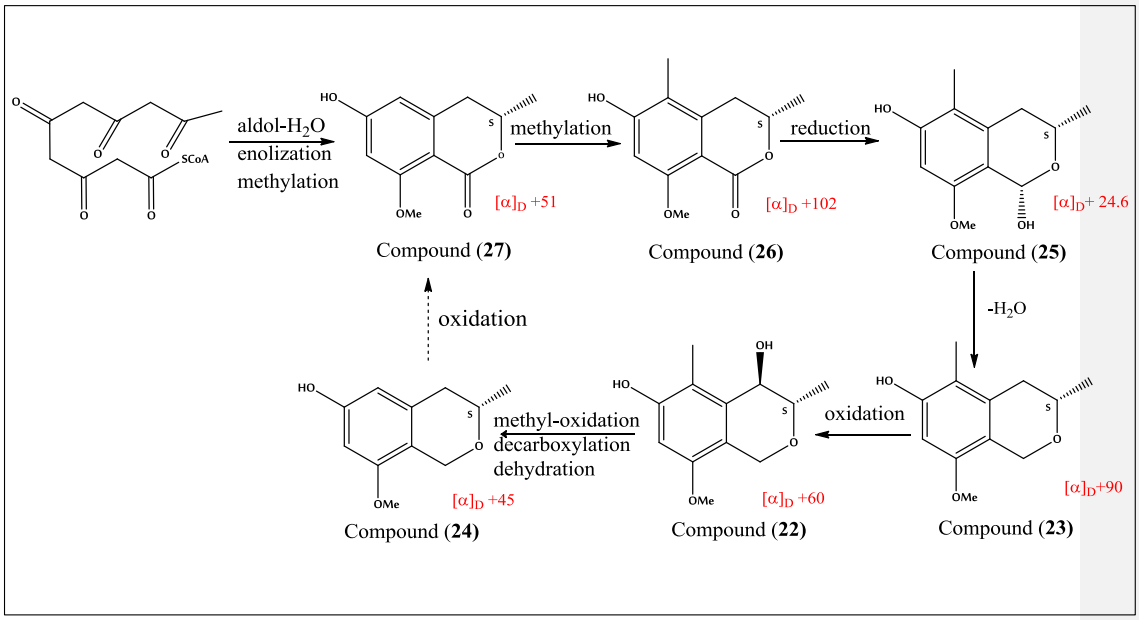


Figure 4.6: Postulated biosynthetic pathway of isochroman and isocoumarin derivatives (**22-27**).

4.4.2.1.2. Biological Activity of isochroman and isocumarin derivatives

Isochroman and isocumarin derivatives **22-27** when tested against L5178Y (mouse lymphoma) cell line showed moderate to weak cytotoxic activities (see Table 3.23). The most active derivative was 6-hydroxy-8-methoxy-3, 5-dimethyl-3,4-dihydro-isocoumarin (**26**). By reduction of the isocoumarin carbonyl the activity will decrease as in 1,6-dihydroxy-3,5-dimethyl-8-methoxyisochroman (**25**). Changing the position of the OH group from C-1 in the former compound to C-4 decreased the activity as in 6-hydroxy-8-methoxy-3,5-dimethylisochromanol (**22**). On the other hand, when the OH group was removed from both positions C-1 and C-4, the activity decreased also as shown in compound arohynapene D (**24**). The oxidation of arohynapene D (**24**) at C-1 to yield the isocoumarin carbonyl in compound 6-hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin (**27**) decreased the activity of this compound. Moreover, the methylation reaction at C-5 of the arohynapene D (**24**) resulted in decrease of the activity as shown in 6-hydroxy-8-methoxy-3,5-dimethylisochroman (**23**). SAR of these related compounds **22-27** have been illustrated in the scheme of (Figure 4.7).

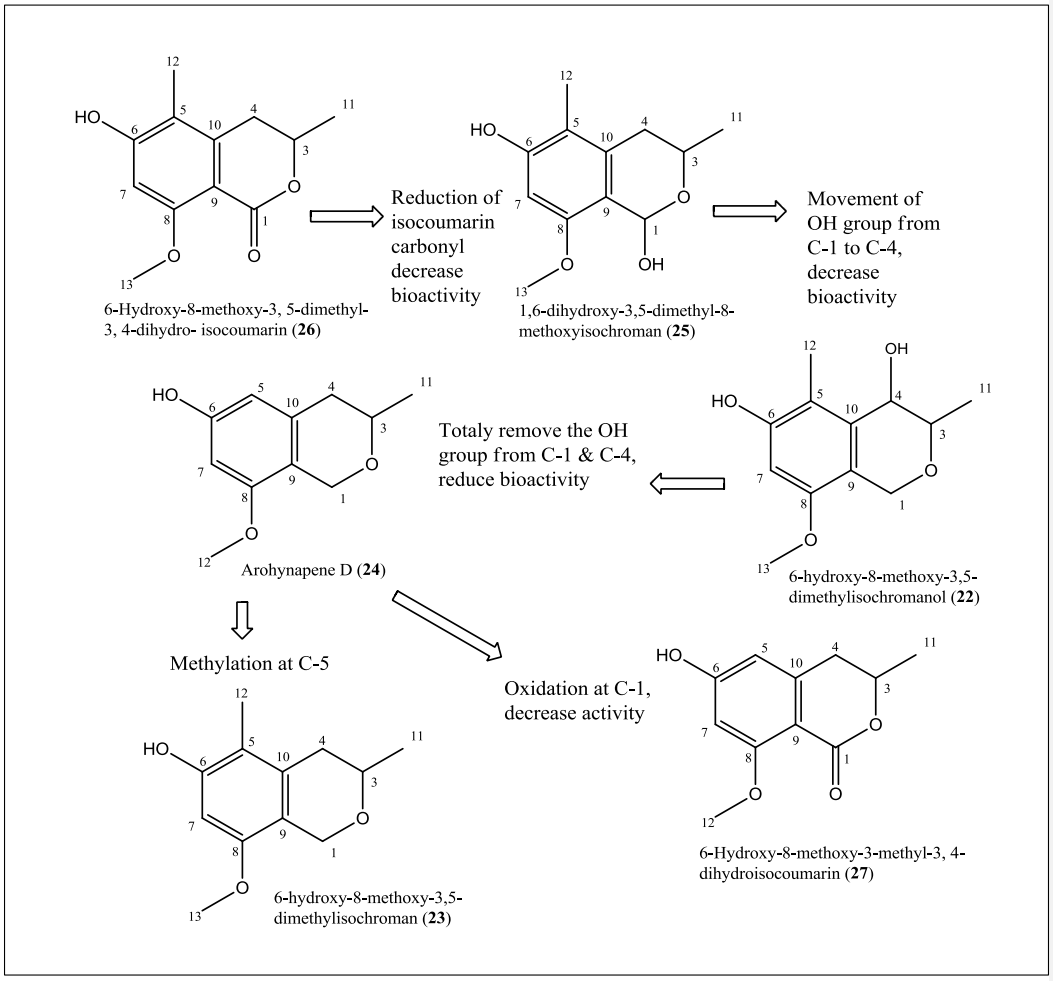


Figure 4.7: Structure-activity relationship of isochroman and isocumarin derivatives.

4.4.2.2. Biosynthesis of 6-methylcurvulinic acid

The 'acetate' is considered as the fundamental unit forming a head-to-tail linkage in the biosynthesis of curvulinic acid (Brich *et al.,* 1959). Moreover, 6-methylcurvulinic acid (**28**) is derived from a pentaketide moiety yielding the curvulinic acid (Peberdy, 1987) and this is followed by methylation at C-6 as shown in (Figure 4.8).

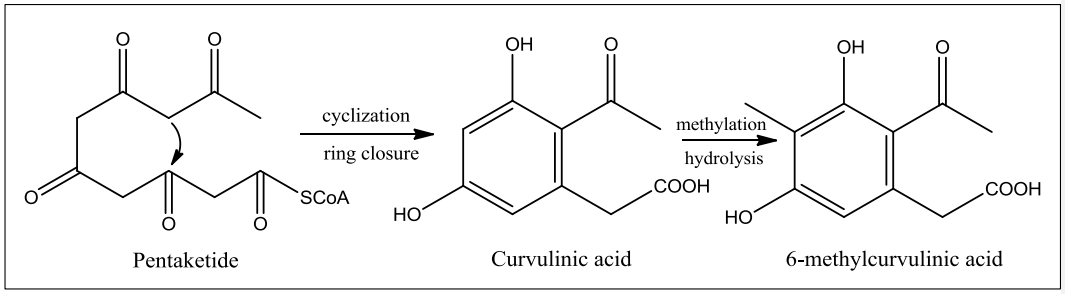


Figure 4.8: Postulated biosynthetic pathway of 6- methylcurvulinic acid (**28**).

4.4.2.3. Pyrrolyl 4-quinolinone alkaloid derivative

4.4.2.3.1. Biosynthesis of 2-methyl penicinoline

Quinolinone alkaloids, a group of secondary metabolites, are found more commonly in terrestrial materials than in marine organisms (Chang-Lun Shao *et al.,* 2010). 2-Methyl penicinoline (**29**) is an unusual pyrrolyl 4-quinolinone alkaloid that has been isolated recently from the fungus *Auxarthron reticulatum* derived from the marine sponge *Ircinia variabilis* (Elsebai *et al.,* 2011) and in this study it was isolated for the first time from *Penicillium* sp. Taking into account the structure of 2-methyl penicinoline (**29**), two amino acids ornithine and tryptophan might be the biosynthetic precursors. A plausible biogenetic pathway for 2-methyl penicinoline (**29**) is proposed in (Figure 4.9) (Chang-Lun Shao *et al.,* 2010).

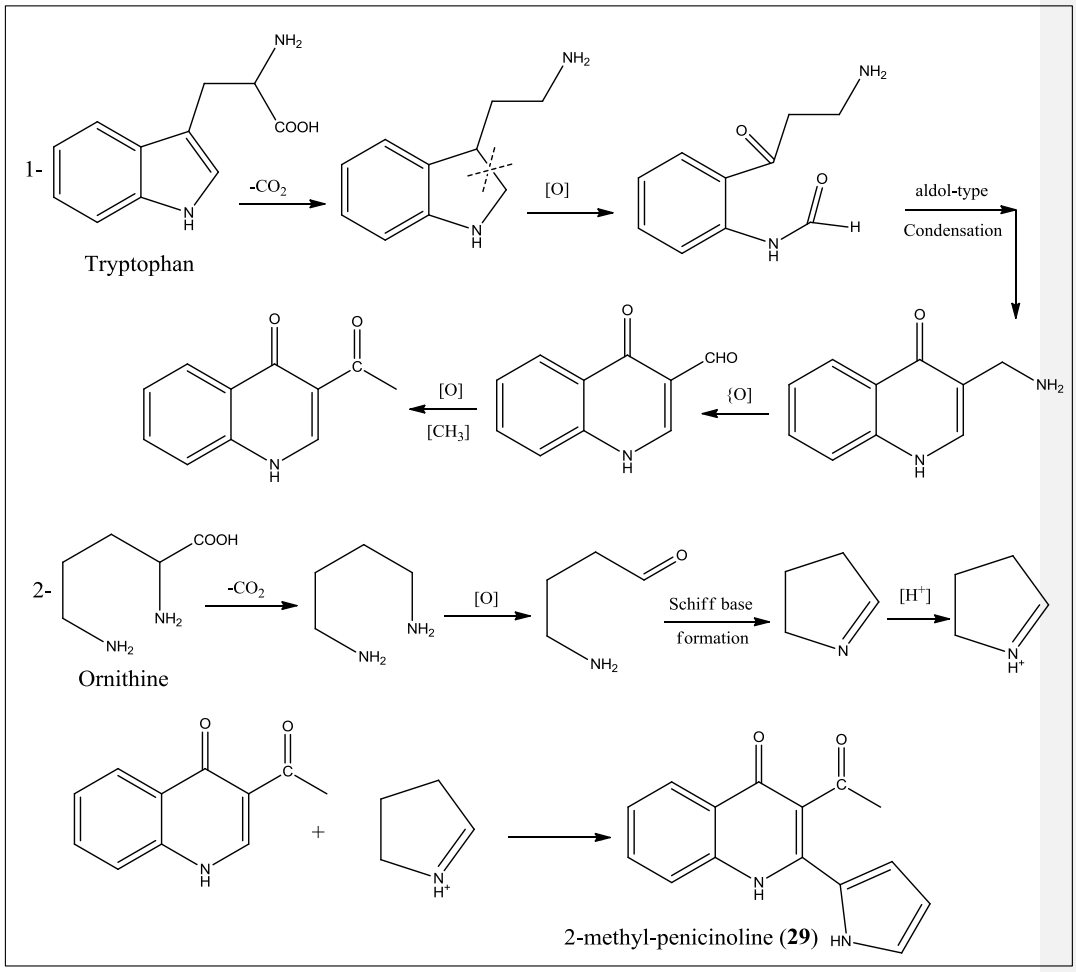


Figure 4.9: Possible biogenetic pathway to 2-methyl penicinoline (**29**).

4.4.2.4. Epipolythiodiketopiperazine alkaloids

4.4.2.4.1. Biosynthesis of pretichodermaide C and N-methylpretichodermamide B

Pretichodermaide C (**30**) and N-methylpretichodermamide B (**31**) are related to epipolythiodiketopiperazine alkaloids which were isolated for the first time in nature. They are the only sulfur containing compounds which were isolated from this study. The source of the sulfur refers to the presence of aerobic sulfide and autotrophic ammonia oxidizers in the lake of Wadi-El Natrun (Imhoff *et al.,* 1979). The structure of these two compounds is closely related to the known antibiotic gliovirin except for the presence of the epoxide moiety between C-4 and C-

5 instead of the free OH group in pretichodermaide C (**30**) and free Cl group in N-methylpretichodermamide B (**31**). Accordingly, the biosynthetic pathway of these former compounds is similar to gliovirin except for some additional reactions. Both halves of the diketopiperazine ring in (**30**) and (**31**) originate from L-phenylalanine. To confirm that hypothesis L-[1-^{13}C] phenylalanine (20% 1-^{13}C) was used in the culture media confirming that that L-phenylalanine is the biosynthetic precursor of gliovirin (Stipanovic *et al.,* 1994), pretichodermaide C (**30**) and N-methylpretichodermamide B (**31**) (Figure 4.10).

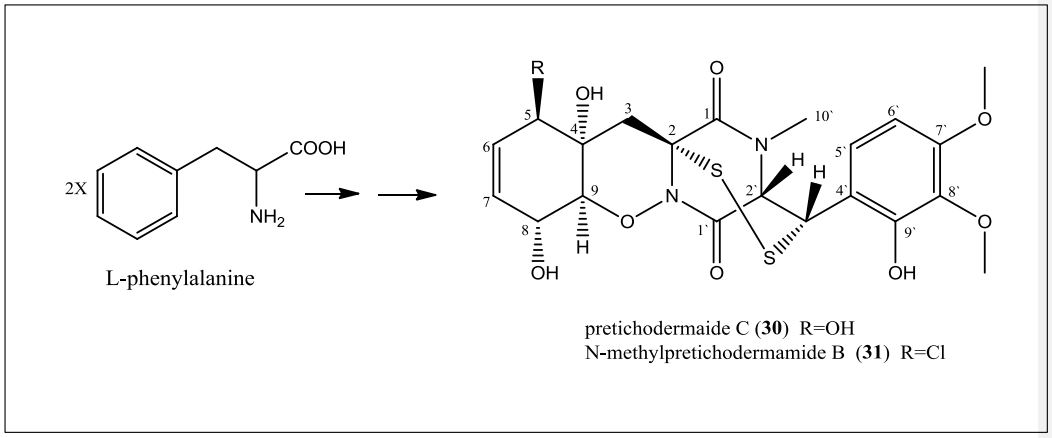


Figure 4.10: Postulated biosynthetic pathway of pretichodermaide C (**30**) and N-methylpretichodermamide B (**31**).

4.4.2.4.2. Bioactivity of N-methylpretichodermamide B

Epidithiodiketopiperazines (ETPs) are a group of diverse natural products that display potent biological effects characterized by a bridged disulfide linkage across the dioxopiperazine ring. These metabolites have been found to possess a broad spectrum of biological activities, including antibacterial (Berg *et al.,* 1976), antiviral (Deffieux *et al.,* 1978) and antifungal activities (Kato *et al.,*1969; Chikara *et al.,*1982).

N-Methylpretichodermamide B (**31**) displayed significant cytotoxicity in vitro against L5178Y mouse lymphoma cell line using the MTT assay with an IC_{50} of 2 µM, comparable with the positive control kahalaide F (IC_{50} 4.3 µM). Pretichodermamide C (**30**) was completely inactive in this bioassay, indicating that the introduction of a chlorine atom into one or more

specific positions of a biologically active molecule may substantially improve the intrinsic biological activity (Klaus, 2000). In fact, the chlorine substituent is crucial for significant biological activity in a number of natural products such as the antibiotics clindamycin (Birkenmeyer and Kagan, 1970), vancomycin (Harris *et al.,* 1985), chloramphenicol and griseofulvin (Crosse *et al.,* 1964), and in the antitumor compounds cryptophycin (Golakoti *et al.,* 1995), rebeccamycin (Bush *et al.,* 1987) and clavulon (Yamada and Nagoka, 1985).

4.5. Halotolerant fungi as a source of bioactive metabolites

Halotolerant fungal species have evolved unique metabolic mechanisms that are responsible to salt concentrations. The most significant challenge for these kinds of fungi is to prevent the loss of water from the cell into the saline environment and the accumulation of excess salt concentrations within the cell (Bugni and Ireland, 2004). For fungi to grow in the hypersaline environment, they must have osmoregulatory mechanisms that signal the production of polyols and amino compounds in conjunction with increasing the concentration of cytoplasmic ions through a so-called "salt-in strategy" which concentrates solutes within the cell in order to balance osmotic pressure. Their proteins are particularly rich in acidic amino acids which are enhancing resistance against structural collapse. Since the biosynthesis of these solutes for osmoregulation is energetically costly, fungi may exhibit decreased secondary metabolite production or slower rates of metabolite production in the presence of high salt concentrations (Bugni and Ireland, 2004).

In fact, some halotolerant fungi have possible important biotechnological applications arising from this adaption mechanisms. These mechanisms are at the level of their secondary metabolites, cell membrane, intracellular and extracellular enzymes, genetic transfer systems and intracellular osmolytes and especially of their compatible solutes which have a wide range of applications because of their ability to stabilize proteins and nucleic acids (Raghukumar, 2012). The extremely halotolerant black yeasts *T. salinum* and *H. werneckii* have been shown to produce extracellular hydrolytic enzymes that are active at high salt concentrations and that could therefore have important roles in different industries (Zalar *et al.,* 2005). *H. werneckii* also produces antibiotics that remain to be commercially exploited (Brauers *et al.,* 2001).

Interestingly, in this study the halotolerant *Penicillium* sp. fungal species can thrive in high sodium chloride concentration since the salt concentration of Wadi El-Natrun lakes is

mostly higher than that of sea water (3.3% total dissolved salts and about 30% w/v sodium chloride). In the present study, two new epipolythiodiketopiperazines were isolated one of which showed very potent cytotoxic activity**.** Indeed halotolerant fungi synthesize specific bioactive metabolites under stress conditions and particularly at increased salt concentrations where higher hemolytic activities are present. Overall these findings suggest that halotolerant fungal metabolites from hypersaline environments could have implications for drug discovery in the future.

5. Conclusions:

Fungi are among the most important groups of eukaryotic organisms that have played a pivotal role as sources for drug leads during the last century. Different environmental factors including different physical conditions and different biological situations in nature may change the behavior of the fungi and favor the production of diverse range of secondary metabolites. Accordingly, this study involved alternative biochemical studies of some unexplored ecological niches.

The first part of the thesis described the chemical investigation of the fungus *Alternaria sp.* an endophytic fungus isolated from the seeds of the traditional medicinal plant *Ziziphus jujube.* The fungus was cultivated in liquid Wickerham and on solid rice media, respectively.

1. *Alternaria* sp.

Twenty one metabolites have been obtained from *Alternaria* sp., isolated from the seeds of Ziziphus *jujube.* Five of them are isocoumarin derivatives that were obtained for the first time from this genus. Citreoisocoumarin proved to be active against Gram positive and MRSA bacteria. The remaining seventeen compounds isolated from the large scale rice cultures media included alternariol derivatives. 7-Methoxyphthalide-3-acetic acid has been isolated for the first time from nature. The alternariol derivatives and some of the structurally related compounds showed high cytotoxic activity when tested against L5178Y mouse lymphoma cell line as well as pronounced antibacterial activities.

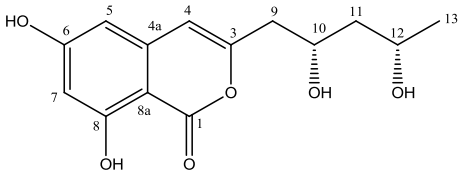
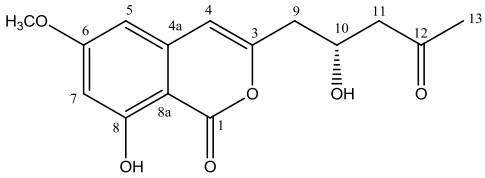
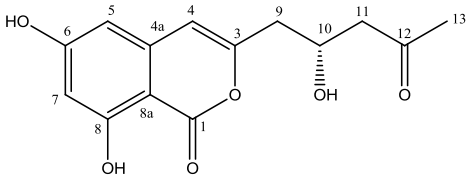
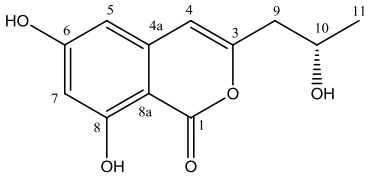
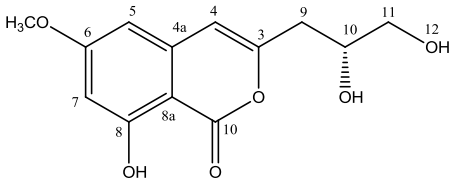
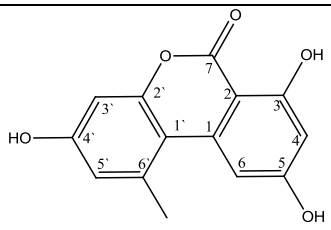
The second part of the thesis was devoted to halophilic fungi as sources of bioactive metabolites. As an example *Penicillium* sp. isolated from the sediments of hypersaline lake of Wadi El-Natrun, Egypt was chosen.

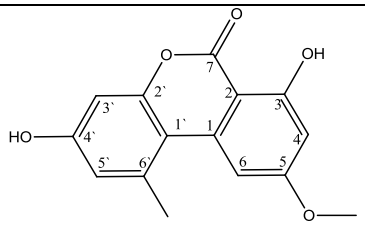
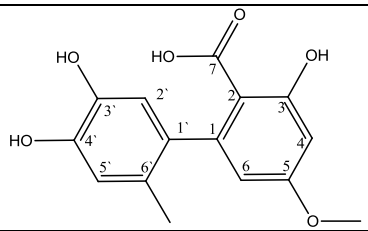
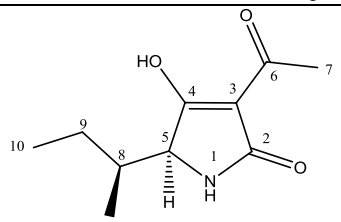
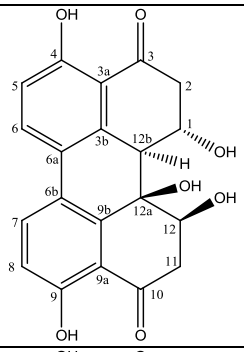
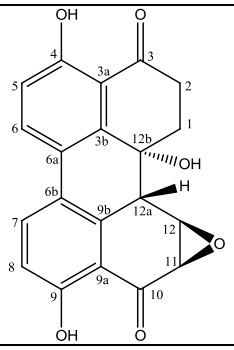
2. *Penicillium* sp.

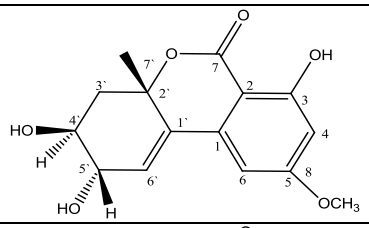
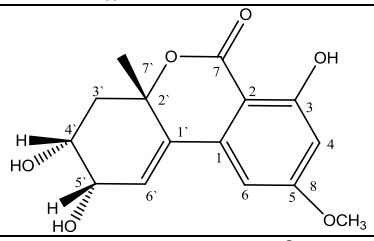
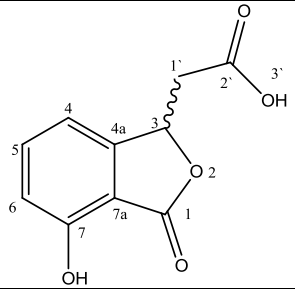
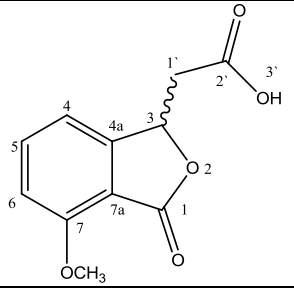
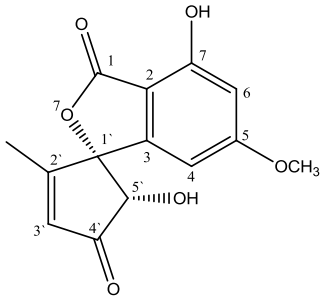
Eleven compounds were isolated from *Penicillium* sp. including five new compounds; two new isochromans, a new isocoumarine, and two new epidithiodiketopiperazine analogues named, pretichodermamide C and N-methylpretichodermamide B. The last compound proved to be highly active against the L5178Y cancer cell line that could be related in different assays.

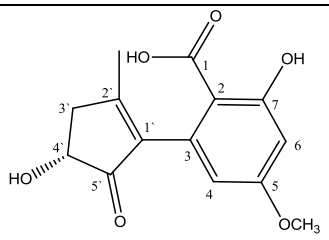
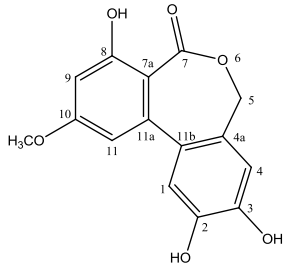
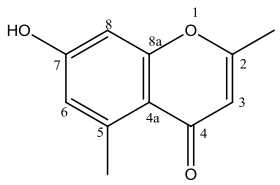
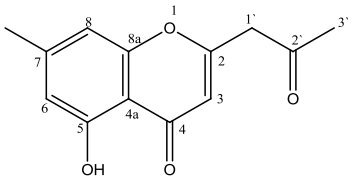
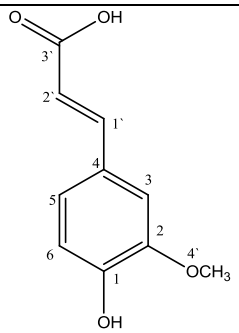
The results presented here support the notion of an ecology based-approach to selection of fungal species for chemical studies, as this demonstrates that such an approach can be effective in the discovery of new bioactive fungal metabolites.

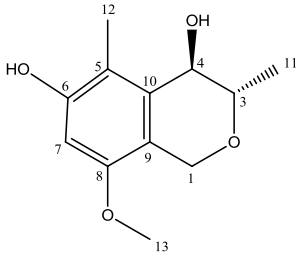
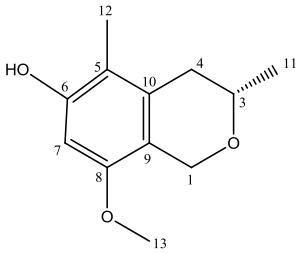
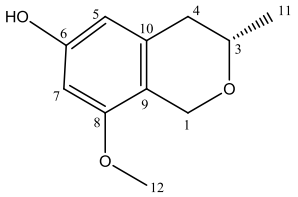
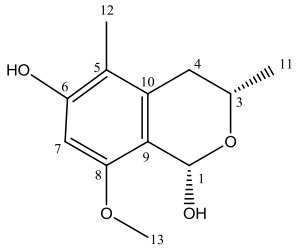
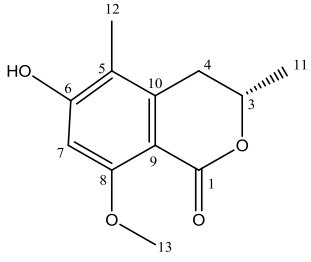
Table 5.1: Summary of the isolated compounds

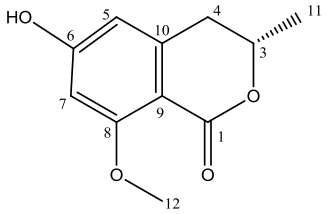
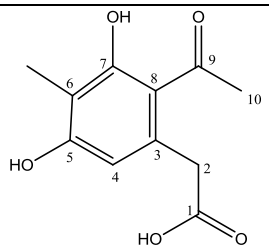
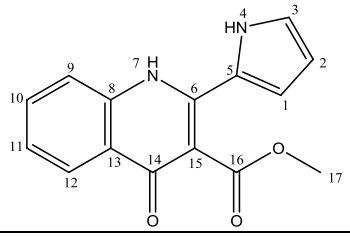
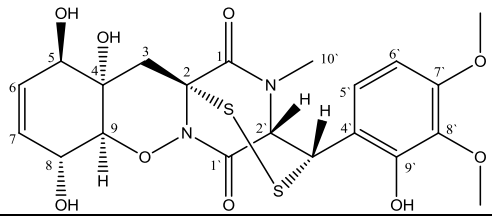
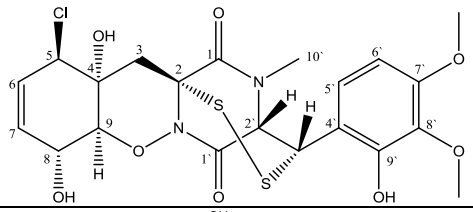
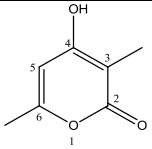
Compound name	Structure	Source	Comment
Citreoisocoumarinol		*Alternaria* sp.	Known
6-methyl-citreoisocoumarin		*Alternaria* sp.	Known
Citreoisocoumarin		*Alternaria* sp.	Known
Orthosporin		*Alternaria* sp.	Known
Diaportinol		*Alternaria* sp.	Known
Alternariol		*Alternaria* sp.	Known

Compound name	Structure	Source	Comment
Alternariol-5-*O*-methyl ether		*Alternaria* sp.	Known
Altenusin		*Alternaria* sp.	Known
Tenuazonic acid		*Alternaria* sp.	Known
Stemphytriol		*Alternaria* sp.	Known
Altertoxin II		*Alternaria* sp.	Known

Compound name	Structure	Source	Comment
Altenuene		*Alternaria* sp.	Known
4`-epialtenuene		*Alternaria* sp.	Known
Isoochracinic acid		*Alternaria* sp.	Known
7-methoxyphthalide-3-acetic acid		*Alternaria* sp.	New
Talaroflavone		*Alternaria* sp.	Known

Compound name	Structure	Source	Comment
Alteric acid		*Alternaria* sp.	Known
Alterlactone		*Alternaria* sp.	Known
2,5-dimethyl-7-hydroxychromone		*Alternaria* sp.	Known
Altechromone B		*Alternaria* sp.	Known
Ferulic acid		*Alternaria* sp.	Known

Compound name	Structure	Source	Comment
6-hydroxy-8-methoxy-3,5-dimethyl isochromanol		*Penicillium* sp.	New
6-hydroxy-8-methoxy-3,5-dimethyl isochroman		*Penicillium* sp.	Known
(+)-Arohynapene D		*Penicillium* sp.	New
1,6-dihydroxy-3,5-Dimethyl-8-methoxy-isochroman		*Penicillium* sp.	Known
6-hydroxy-8-methoxy-3,5-dimethyl 3,4-dihydroisocoumarin		*Penicillium* sp.	Known

Compound name	Structure	Source	Comment
6-hydroxy-8-methoxy-3-methyl 3,4-dihydro-isocoumarin		*Penicillium* sp.	New
6-methylcurvulinic acid		*Penicillium* sp.	Known
2-methyl penicinoline		*Penicillium* sp.	Known
Pretichodermamide C		*Penicillium* sp.	New
N-methylpreticho-dermamide B		*Penicillium* sp.	New
Methyltriacetic lactone		*Penicillium* sp.	Known

6. References:

Abe, I., Watanabe,T., Lou, W., and Noguchi, H. (2006). Active site resides governing substrate selectivity and polyketide chain length in aloesone synthase. *FEBS Journal*., **273**, 208-218.

Abell, C., Sutkowski, A. C., and Staunton, J. (1987). Studies on the biosynthesis of hydroxymellein using ^{17}O NMR and ^{2}H NMR spectroscopy to determine the origin of the C-4 hydroxy group. *J. Chem. Soc. Chem. Commun.*, **8**, 586-587.

Akone, S. H., El Amrani, M., Lin, W. H., Lai, D., and Proksch, P. (2013). Cytosporins F–K, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*. *Tetrahedron Letters*., **54**, 6751-6754.

Almeida, C., Ortega, H., Higginbotham, S., Spadafora, C., Arnold, A. E., Coley, P. D., Kursar, T. A., Gerwick, W. H., and Cubilla-Rios, L. (2014). Chemical and bioactive natural products from *Microthyriaceae* sp., an endophytic fungus from a tropical grass. *Letters in Applied Microbiology*., **59**, 58–64.

Alvin, A., Miller, K. I., and Neilan, B. A. (2014). Exploring the potential of endophytes from medicinal plants assources of antimycobacterial compounds. *Microbiological Research*., **169**, 483–495.

Aly, A. H., Debbab, A., Kjer, J., and Proksch, P. (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Divers.*., **41**, 1–16.

Aly, A. H., Debbab, A., and Proksch. P. (2011). Fifty years of drug discovery from fungi review. *Fungal Divers*., **50**, 3–19.

Aly, A. H., Debbab, A., and Proksch, P. (2011). Fungal endophytes: unique plant inhabitants with great promises. *Appl. Microbiol. Biotechnol.*, **90**, 1829–1845.

Aly, A. H., Debbab, A., and Proksch, P. (2013). Fungal endophytes secret producers of bioactive plant metabolites. *Die Pharmazie*., **68**, 499-505.

Arnone, A., Nasini, G., Merlini, L., and Assante, G. (1986). Secondary mould metabolites. Part 16. Stemphyltoxins, new reduced perylenquinone metabolites from *Stemphlium botryosum* Var. Lactucum. *J. Chem. Soc. Perkin Trans.*, **1**, 525-530.

Asai, T., Yamamoto, T., and Oshima, Y. (2011). Histone deacetylase inhibitor induced the production of three novel prenylated tryptophan analogs in the entomopathogenic fungus, *Torrubiella luteorostrata*. *Tetrahedron Lett.*, **52**, 7042–7045.

Asai, T., Chung, Y. M., Sakurai, H., Ozeki, T., Chang, F. R., Wu, Y. C., Yamashita, K., and Oshima, Y. (2012). Highly oxidized ergosterols and isariotin analogs from an entomopathogenic fungus, *Gibellula formosana*, cultivated in the presence of epigenetic modifying agents. *Tetrahedron*., **68**, 5817-5823.

Ayar, W. A., and Racok, J. S. (1990). The metabolites of *Talaromyces flavus*: Part 1. Metabolites of the organic extracts. *Can. J. Chem.*, **68**, 2085-2094.

Bacon, C. W., and White, J. F. (2000). *Microbial endophytes*. Marcel Dekker, Inc., New York, pp. 341–388.

Baraban, E. G., Morin, J. B., Phillips, G. M., Phillips, A. J., and Strobel, S. A. (2013). Xyolide, a bioactive nonenolide from an Amazonian endophytic fungus, *Xylaria feejeensis*. *Tetrahedron Letters*., **30**, 1-4.

Barry, M., Gordon, T. and Jeffery, M. (1980). Regiocontrolled synthesis of hydroxyphathalides. syntesis of (±)-Isoochracinic acid and a zealeranone Intermediate. *J. Org. Chem.*, **45**, 1835-1838.

Barton, D., and Meth-Cohn, O. (1999). *Comprehensive natural products chemistry*, Elsevier, Inc., Philadelphia, U.S, p. 367.

Bashyal, B., Li, J. Y., and Strobel, G. A., and Hess, W. M. (1999). Seimatoantlerium nepalense, an endophytic taxol producing coelomycete from Himalayan yew (*Taxus wallachiana*). *Mycotaxon*., **72**, 33–42.

Bensassi, F., Gallerne, C., Sharaf El-Dein, O., Hajlaoui, M. R., Bacha, H., and Lemaire, C. (2012). Cell death induced by the *Alternaria* mycotoxin alternariol. *Toxicol. In Vitro*., **26**, 915–923.

Berg, D. H., Massing, R. P., Hoehn, M. M., Boeck, L. D. and Hamil, R. L. (1976). A 30641, a new epidithiodiketopiperazine with antifungal activity. *J. Antibiotics*., **29**, 394-397.

Berg, G., and Hallmann, J. (2006). *Control of plant pathogenic fungi with bacterial endophytes*, Springer-Verlag, Inc., Berlin, pp. 53–69.

Birch, A. J., Musgrave, O. C., Richards, R. W. and Smith, H. (1959). Studies in relation to biosynthesis. Part XX. The structure and biosynthesis of curvularin. *J. Chem. Soc.*, 3146-3152.

Birkenmeyer, R. D., and Kagan, F. (1970). Lincomycin. XI. Synthesis and structure of clindamycin. A potent antibacterial agent. *J. Med. Chem.*, **13**, 616-619.

Bode, H. B., Bethe, B., Hofs, R., and Zeeck, A. (2002). Big effects from small changes: Possible ways to explore nature's chemical diversity. *Chem. Bio. Chem.*, **3**, 619-627.

Bottalico, A., and Logrieco, A. (1998). *Mycotoxins in agriculture and food safety*, Marcel Dekker, Inc., New York, p.65.

Bradburn, N., Coker, R. D., Blunden, G., Turner, C. H., and Crabb, T. A. (1994). 5`-Epialtenuene and neoaltenuene, dibenzo-alpha-pyrones from *Alternaria alternata* cultered on rice. *Phytochemistry*, **35**, 665-669.

Brahmachari, G. (2013). *Natural products in drug discovery: Impacts and opportunities-an assessment*, world scientific publishing Co., Inc., Singapore, pp. 11-12.

Brauers, G., Ebel, R., Edrada, R., Wray, V., Berg, A., Grafe, U., and Proksch, P. (2001). Hortein, a new natural product from the fungus *Hortaea werneckii* associated with the sponge *Aplysina aerophoba*. *J. Nat. Prod.,* **64**, 651-652.

Brugger, E. M., Wagner, J., Schumacher, D. M., Koch, K., and Podlech, J. (2006). Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. *Toxicol Lett*., **164**, 221–230.

Bugni, T. S. and Ireland, C. M. (2004). Marine derived fungi: a chemically and biologically diverse group of mucroorganisms. *Nat. Prod. Rep.*, **21**, 143-163.

Bush, J. A., Long, B. H., Catino, J. J., and Bradner, W. T. (1987). Production and biological activity of rebeccamycin, a novel antitumor agent. *J. Antibiot.*, **40**, 668-678.

Butinar, L., Santos, S., Spencer-Martins, L., Oren, A. and Gunde-Cimerman, N. (2005a). Yeast diversity in hypersaline habitats. *FEMS Microbiology Letters*., **244**, 229–234.

Butinar, L., Sonjak, S., Zalar, P., Plemenitas, A., and Gunde-Cimerman, N. (2005b). Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns. *Bot. Mar.*, **1**, 73– 79.

Butler, M. S. (2004). The role of natural product chemistry in drug discovery. *J. Nat. Prod.*, **67**, 2141–2153.

Butler, M. S. (2005). Natural products to drug: Natural product-derived compounds. *Nat. Prod. Rep.*, **22**, 162–195.

Canfield, D. E., Sørensen, K. B. and Oren, A. (2004). Biogeochemistry of a gypsum-encrusted microbial ecosystem. *Geobiol.*, **2**, 133-150.

Cannall, R. J. P. (1998). *Methods in biotechnology, natural products isolation*, NJ: Humana Press, Inc., Totowa, p.1.

Cantrell, S. A., Dianese, J. C., Fell, J., Gunde-Cimerman, N., and Zalar, P. (2011). Unusual fungal niches. *Mycologia*., **103**, 1161-1174.

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of radiosensitivity. *Cancer Res.*, **47**, 943-946.

Caumette, P. (1993). Ecology and physiology of phototrophic bacteria and sulphate-reducing bacteria in marine salterns. *Experientia*., **49**, 473-481.

Chang-lun, S., Wang, C. Y., Gu, Y. C., Wei, M. Y., Pan, J. H., Deng, D. S., She, Z. G., and Lin, Y. C. (2010). Penicinoline, a new pyrrolyl 4-quinolinone alkaloid with an unprecedented. *Bioorganic & Medicinal Chemistry Letters*., **20**, 3284-3286.

Chen, J., and Chen, T. (2003). *Chinese medical herbology and pharmacology*, Art of Medicine Press., Inc., England, pp. 770-771.

Chen, H. J., Awakawa, T., Sun, J. Y., Wakimoto, T., and Abe, I. (2013). Epigenetic modifier-induced biosynthesis of novel fusaric acid derivatives in endophytic fungi from *Datura stramonium* L. *Natural Products and Bioprospecting*, **3**, 20-23.

Chikara, M., Kazuteru, Y., Tamotsu, F., and Hiromi, B. M. (1982). A new epidithiodiketopiperazine. *J. Antibiotics*, **35**, 374-377.

Chong, Y. S. and Huang, H. H. (1986). A physicochemical study of isocoumarin, 3,4-dihydroisocoumarin, and bis-3,4-dihydroisocoumarin by dipole moment, infrared, ultraviolet, and polarographic measurements. *J. Chem. Soc., Perkin Trans. 2*, **12**, 1875-1879.

Christensen, M. J., Bennett, R. J., and Schmid, J. (2002). Growth of Epichloë/Neotyphodium and p-endophytes in leaves of *Lolium* and *Festuca* grasses. *Mycol. Res.*, **106**, 93–106.

Chung, Y. M., Wei, C. K., Chuang, D. W., El-Shazly, M., Hsieh, C. T., Asai, T., Oshima, Y., Hsieh, T. J., Hwang, T. L., Wu, Y. C., and Chang, F. R. (2013). An epigenetic modifier enhances the production of anti-diabetic and anti-inflammatory sesquiterpenoids from *Aspergillus sydowii. Bioorganic & Medicinal Chemistry*, **21**, 3866-3872.

Cole, R. J. (1981). *Handbook of toxic fungal metabolites*. Academic Press., Inc., New York-London, pp.614-645.

Coombe, R. G., Jacobs, J. J., and Watson, T. R. (1970). Metabolites of some *Alternaria* species. The structures of altenusin and dehydroaltenusin. *Aust. J. Chem.*, **23**, 2343-2351.

Cota, B. B., Rosa, L. H., Caligiorne, R. B., Rabello, A. L. T., Alves, T. M. A., Rosa, C. A., and Zani, C. L. (2008). Altenusin, a biphenyl isolated from the endophytic fungus *Alternaria* sp., inhibits trypanothione reductase from *Trypanosoma cruzi*. *FEMS Microbiol. Lett.*, **285**, 177-182.

Cragg, G. M., and Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochim. Biophys. Acta.*, **1830**, 3670–3695.

Crawford, J. M., and Townsend, C. A. (2010). New insights into the formation of fungal aromatic polyketides. *Nat Rev Microbiol*., **8**, 879–889.

Crosse, R., McWilliams, R., and Rhodes, A. (1964). Some relations between chemical structure and antifungal effects of griseofulvin analogues. *J. Gen. Microbiol.*, **34**, 51-65.

Cynthia, M. H., Yali, F. H., Jon, C., Doug, S. K. and Gary, S. (1989). Phytotoxins from *Alternaria Cassiae*. *Phytochemistry*, **28**, 73-75.

Das sarma, S., and Arora, P. (2001). *A general review on halophiles. In encyclopedia of life sciences*, Nature Publishing Group, Inc., London, pp. 458-466.

David, W., and David, P. (1977). A total synthesis of (±)-Iso-ochranic acid, a hydroxy phthalide from *Alternaria kikuchiana*. *Tetrahydron Letters*, **51**, 4543-4544.

Debbab, A., Aly, A. H., and Proksch, P. (2012). Endophytes and associated marine derived-ecological and chemical perspectives. *Fungal Diversity*, **57**, 45–83.

Debbab, A., Aly, A. H., and Proksch, P. (2013). Mangrove derived fungal endophytes–a chemical and biological perception. *Fungal Diversity*, **61**, 1-27.

Deffieux, G., Baut, M. J., and Filleau, R. (1978). New antibiotics from the fungus *Epicoccum nigrum*. III. Epicorazine B: Structure elucidation and absolute configuration. *J. Antibiotics*, **31**, 1106-1109.

Dethoup, T., Manoch, L., Kijjoa, A., Pinto, M., Gales, L., Damas, A. M., Silva, A. M. S., Eatpn, G., and Herz, W. (2007). Merodrimanes and other constituents from *Talaromyces thailandiasis*. *J. Nat. Prod.*, **70**, 1200-1202.

Devys, M., and Barbier, M. (1992). Isolation of the new (-)-(3*R*,4*S*)-4-hydroxymellein from the fungus *Septoria nodorum* berk. *Z. Naturforsch*, **47**, 779-781.

Dewick, P. M. (2006). *Medicinal natural products. A biosynthetic approach*, John Wiley & Sons Ltd, Baffins Lane, Chichester, England.

Dias, D. A., Urban, S. and Roessner, U. (2012). A historical overview of natural products in drug discovery. *Metabolites*, **2**, 303-336.

Dimitriadis, C., Gill, M. and Harte, M. F. (1997). The first stereospecific approach to both enantiomers of mellein. *Tetrahedron*, **8**, 2153-2158.

Du, L., Zhu, T., Fang, Y., Liu, H., Gu, Q., and Zhu, W. (2007). Aspergiolide A, a novel anthraquinone derivative with naphtho[1,2,3-de]chromene-2,7-dione skeleton isolated from a marine-derived fungus *Aspergillus glaucus*. *Tetrahedron*, **63**, 1085–1088.

Ebada, S. S., and Proksch, P. (2013). *Bioactive secondary metabolites from marine-derived fungi. In: Marine Pharmacognosy: Trends and Applications*, CRC Press Taylor & Francis Group, LLC, Boca Raton, pp. 27-51.

Ebrahim, W., Aly, A., Wray, V., Proksch, P., and Debbab, A. (2013). Unusual octalactones from *Corynespora cassiicola*, an endophyte of *Laguncularia racemosa*. *Tetrahedron Letters*, **54**, 6611-6614.

El-neketi, M., Ebrahim, W., Lin, W., Gedara, S., Badria, F., Saad, H. A., Lai, D. and Proksch, P. (2013). Alkaloids and polyketides from *Penicillium citrinum*, an endophyte isolated from the Moroccan plant *Ceratonia siliqua*. *J. Nat. Prod.*, **76**, 1099-1104.

Elsebai, M. F., Rempel, V., Schnakenburg, G., Kehraus, S., Muller, C. E., and Konig, G. M. (2011). Identification of a potent and selective cannabinoid CB1 receptor antagonist from *Auxarthron reticulatum*. *ACS Medicinal Chemistry Letters*, **2**, 866-869.

Eyberger, A. L., Dondapati, R., and Porter, J. R. (2006). Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *J. Nat. Prod.*, **69**, 1121–1124.

Fabbretti, A., Gualerzi, C. O., and Brandi, L. (2011). How to cope with the quest for new antibiotics. *FEBS Lett.*., **585**, 1673–1681.

Fernanda, O. C., Luís, G. D., and Mônica, T. P. (2013). A mixed culture of endophytic fungi increases production of antifungal polyketides. *Journal of Chemical Ecology*, **39**, 1335-1342.

Fiedurek, J., Gromada, A., and Jamroz, J. (1996). Effect of medium components and metabolic inhibitors on β-galactosidase production and secretion by *Penicillium notatum* 1. *J Basic Microb*., **36**, 27-32.

Firn, R. D. and Jones, C. G. (2003). Natural products a simple model to explain chemical diversity. *Nat. Prod. Rep.*, **20**, 382-391.

Fleck, S. C., Burkhardt, B., Pfeiffer, E., and Metzler, M. (2012). Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. *Toxicol. Lett.*, **214**, 27−32.

Flores-bustamante, Z. R., Orduña, F. N., Cárdenas, A. M. and Cotera, L .B. (2010). Microbial paclitaxel: advances and perspectives. *J. Antibiot.*, **63**, 460–467.

Freeman, G. (1965). Isolation of alternariol and alternariol monomethyl ether from *Alternaria Dauci* (Kuhn) Groves and Skolko. *Phytochemisry*, **5**, 719-725.

Frisvad, J. C. and Samson, R. A. (2004). Polyphasic taxonomy of *Penicillium subgenus Penicillium*: A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Studies in Mycology*, **49**, 1-174.

Gabardi, S. and Cerio, J. (2004). Future immunosuppressive agents in solid-organ transplantation. *Prog. Transplant.*, **14**, 148-156.

Gamboa, M. A., Laureano, S., and Bayman, P. (2002). Measuring diversity of endophytic fungi in leaf fragments: does size matter? *Mycopathologia*, **156**, 41–45.

Gatenbeck, S., and Hermodsson, S. (1965). Enzymic synthesis of the aromatic product alternariol. *Acta. Chem. Scand.,* **19**, 65–71.

Gatenbeck, S., and Sierankiewicz, J. (1973). On the biosynthesis of tenuazonic acid in *Alternaria tenuis*. *Acta Chem. Scand.*, **27**, 1825-1827.

George, A. E., Maurice, L., Nancy, A. P., Ronald, T. H. and William, J. M. (1978). New zearlenone related macrolides and isocoumarins from an unidentified fungus. *J. Org. Chem.*, **43**, 2339-2343.

Gitterman, C. O. (1965). Antitumor, cytotoxic, and antibacterial activities of tenuazonic acid and congeneric tetramic acids. *J. Med. Chem.*, **8**, 483–486.

Godtfredsen, W. O., Jahnsen, S., Lorck, H., Roholt, K., and Tybring, L. (1962). Fusidic acid; a new antibiotic. *Nature*, **193**, 987.

Golakoti, T., Ogino, J., Heltzel, C. E., Le Husabo, L., Graig, C. M., Larsen, L. K., Patterson, G., Moore, R. E., and Mooberry, S. L. (1995). Structure determination, conformational analysis, chemical stability studies, and antitumor evaluation of the cryptophycins. Isolation of 18 new analogs from *Nostoc* Sp. strain GSV224. *J. Am. Chem. Soc*., **117**, 12030-12040.

Gonçalves, V. N., Vaz, A. B., Rosa, C. A., and Rosa, L. H. (2012). Diversity and distribution of fungal communities in lakes of Antarctica. *FEMS Microbiol Ecol.*, **82**, 459-471.

Griffin, G. F., and Chu, F. S. (1983). Toxicity of the *Alternaria* metabolites alternariol, alternariol methyl ether, altenuene, and tenuazonic acid in the chicken embryo assay. *Appl. Environ. Microbiol.*, **46**, 1420–1422.

Grove, J. F., MacMillan, J., Mulholland, T. P. C., and Rogers, M. A. T. (1952). Griseofulvin. Part IV. Structure. *J. Chem. Soc.*, 3977–3987.

Gulder, T. A., Freeman, M. F., and Piel, J. (2011). *The catalytic diversity of multimodular polyketide synthases: Natural product biosynthesis beyond textbook assembly rules*, Top Curr Chem, pp. 1–53.

Gunatilaka, A. A. L. (2006). Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *Journal of Natural Products*, **69**, 509-526.

Gunde-cimerman, N., Zalar, P., de Hoog, S., and Plemenitas, A. (2000). Hypersaline waters in salterns: natural ecological niches for halophilic black yeasts. *FEMS Microbiol. Ecol.*, **32**, 235–240.

Gunde-cimerman, N., and Plemenitas, A. (2006). Ecology and molecular adaptation of the halophilic black yeast *Hortaea werneckii*. *Rev. Environ. Sci. Biotechnol.*, **5**, 323–331.

Guochun, H., Hideyuki, M., Tetsuya, T., Shinji, K. and Teruhiko, Y. (2004). A new antifungal metobolite from *Penicillium expansum*. *J.Nat. Prod.*, **67**, 1084-1087.

Hallock, Y. F., Clardy, J., kenfield, D. S., and Strobel, G. (1988). De-O-methyldiaporthin, a phytotoxin frm *Drechslera siccans*. *Phytochemistry*, **27**, 3123-3125.

Hao, X., Pan, J., and Zhu, X. (2013). *Taxol producing fungi. In natural products*, Springer-Verlag Berlin Heidelberg, pp. 2797–2812.

Harris, C., Kannan, R., Kopecka, H., and Harris, T. M. (1985). The role of chlorine substituents in the antibiotic vancomycin: preparation and characterization of mono- and di-dechlorovancomycin. *J. Am. Chem. Soc.*, **107**, 6652-6658.

Hartley, S. E., and Gange, A. C. (2009). Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. *Annu. Rev. Entomol.*., **54**, 323–342.

Hassan, A. (2007). Novel natural products from endophytic fungi of Egyptian medicinal plants, chemical and biological characterization. *Dissertation*.

Hawksworth, D. L. (2004). Fungal diversity and its implications forgenetic resource collections. *Studies in Mycology*., **50**, 9–18.

Heinig, U., Scholz, S., and Jennewein, S. (2013). Getting to the bottom of taxol biosynthesis by fungi. *Fungal Diversity*, **60**, 161–70.

Herrera-carillo, Z., Torres, M. S., Singh, A. P., Vorsa, N., Gianfagna, T., Meyer,W, and White, J. F. (2009). *Phenolic, flavonoid and antioxidant profiling for cool-season grasses with and without endophyte. Proceedings of the 18th annual rutgers Turfgrass Symposium,* New Brunswick, Rutgers University, p. 43.

Hirofumi, N., Mitsunobu, H., Tsuyoshi, M., Katsuhiko, A., Yutaka, S., and Makoto, M. (1990). DC1149B, DC1149R, and their manufacture with Trichoderma. *Jpn. Kokai Tokkyo Koho*., JP 02218686 A 19900831.

Hocking, A. D. (1993). *Responses in xerophilic fungi to changes in water activity tolerance of Fungi*, Marcel Dekker, Inc. New York, pp.233-243.

Huang, W. Y., Cai, Y. Z., Hyde, K. D., Corke, H., and Sun, M. (2007a). Endophytic fungi from *Nerium oleander* L (Apocynaceae): main constituents and antioxidant activity. *World J Microbiol Biotechnol*., **23**, 1253–1263.

Huang, Y. F., Tian, L., Hua, H. M. and Pei, Y. H. (2007b). Two diketopiperazines from marine fungus Gliocladium sp. YUP08. *J. Asian Nat. Prod. Res.*, **9**, 197–201.

Hyde, K. D. (2005). *Tropical fungi*, Taylor and Francis, Boca Raton, pp.93–115.

Hyde, K. D., and Soytong, K. (2008). The fungal endophyte *dilemma*. *Fungal Divers*., **33**, 163–173.

Ichihara, A., Hashimoto, M., Hirai, T., Takeda, I., Sasamura, Y., Sakamura, S., Sato, R., and Tajimi, A. (1989). Structure, synthesis and stereochemistry of (+)-Orthosporin, a phytotoxic metabolite of *Rhynchosporium Orthosporum*. *Chemistry Letters*., **18**, 1495-1498.

Imhoff, J. F., Sahl, H. G., Soliman, G. S., and Truper, H. G. (1979). The Wadi Natrun: Chemical composition and microbial mass developments in alkaline brines of eutrophic desert lakes. *Geomicrobiol J.*, **1**, 219–234.

In-kyoung, L., Soon-Ja, S., Wan-Gyu, K. and Bong-Sik, Y. (2006). Diaporthin and orthosporin from the fruiting body of *Daldinia concentrica*. *Mycobiology*, **34**, 38-40.

James, J. P. (2009). Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *Clin. Biochem. Rev.*, **30**, 19-34.

Jeewon, R., Ittoo, J., Mahadeb, D., Jaufeerally-Fakim, Y.,Wang, H. K., and Liu, A. (2013). DNA based identification and phylogenetic characterisation of endophytic and saprobic fungi from *Antidesma madagascariense*, a medicinal plant in Mauritius. *Journal of Mycology*, **2013**, 1-10.

Kalpesh, N. P., Jayvadan, K. P., Manish, P. P., Ganesh, C. R., and Hitesh, A. P. (2010). Introduction to hyphenated techniques and their applications in pharmacy. *Pharm. Methods*, **1**, 2-13.

Karsten, K., Markus, J., Hans, J.A., Siegfried, D., and Barbara, S. (1999). Biologically active metabolites from fungi 13 stemphytriol, a new perylene derivative from *Monodictys fluctuata*. *J. Natural Product Letters*, **14**, 31-34.

Kashiwada, Y., Nonaka, G. and Nishioka, I. (1984). Studies on rhubarb (Rhei rhizome). V. Isolation and characterization of chromone and chromanone derivatives. *Chem. Pharm. Bull.*, **32**, 3493-3500.

Kato, A., Saeki, T., Suzuki, S., Ando, K., Tamura, G. and Arima, K. (1969). Oryzachlorin, a new antifungal antibiotic (Studies on antiviral and antitumor antibotics. XVIII). *J. Antibiotics*, **22**, 322-326.

Kaul, S., Gupta, S., Ahmed, M., and Dhar, M. K. (2012). Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. *Phytochemistry Reviews*, **11**, 487-505.

Kharwar, R. N., Mishra, A., Gond, S. K., Stierle, A. and Stierleb, D. (2011). Anticancer compounds derived from fungal endophytes: their importance and future challenges. *Nat. Prod. Rep.*, **28**, 1208-1228.

Kimura, Y., Kozawa, M., Baba, K. and Hata, K. (1983). New constitutents of roots of *Polygonum cuspidatum*. *Planta Med.*, **48**, 164-168.

Kirby, G. W., and Robins, D. J. (1980). *Thenbiosynthesis of gliotoxin and related epipolythiodioxopiperazines. In the biosynthesis of Mycotoxins*, Academic Press, New York, pp. 301-326.

Kiyoshi, N., and Mitsuo, K. (1974). A new phathalidr from a fungus, *Alternaria kikuchiana*. *Chemistry Letters*, **1974**, 1491-1492.

Kladwang, W., Bhumirattana, A. and Hywel-Jones, N. (2003). Alkaline-tolerant fungi from Thailand. *Fungal Diversity*, **13**, 69-83.

Klaus, N. (2000). Influence of chlorine substituents on biological activity of chemicals: a review. *Pest Manag. Sci.*, **56**, 3-21.

Knight, V., Sanglier, J. J., DiTullio, D., Braccili, S., Bonner, P., Waters, J., Hughes, D., and Zhang, L. (2003). Diversifying microbial natural products for drug discovery. *Appl. Microbiol. Biotechnol.*, **62**, 446-458.

Kogel, K. H., Franken, P. and Huckelhoven, R. (2006). Endophyte or parasite-what decides?. *Current Opinion in Plant Biology*, **9**, 358–363.

Kozyrovska, N. O. (2013). Crosstalk between endophytes and a plant host within information processing networks. *Biopolymers & Cell*, **29**, 234-243.

Kusari, S., Lamshöft, M., Zühlke, S., and Spiteller, M. (2008). An endophytic fungus from *Hypericum perforatum* that produces hypericin. *J. Nat. Prod.*, **71**, 159-162.

Kusari, S., Zühlke, S., and Spiteller, M. (2009a). An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. *J. Nat. Prod.*, **72**, 2-7.

Kusari, S., Lamshöft, M., and Spiteller, M. (2009b). *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. *J. Appl. Microbiol.*, **107**, 1019-1030.

Kusari, S., Zühlke, S., Kosuth, J., Cellarova, E., and Spiteller, M. (2009c). Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. *J. Nat. Prod.*,**72**, 1825-1835.

Kusari, S. and Spiteller, M. (2011). Are we ready for industrial production of bioactive plant secondary? *Nat. Prod. Rep.*, **28**, 1203-1207.

Kusari, S., Zühlke, S., and Spiteller, M. (2011). Effect of artificial reconstitution of the interaction between the plant *Camptotheca acuminata* and the fungal endophyte *Fusarium solani* on camptothecin biosynthesis. *J. Nat. Prod.*,**74**, 764-775.

Kusari, S. and Spiteller, M. (2012). *Biochemistry, genetics and molecular biology "Metabolomics of endophytic fungi producing associated plant secondary metabolites: progress, challenges and opportunities"*, Ute Roessner, Germany, pp.242-266.

Kusari, S., Hertweck, C., and Spiteller, M. (2012). Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chemistry & Biology*, **19**, 792-798.

Kusari, S., Singh, S., and Jayabaskaran, G. (2014). Rethinking production of taxol. *Trends in Biotechnology*, **32**, 304-311.

Kwon, O. E., Rho, M. C., Song, H. Y., Lee, S. W.,Chung, M. Y., Lee, J. H., Kim, Y. H., Lee, H. S., and Kim, Y. K. (2002). Phenylpyropene A and B, new inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Penicillium griseofulvum* F1959. *Journal of Antibiotics*, **55**, 1004-1008.

Lai, S., Shizuri, Y., Yamamura, S., Kawai, K., and Furukawa, H. (1991). Three new phenolic metabolites from *Penicillium* species. *Heterocycles*, **32**, 297-305.

Larsen, T. O., Smedsgaard, J., Nielsen, K. F., Hansen, M. E., and Frisvad, J. C. (2005). Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat. Prod. Rep.*, **22**, 672-695.

Leathers, T. D. (2003). Biotechnological production and applications of pullulan. *Appl. Microbiol. Biotechnol.*, **62**, 468-473.

Lebrun, M. H., Nicolas, L., Boutar, M., Gaudemer, F., Ranomenjanahary, S., and Gaudemer, A. (1988). Relationships between the structure and the phytotoxicity of the fungal toxin tenuazonic acid. *Phytochemistry*, **27**, 77-84.

Lee, J., Lobkovsky, E., Pliam, N. B., Strobel, G. A., and Clardy, J. (1995). Subglutinols A & B: immunosuppressive compounds from the endophytic fungus *Fusarium subglutinans*. *J. Org. Chem.*, **60**, 7076-7077.

LI, J., Strobel, Y., Harper, J., Lobkovsky, E., and Clardy, J. (2000). Cryptocin, a potent tetramic acid antimycotic from the endophytic fungus *Cryptosporiopsis cf. quercina*. *Org. Lett.*, **2**, 767-770.

LI, Y. B., Liu, R. M., and Zhong, J. J. (2013). A new ganoderic acid from *Ganoderma lucidum* mycelia and its stability. *Fitoterapia*, **84**, 115–122.

LIA, D., Chen, L., Zhu, T., Kurtánd, T., Mándi, A., Zhao, Z., Li, J. and Gu, Q. (2011). Chloctanspirones A and B, novel chlorinated polyketides with an unprecedented skeleton, from marine sediment derived fungus *Penicillium terrestre*. *Tetrahedron*, **67**, 7913–7918.

Lindequist, U., Rausch, R., Füssel, A., and Hanssen, H. P. (2010). Higher fungi in traditional and modern medicine. *Med. Monatsschr. Pharm.*, **33**, 40–48.

Liu, K., Ding, X., Deng, B., and Chen, W. (2009). Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis*. *J. Ind. Microbiol. Biotechnol*., **36**, 1171–1177.

Liu, G. Q.,Wang, X. L., Han,W. J., and Lin, Q. L. (2012). Improving the fermentation production of the individual key triterpene ganoderic acid me by the medicinal fungus *Ganoderma lucidum* in submerged culture. *Molecules*, **17**, 12575–12586.

Lucas, M. F., Castro, C. M. and Takahashi, J. A. (2007). Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a Brazilian cerrado isolate of *Penicillium sclerotiorum* Van Beyma. *Brazilian Journal of Microbiology*, **38**, 785-789.

Macadam, J. H., and Grabber, J. W. (2002). Relationship of growth cessation with the formation of diferulate cross-links and p-coumarolyted lignins in tall fescue leaf blades. *Planta*, **215**, 783-793.

Mahajan, R. T., and Chopda, M. Z. (2009). Phyto pharmacology of *Ziziphus jujuba* mill- a plant review. *Pharmacognosy Review*, **3**, 320-329.

Mansour, G., Authman, M. and Gaber, N. (2011). Ciliated protozoan diversity at El-hammra hypersaline lake, Wadi Al-Natrun, Egypt. *African J. Biol. Sci.*,**7**, 1-12.

Marmann, A., Aly, A., Lin, W.,Wang, B. and Proksch, P. (2014). Co-Cultivation—A powerful emerging tool for enhancing the chemical diversity of microorganisms. *Mar. Drugs*, **12**, 1043-1065.

Masuma, R., Tabata, N., Tomoda, H., Haneda, K., Iwai, Y., and Omura, S. (1994). Arohynapenes A and B, new anticoccidial agents produced by *Penicillium* sp. *The Journal of Antibiotics*, **47**, 46-53.

Mathias, J. F., Giambattista, C., Michelangelo, S. and Rudolf, S. (1999). Asymmetric hydrogenation of substituted 2-Pyrones. *J. Org. Chem.*, **64**, 5768-5776.

Mcphail, A. T., Miller, R. W., Harvan, D., and Pero, R. W. (1973). X-ray crystal structure revision of the fungal metabolite (±)-altenuene. *J. Chem. Soc.*, *Chem. Commun.*, **18**, 682.

Meronuck, R. A., Steele, J. A., Mirocha, C. J., and Christensen, C. M. (1972). Tenuazonic acid, a toxin produced by *Alternaria alternata*. *Appl. Microbiol.*, **23**, 613-617.

Metz, A., Haddad, A.,Worapong, J., Long, D., Ford, E., Hess, W. M., and Strobel, G. A. (2000). Induction of the sexual stage of *Pestalotiopsis microspora*, a taxol producing fungus. *Microbiology*, **146**, 2079–2089.

Miao, L., Kwong, T. F. N., and Qian, P.Y. (2006). Effect of culture conditions on mycelial growth, antibacterial activity, and metabolite profiles of the marine-derived fungus *Arthrinium c.f. saccharicola*. *Appl. Microbiol. Biotechnol.*,**72**, 1063-1073.

Mikami, Y., Nishijima, N., Limura, H., Suzaki, A., and Tamura, S. (1971). Chemical studies on brown-spot disease of tobacco plants, Part 1. Tenuzonic acid as a vivotoxin of *Alternaria longipes*. *Agr. Biol. Chem.*, **35**, 9-19.

Miller, F. A., Rightsel, W. A., Sloan, B. J., Ehrlich, J., French, J. C., and Bartz, Q. R. (1963). *Antiviral activity of tenuazonic acid*, Nature, London, U.K., p. 1338.

Mishra, B. B., and Tiwari, V. K. (2011). Natural products: An evolving role in future drug discovery. *European Journal of Medicinal Chemistry*, **46**, 4769-4867.

Mohanty, S. S. and Prakash, S. (2009). Effects of culture media on larvicidal property of secondary metabolites of mosquito pathogenic fungus *Chrysosporium lobatum* (Moniliales: Moniliaceae). *Acta. Trop.*, **109**, 50-54.

Moricca, S., and Ragazzi, A. (2008). Fungal endophytes in Mediterranean oak forests: a lesson from *Discula quercina*. *Phytopathology*, **98**, 380–386.

Negishi, S., Cai-Huang, Z., Hasumi, K., Murakawa, S., and Endo, A. (1986). Productivity of molacolin K (mevilonin) in the genus *Monascus*. *Hakko Kogaku Kaishi*, **64**, 509–512.

Newman, D. J., and Cragg, G. M., and Snader, K. M. (2003). Natural products as sources of new drugs over the period 1981–2002. *J. Nat. Prod.*, **66**, 1022–1037.

Newman, D. J., and Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.*, **70**, 461–477.

Newman, D. J. and Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.*, **75**, 311-355.

Newton, G. G. F., and Abraham, E. P. (1955). Cephalosporin C, a new antibiotic containing sulphur and D-α-aminoadipic acid. *Nature*, **175**, 548.

Nicoetti, R., Lopez-Gresa, M. P., Manzo, E., Carella, A. and Ciavatta, M. L. (2007). Production and fungitoxic activity of Sch642305, a secondary metabolite of *Penicillium canescens*. *Mycopathologia*, **163**, 295-301.

Niu, S., Liu, D., Hu, X., Proksch, P., Shao, Z., and Lin, W. H. (2014). Spiromastixones A-O, antibacterial chlorodepsidones from a deep-sea-derived *Spiromastix* sp. fungus. *J. Nat. Prod.*, **77**, 1021-1030.

Nolte, M. J., Steyn, P. S., and Wessels, P. L. (1980). Structural investigation of 3-acylpyrrolidine-2,4-diones by nuclear magnetic resonance spectroscopy and x-ray crystallography. *J. Chem. Soc. Perkin Trans*. **1**, 1057-1065.

Noriko, T., Hiroshi, T., Yuzuru, I., and Satoshi, O. (1994). Arohynapene D, a new anticoccidial agent produced by *Penicillium* sp. FO-2295. *The Journal of Antibiotics*, **48**, 83-84.

Northolt, M. D., Frisvad, J. C., and Samson, R. A. (1995). *Occurrence of foodborne fungi and factors for growth Introduction to foodborne fungi*, Delft, the Netherlands, pp. 243-250.

Okuno, T., Natsume, I., Sawai, K., Sawamura, K., Furusaki, A., and Matsumoto, T. (1983). Structure of antifungal and phytotoxic pigments produced by *Alternaria* sp. *Tetrahedron Lett.*, **24**, 5653-5656.

Ola, A. R., Thomy, D., Lai, D., Brötz-Oesterhelt, H., and Proksch, P. (2013). Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through coculture with *Bacillus subtilis*. *J. Nat. Prod.*, **22**, 2094-2099.

Onocha, P. A., Okorie, D. A., Connolly, J. D., and Roycroft, D. S. (1995). *Phytochemistry*, **40**, 1183-1189.

Oren, A. (1999). *Microbiology and biogeochemistry of hypersaline environments*, Boca Raton, Florida, pp. 1-12.

Oren, A., and Gunde-Cimerman, N. (2012). *Fungal life in the Dead Sea*, Springer verlag, Berlin, pp. 115-132.

Ostry, V. (2008). *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.*, **1**, 175–188.

Padhi, L., Mohanta, Y. K., and Panda, S. K. (2013). Endophytic fungi with great promises: A review. *J. Adv. Pharm. Edu. & Res.*, **3**, 152-170.

Paz, Z., Komon-Zelazowska, M., Druzhinina, I. S., Aveskamp, M. M., Shnaiderman A., Aluma Y., Carmeli, S., Ilan, M., and Yarden, O. (2010). Diversity and potential antifungal properties of fungi associated with a Mediterranean sponge. *Fungal Divers*., **42**, 17–26.

Peberdy, J. F. (1987). *Penicillium and Acremonium*, Springer, New York, p. 178.

Pero, R. W., Posner, H., Blois, M., Harvan, D., and Spalding, J. W. (1973). Toxicity of metabolites produced by the ‘‘*Alternaria*’’. *Env Health Perspect*, **4**, 87–94.

Petit, P., Lucas, E. M. F., Abreu, L. M., Pfenning, L. H., and Takahashi, J. A. (2009). Novel antimicrobial secondary metabolites from a *Penicillium* sp. isolated from Brazilian cerrado soil. *Electronic Journal of Biotechnology*, **12**, 1-9.

Pettit, R. K. (2009). Mixed fermentation for natural product drug discovery. *Appl Microbiol Biotechnol*., **83**, 19–25.

Pfeiffer, E., Schebb, N. H., Podlech, J., and Metzler, M. (2007). Novel oxidative in vitro metabolites of the mycotoxins alternariol and alternariol methyl ether. *Mol. Nutr. Food Res.*, **51**, 397–316.

Pham, C. D., Weber, H., Hartmann, R., Wray, V., Lin, W. H., Lai, D. W., and Proksch, P. (2013). New cytotoxic 1, 2, 4-thiadiazole alkaloids from the ascidian *Polycarpa aurata*. *Organic Letters*, **15**, 2230–2233.

Pieter, S. S. and Christiaan, J. (1976). Characterization of magnesium and calcium tenuazonate from *Phoma Sorghina*. *Phytochemistry*, **15**, 1977-1979.

Pitt, J. I., and Hocking, A. D. (1997). *Fungi and food spoilage*, Blackie Academic & Professional, London, p. 548.

Plemenitas, A., Vaupotic, T., Lenassi, M., Kogej, T., Gunde-Cimerman, N. (2008). Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: a molecular perspective at a glance. *Stud. Mycol.*, **61**, 67–75.

Puri, S. C., Verma, V., Amna, T., Qazi, G. N., and Spiteller, M. (2005). An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *Journal of Natural Products*, **68**, 1717–1719.

Puri, S. C., Nazir, A., Chawla, R., Arora, R., Riyaz-ul Hasan, S., Amna, T., Ahmed, B., Verma, V., Singh,S., Sagar, R., Sharma, A., Kumar, R., Sharma, R. K., and Qazi, G. N. (2006). The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *J. Biotechnol.*, **122**, 494–510.

Raghukumar, C. (2012). *Biology of marine fungi*, Springer Heidelberg Dodrecht, London, p. 152.

Rateb, M. E., and Ebel, R. (2011). Secondary metabolites of fungi from marine habitats. *Nat. Prod. Rep.*, **28**, 290-344.

Redman, R. S., Sheehan, K. B., Stout, T. G., Rodriguez, R. J., and Henson, J. M. (2002). Thermotolerance generated by plant/fungal symbiosis. *Science*, **298**, 1581.

Rodriguez, R. J., White, J .F., Arnold, A. E., and Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New Phytol.*, **182**, 314–330.

Rudgers, J. A., Koslow, J. M., and Clay, K. (2004). Endophytic fungi alter relationships between diversity and ecosystem properties. *Ecol.Lett.*, **7**, 42–51.

Saha, D., Fetzner, R., Burkhardt, B., Podlech, J., Metzler, M., Dang, H., Lawrence, C., and Fischer, R. (2012). Identification of a polyketide synthase required for Alternariol (AOH) and Alternariol-9-Methyl Ether (AME) formation in *Alternaria alternata*. *PLoS ONE*, **7**, 1-14.

Saikkonen, K., Faeth, S .H., Helander, M., and Sullivan, T. J. (1998). Fungal endophytes: A continuum of interactions with host plants. *Annu. Rev. Ecol. Syst.*, **29**, 319–343.

Sajjadi, S. E., Shokoohinia, Y., and Moayedi, N. (2012). Isolation and Identification of ferulic acid from aerial parts of *Kelussia odoratissima* Mozaff. *Natural Pharmaceutical Products*, **4**, 159-162.

Savard, M. E., Miller, J. D., Blais, L. A., Seifert, K. A., and Samson, R. A. (1994). Secondary metabolites of *Penicillium bilaii* Strain PB-50. *Mycopathologia*, **127**, 19-27.

Schreck, I., Deigendesch, U., Burkhardt, B., Marko, D., and Weiss, C. (2012). The *Alternaria* mycotoxins alternariol and alternariol methyl ether induce cytochrome P450 1A1 and apoptosis in murine hepatoma cells dependent on the aryl hydrocarbon receptor. *Arch. Toxicol.*, **86**, 625–632.

Schroeckh, V., Scherlach, K., Nützmann, H. W., Shelest, E., Schmidt-Heck,W., Schuemann, J., Martin, K., Hertweck, C., and Brakhage, A. A. (2009). Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. USA*, **106**, 14558–14563.

Schulz, B., and Boyle, C. (2005). The endophytic continuum. *Mycol. Res.*, **109**, 661--686.

Scott, P. M. (2001). Analysis of agricultural commodities and foods for *Alternaria mycotoxins*. *J. AOAC Int.*, **84**, 1809–1817.

Seephonkai, P., Kongsaeree, P., Prabpai, S., Isaka, M., Thebtaranonth, Y. (2006). Transformation of an irregularly bridged epidithiodiketopiperazine to Trichodermamide A. *Organic Letters*, **8**, 3073-3075.

Selim, K. A., El-Beih, A. A., AbdEl-Rahman, T. M. and El-Diwany, A. I. (2012). Biology of Endophytic Fungi. *Current Research in Environmental & Applied Mycology*, **2**, 31-82.

Seo, C., Sohn, J. H., Oh, H., Kim, B. Y., and Ahn, J. S. (2009). Isolation of the protein tyrosine phosphatase 1B inhibitory metabolite from the marine-derived fungus *Cosmospora* sp. SF-5060. *Bioorg Med Chem Lett*., **19**, 6095–6097.

Sepcic, K., Zalar, P. and Gunde-Cimerman, N. (2011). Low water activity induces the production of bioactive metabolites in halophilic and halotolerant fungi. *Mar. Drugs*, **9**, 43-58.

Shaw, L. M. (1989). Advances in cyclosporine pharmacology, measurement,and therapeutic monitoring. *Clin. Chem.*, **35**, 1299–1308.

Sheng, L., Yoshikazu, S., Shosuke, Y., Kazuaki, K., Yukimasa, T. and Hideyuki, F. (1990). New metabolites of two hybrid strains ME 0004 and 0005 deerived from *Penicillium citreo-viride* B. IFO 6200 and 4692. *Chemistry Letters*, **19**, 589-592.

Sheng, L., Yoshikazu, S., Shosuke, Y., Kazuaki, K., and Hideyuki, F. (1991). Three new phenolic metabolites from *Penicillium* Species. *Heterocycles*, **32**, 297-305.

Shiao, M. (2003). Natural products of the medicinal fungus *Ganoderma lucidum*: Occurrence, biological activities, and pharmacological functions. *Chem. Rec.*, **3**, 72-180.

Shweta, S., Zühlke, S., Ramesha, B. T., Priti, V., Kumar, P. M., Ravikanth, G., Spiteller, M., Vasudeva, R., and Shaanker, R. U. (2010). Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiata* E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. *Phytochemistry*, **71**, 117–122.

Silva, S., Reed, J., Billedeau, R., Norris, D. and Snieckus, V. (1992). Directed *ortho* metalation of N, N-diethyl benzamodes. *Tetrahedron*, **48**, 4863-4878.

Singh, M. P., Janso, J. E., Luckman, S. W., Brady, S. F., Clardy, J., Greenstein, M., and Maiese, W. M. (2000). Biological activity of guanacastepene, a noval diterpenoid antibiotic produced by an unidentified fungus CR115. *J.Antibiot.*, **53**, 256-261.

Singh, S. B., Ondeyka, J. G., Tsipouras, N., Ruby, C., Sardana, V., Schulman, M., Sanchez, M., Pelaez, F., Stahlhut, M. W., Munshi, S., Olsen, D. B., and Lingham, R. B. (2004). Hinnuliquinone, a C2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease. *Biochem. Biophys. Res. Commun.*, **324**, 108-113.

Singh, R. S., Saini, G. K., and Kennedy, J. F. (2008). Pullulan: Microbial sources, production and applications. *Carbohydr Polymer*., **73**, 515-531.

Singh, P., Raghukumar, C., Meena, R. M., Verma, P., and Shouche,Y. (2012). Fungal diversity in deep-sea sediments revealed by culture-dependent and culture-independent approaches. *fungal ecology*, **5**, 543-553.

Sliva, D. (2003). Ganoderma lucidum (Reishi) in cancer treatment. *Integr. Cancer Ther.*, **2**, 358–364.

Smedsgaard, J., and Frisvad, J. C. (1996). Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. *J. Microbiol. methods*, **25**, 5-17.

Smith, E .R., Fredrickson, T. N. and Hadidian, Z. (1968). Toxic effects of the sodium and the N, N'-dibenzylethylenediamine salts of tenuazonic acid (NSC-525816 and NSC-82260). *Cancer Chemother. Rep.*, **52**, 579-585.

Smith, J. E., and Berry, D. R. (1975). *An introduction to the biochemistry of fungal development*, Academic Press.,London-New York-San Francisco, p.189.

Sonaimuthu, V., Parihar, S, Thakur, J. P., Luqman, S., Saikia, D., Chanotiya, C. S., Jhonpaul, M., and Negi, A. S. (2010). Tenuazonic acid: A promising antitubercular principle from *Alternaria alternata*. *Microbiol. Res.*, **2**, 63–65.

Spiteller, P. (2008). Chemical defence strategies of higher fungi. *Chem. Eur. J.*, **14**, 9100–9110.

Stack, M. E., Mazzola, E. P., Page, S. W., and Pohland, A. E. (1986). Mutagenic perylenequinone metabolites Of *Alternaria alternata*: Altertoxins I,II and III. *Journal of Natural Products*, **49**, 866-871.

Stack, M. E., and Prival, M. J. (1986). Mutagenicity of the *Alternaria* metabolites altertoxins I, II, and III. *Appl. Environ. Microbiol.*, **52**, 718−722.

Statoshi, N., Shinichiro, T., Yutaka, S., Eiji, T., Kiyotaka, K., Katsuhiko, A. and Yuzuru, M. (1995). Isolation of myosin light chain kinase inhibitors from microorganisims: Dehydro altenusin, altenusin, atrovenetinone and cyclooctasulfur. *Biosci. Biotech. Biochem.*, **59**, 1333-1335.

Steyn, P. S., and Rabie, C. J. (1976). Characterization of magnesium and calcium tenuazonate from *Phoma sorghina*. *Phytochemistry*, **15**, 1977-1979.

Stickings, C. E., and Townsend, R. J. (1961). Studies in the biochemistry of micro-organisms. 108. Metabolites of *Alternaria tenuis* Auct.: the biosynthesis of tenuazonic acid. *Biochem. J.*, **78**, 412-418.

Stierle, A., Strobel, G. A., and Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of *Pacific yew*. *Science*, **260**, 214–216.

Stierle, A., Stobel, G., Stierle, D., Grothaus, P., and Bignami, G. (1995). The search for a taxol-producingmicroorganism among the endophytic fungi of the *Pacific yew*, *Taxus brevifolia*. *J Nat Prod*., **58**, 1315–1324.

Stinson, E. E., Wise, W. B. , Moreau, R. A., Jurewicz, A. J. and Pfeffer, P. E. (1986). Alternariol: evidence for biosynthesis via norlichexanthone. *Canadian Journal of Chemistry,* **64**, 1590-1594.

Stipanovic, R. D., and Howell, C. R. (1982). The Structure of Gliovirin, a new antibiotic from *Gliocladium Virens*. *The Journal of Antibiotics*, **35**, 1326-1330.

Stipanovic, R. D., Howell, C. R., and Hedin, P. A. (1994). Biosynthesis of gliovirin: incorporation of L-phenylalanine (1-13C). *J. Antibiot.*, **47**, 942-944.

Strobel, G. A., and Pliam, N. B. (1997). Immunosuppressant diterpene compound. *US Patent 5648376.*

Strobel, G. A. (2002a). Microbial gifts from rain forests. *Can. J. Plant. Pathol.*, **24**, 14–20.

Strobel, G. A. (2002b). Rainforest endophytes and bioactive products. *Crit. Rev. Biotechnol.*, **22**, 315–333.

Strobel, G., and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*., **67**, 491-502.

Strobel, G. (2003). Endophytes as sources of bioactive products. *Microbes and Infection*, **5**, 535-544.

Strobel, G. A., Daisy, B., Castillo, U. and Harper, J. (2004). Natural products from endophytic microorganisms. *J. Nat. Prod.*, **67**, 257-268.

Suffness, M. (1995). *Taxol, science and applications*, CRC Press, Boca Raton, Fla., p. 62.

Sullivan, R., Smith, J. E., and Rowan, N. J. (2006). Medicinal mushrooms and cancer therapy: translating a traditional practice into Western medicine. *Perspect Biol Med*., **49**, 159–170.

Sun, X., Zhou, X., Cai, M., Tao, K., and Zhang, Y. (2009). Identified biosynthetic pathway of aspergiolide A and a novel strategy to increase its production in a marine derived fungus *Aspergillus glaucus* by feeding of biosynthetic precursors and inhibitors simultaneously. *Bioresour Technol*., **100**, 4244–4251.

Suryanarayanan, T. S., Thirunavukkarasu, N., Govindarajulu, M. B., Sasse, F., Jansen, R., and Murali, T. S. (2009). Fungal endophytes and bioprospecting. *fungal biiology reviews*, **30**, 1-11.

Sweetman, S. C. (2009). *Cardiovascular drugs. Martindale: the complete drug reference*, Pharmaceutical Press, London, pp. 1155–1434.

Taghavi, S., Barac, T., Greenberg, B., Borremans, B., Vangronsveld, J., and van der Lelie, D. (2005). Horizontal gene transfer to endogenous endophytic bacteria from poplarimproves phytoremediation of toluene. *Applied and Environmental Microbi-ology*, **71**, 8500–8505.

Tan, R. X.,and Zhou, W. X. (2001). Endophytes: a rich source of functional metabolites. *Natural Product Reports*, **18**, 448-459.

Tang, W., Liu, J. W., Zhao, W. M., Wei, D. Z., and Zhong, J. J. (2006). Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. *Life Sci.*, **80**, 205–211.

Tejesvi, M. V., Tamhanka, S. A., Kini, K .R., Rao, V. S., and Prakash, H. S. (2009). Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmaceutically important medicinal trees. *Fungal Divers.*, **38**, 167–183.

Tejesvi, M. V., Kajula, M., Mattila, S., and Pirttilä, A. M. (2011). Bioactivity and genetic diversity of endophytic fungi in *Rhododendron tomentosum* Harmaja. *Fungal Divers.*, **47**, 97–107.

Thomas, R. (1961). Studies in the biosynthesis of fungal metabolites. *Biochem. J.*, **80**, 234–240.

Thomas, O. L. and Jens, B. (1999). Dichlorodiaportin, diaportinol and diaportinic acid: Three novel isocoumarins from *Penicillium nalgiovense*. *J. Nat. Prod.*, **62**, 1182-1184.

Torres, M. S., Singh, A. P., Shah, S., Herrera-Carrillo, Z., Gianfagna, T., White, J. F. and Vorsa, N.(2009). *LC-MS-MS identification and quantification of phenolics in symbiotic tall fescue. Proceedings of the 18th Annual Rutgers Turfgrass Symposium*, Rutgers University, New Brunswick, p. 54.

Verma, V. C., Kharwar, R. N., Strobel, G. A. (2009). Chemical and functional diversity of natural products from plant associated endophytic fungi. *Nat Prod Commun*., **4**, 1511–1532.

Verma, V. C., Prakash, S., Singh, R. G., and Gange, A. C. (2014). *Host-mimetic metabolomics of endophytes: Looking back into the future*, Springer, pp. 203-218.

Vincent, P. G., McAlpine, J., Lam, K. S., Baker, D. and Petersen, F. (2006). Drug discovery from natural products. *J. Ind. Microbiol. Biotechnol.*, **33**, 523-531.

Wang, J., Li, G., Lu, H., Zheng, Z., Huang, Y., and Su, W. (2000). Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei*. *FEMS Microbiol Lett*., **193**, 249–253.

Wang, W. L., Wang,Y., Tao, H. W., Peng, X. P., Liu, P. P. and Zhu, W. M. (2009). Cerebrosides of the halotolerant fungus *Alternaria raphani* isolated from a sea salt field. *J. Nat. Prod.*,**72**, 1695–1698.

Wang, X., Sena Filho, J. G., Hoover, A. R., King, J. B., Ellis, T. K., Powell, D. R., and Cichewicz, R. H. (2010). Chemical epigenetics alters the secondary metabolite composition of guttate excreted by an atlantic-forest-soil-derived *Penicillium citreonigrum*. *J. Nat. Prod.*, **73**, 942–948.

Wang, Y., Wray, V., Tsevegsuren, N., Lin, W.H., and Proksch, P. (2012). Phenolic compounds from the Mongolian medicinal plant *Scorzonera radiata*. *Z. Naturforsch.*, **67c**, 135-143.

Wang, M. H., Li, X. M., Li, C. S., Ji, N. Y., and Wang, B. G. (2013). Secondary metabolites from *Penicillium pinophilum* SD-272, a marine sediment-derived fungus. *Marine Drugs*, **11**, 2230-2238.

Wang, X., Wang, H., Liu, T., and Xin, Z. (2014). A PKS I gene-based screening approach for the discovery of a new polyketide from *Penicillium citrinum* Salicorn 46. *Appl. Microbiol. Biotechnol.*, **98**, 4875-85.

Watanabe, A., Ono, Y., Fujii, I., Sankawa, U., Mayoorga, M. E., Timberlake, W. E., and Ebizuka, Y. (1998). Product identification of polyketide synthase coded by *Aspergillus nidulans* wA Gene. *Tetrahedron Lett.*, **39**, 7733-7736.

Watanabe, A., Fujii, I., Sankawa, U., Mayoorga, M. E., Timberlake, W. E., and Ebizuka, Y. (1999). Re-identification of *Aspergillus nidulans* wA gene to code for polyketide synthase of naphthopyrone. *Tetrahedron Lett.*, **40**, 91-94.

Wen-huang, P., Ming-Tsuen, H., YiShung, L.,YiChin, L. and Jen, L. (2000). Anxiolytic effect of seed of *Ziziphus jujuba* in mouse models of anxiety. *Journal of Ethnopharmacology*, **72**, 435-441.

White, J. F. and Torres, M. S. (2010). Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection?. *Physiol. Plant*., **138**, 440–446.

Xin, Z. H., Tian, L., Zhu, T. J., Wang, W. L., Du, L., Fang, Y. C., Gu, Q. Q., and Zhu, W. M. (2007). Isocoumarin derivatives from the sea squirt-derived fungus *Penicillium stoloniferum* QY2-10 and the halotolerant fungus *Penicillium notatum* B-52. *Arch. Pharm. Res.*. **30**, 816-819.

Yamada, Y., and Nagoka, I. (1985). Synthesis of a halogenated clavulone Analog. *Tetrahedron Lett.*, **26**, 5053-5056.

Yao, Y., Tian, L., Cao, J. Q. and Pei, Y. H. (2007). A new piperazine-2,5-dione from the marine fungus *Gliocladium* sp. *Pharmazie*., **62**, 478–479.

Yasuo, K., Takashi, M., Hiromitsu, N., and Takashi, H. (1992). Altechromones A and B, new plant growth regulators produced by the fungus, *Alternaria* sp. *Biosci. Biotech. Biochem.*, **56**, 1664-1665.

Yokose, K., Nakayama, N., Miyamoto, C., Furumai, T., Maruyama, H. B., Stipanovic, R. D., Howell, C. R. (1984). Structure of FA-2097, a new member of the dioxopiperazine antibiotics. *The Journal of Antibiotics*, **37**, 667-669.

You, J., Dai, H., Chen, Z., Liu, G., He, Z., Song, F., Yang, X., Fu, H., Zhang, L., and Chen, X. (2010). Trichoderone, a novel cytotoxic cyclopentenone and cholesta-7, 22-diene-3 beta, 5 alpha, 6 beta-triol, with new activities from the marine-derived fungus *Trichoderma* sp. *J. Ind. Microbiol. Biotechnol*., **37**, 245-252.

Yuan, Y. J., Li, C., Hu, Z. D., and Wu, J. C. (2001). Signal transduction pathway for oxidative burst and taxol production in suspension cultures of Taxus chinensis var.mairei induced by oligosaccharide from *Fusarium oxysprum*. *Enzyme Microb. Technol.*, **29**, 372–379.

Zalar, P., Kocuvan, M. A., Plemenitas, A., and Gunde-Cimerman, N. (2005). Halophilic black yeasts colonize wood immersed in hypersaline water. *Bot. Mar.*, **48**, 323-326.

Zalar, P., de Hoog, G. S., Schroers, H. J.,Frank, J. M., and Gunde-Cimerman, N. (2005). Taxonomy and phylogeny of the xerophilic genus *Wallemia* (Wallemiomycetes and Wallemiales). *Antonie van Leeuwenhoek*, **87**, 311-328.

Zalar, P., de Hoog, G. S., Schroers, H. J., Crous, J., Groenewald, J. Z., and Gunde-Cimerman, N. (2007). Phylogeny and ecology of the ubiquitous saprobe *Cladosporium sphaerospermum*, with descriptions of seven new species from hypersaline environments. *Stud. Mycol*., **58**, 157–183.

Zhang, H. W., Song, Y. C., and Tan, R. X. (2006). Biology and chemistry of endophytes. *Nat. Prod. Rep.*, **23**, 753–771.

Zhou, Y., Debbab, A., Wray, V., Lin, W. H., Schulz, B., Trepos, R., Pile, C., Hellio, C., Proksch, P., and Aly, A. H. (2014). Marine bacterial inhibitors from the sponge-derived fungus *Aspergillus* sp.. *Tetrahedron Letters*., **55**, 2789-2792.

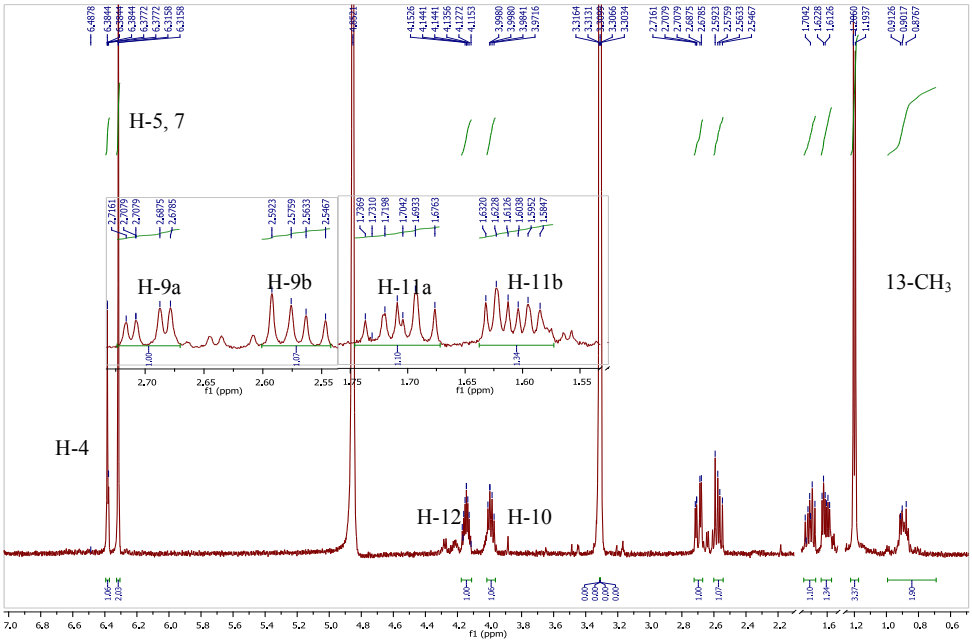
7. List of Abbreviations:

$[\alpha]_D$	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	electronvot
g	gram
HMBC	heteronuclear multiple bond connectivity
HMQC	heteronuclear multiple quantum coherence
H_2O	water
HPLC	high performance liquid chromatography
hr	hour
HR-MS	high resolution mass spectrometry
Hz	Herz
L	liter
LC	liquid chromatography
LC/MS	liquid chromatography-mass spectrometery
m	multiplet

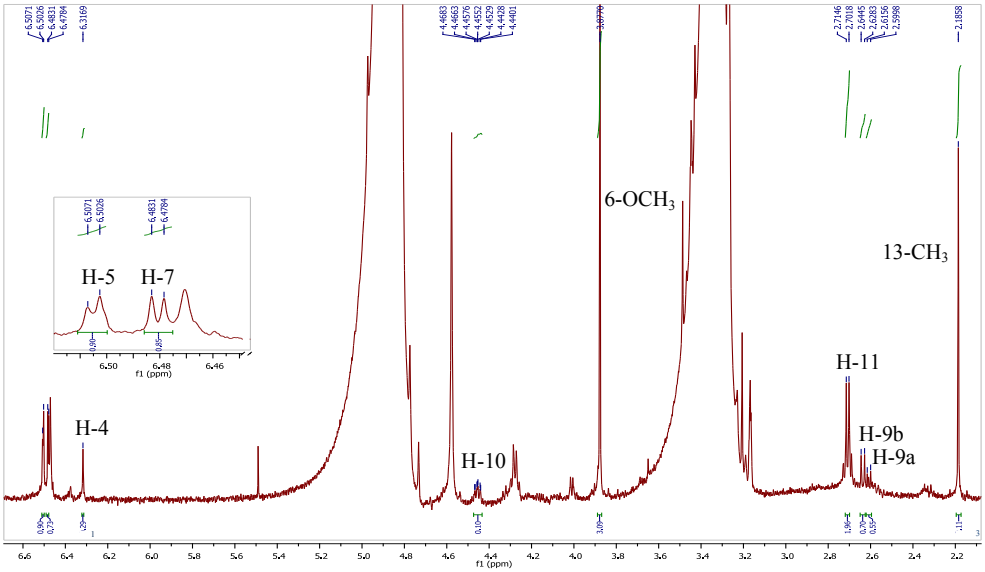
M	molar
MeOD	deuterated methanol
MeOH	methanol
mg	miligram
MHz	mega Herz
min	minute
mL	milliliter
mm	millimeter
MS	mass spectrometry
MTT	microculture tetrazolium assay
m/z	mass per charge
µg	microgram
µL	microliter
µM	micromole
MRSA	*methicillin-resistant Staphylococcus aureus*
NaCl	sodium chloride
ng	nanogram
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser and exchange spectroscopy
PCR	polymerase chain reaction
ppm	parts per million
q	quartet
ROESY	rotating frame Overhauser enhancement spectroscopy
s	singlet
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultra-violet
VLC	vacuum liquid chromatography

8. Attachments:

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

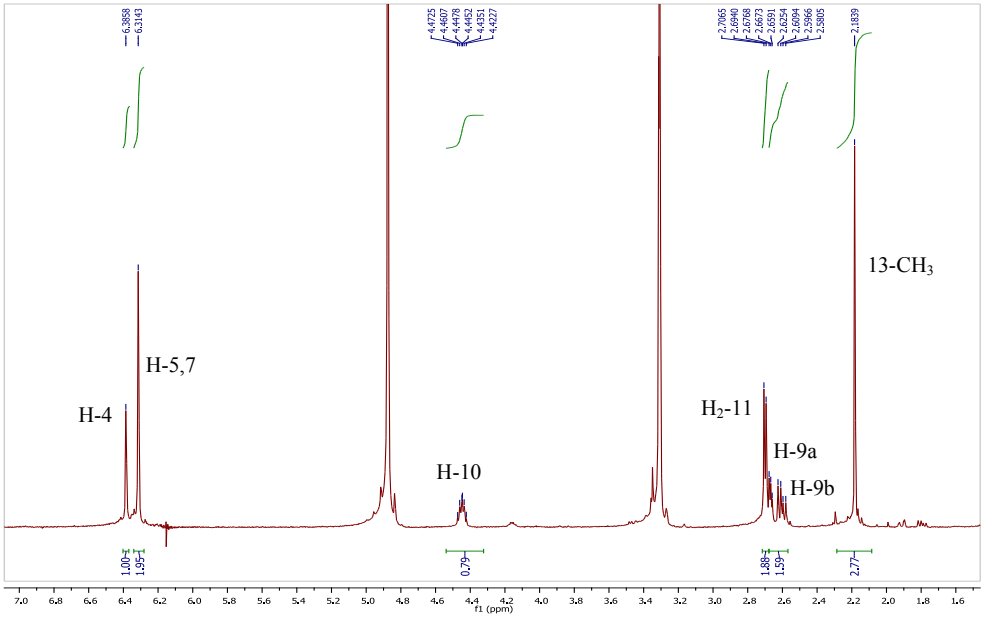


Attachment: The ^{1}H NMR spectrum of 6-methyl-Citreoisocoumarin (**2**).

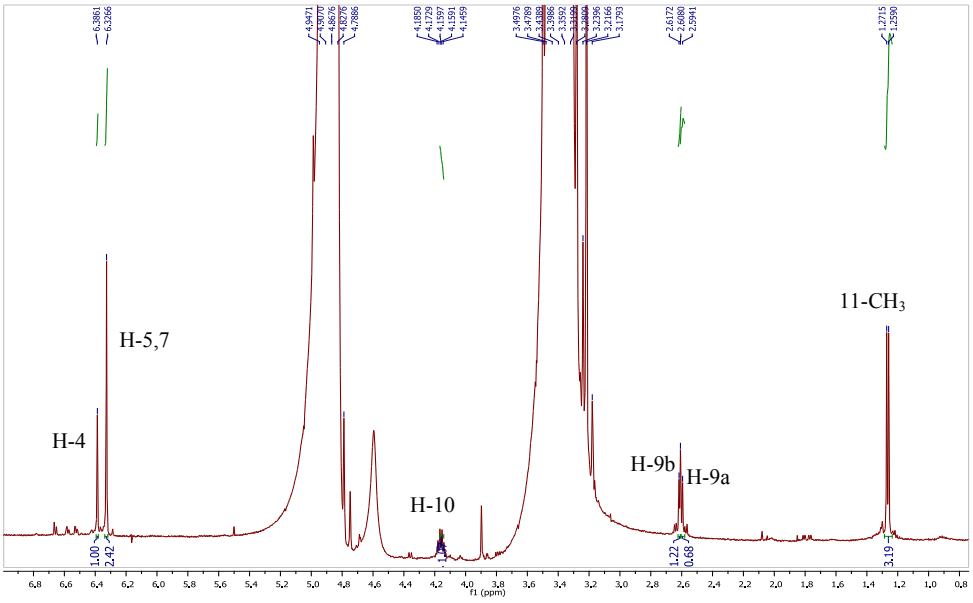


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Citreoisocoumarin (**3**).

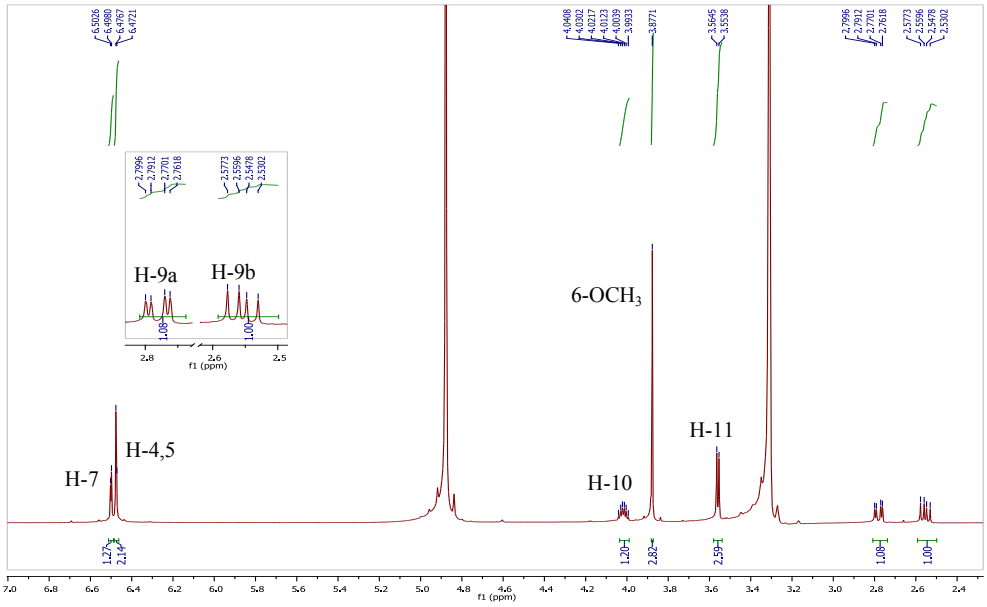


Attachment: The ^{1}H NMR spectrum of Orthosporin (**4**).

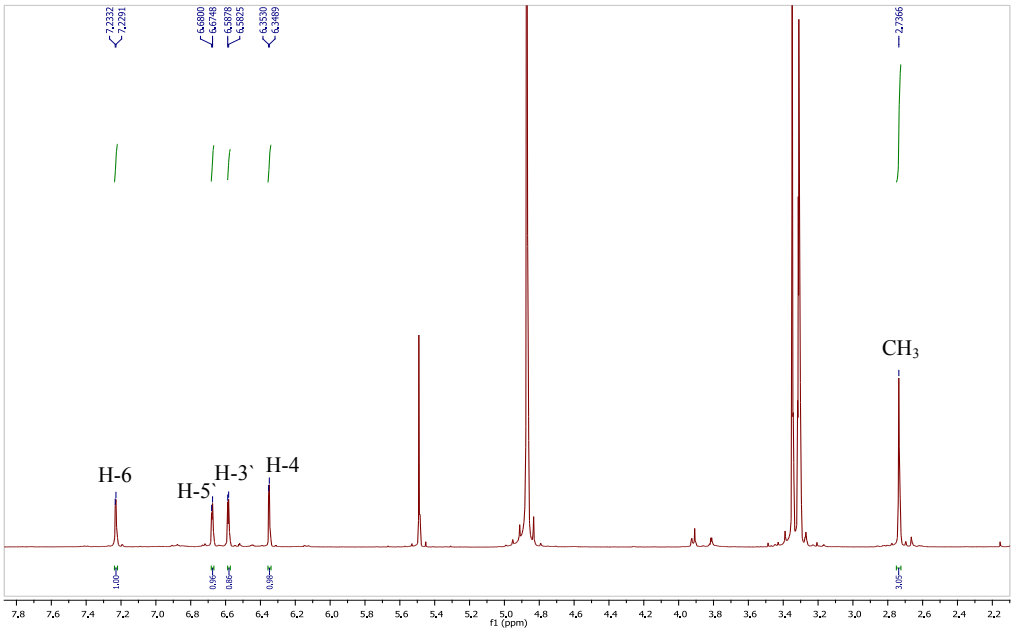


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Diaportinol (**5**).

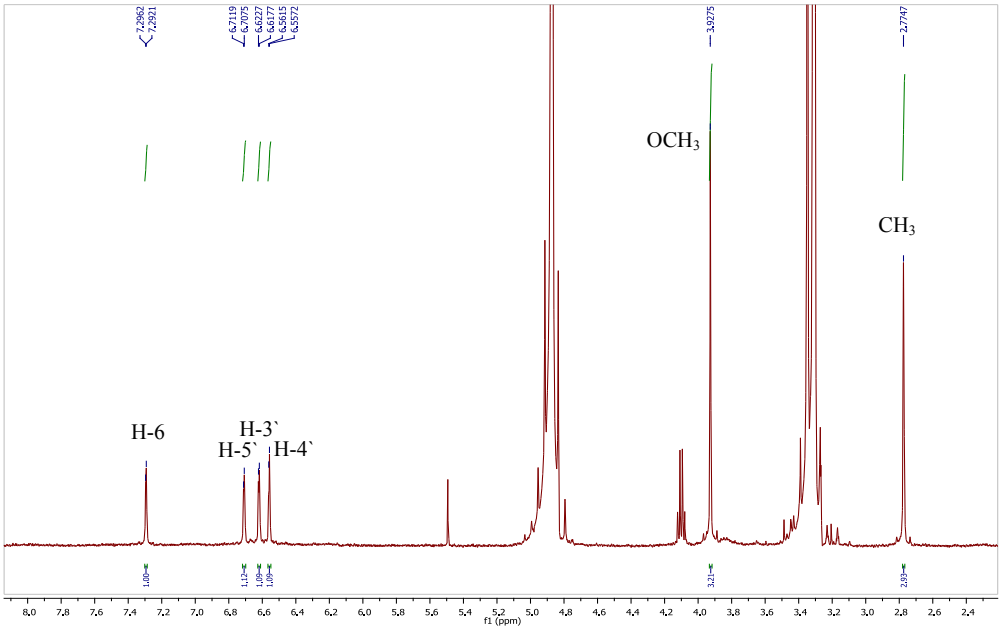


Attachment: The ^{1}H NMR spectrum of Alternariol (**6**).

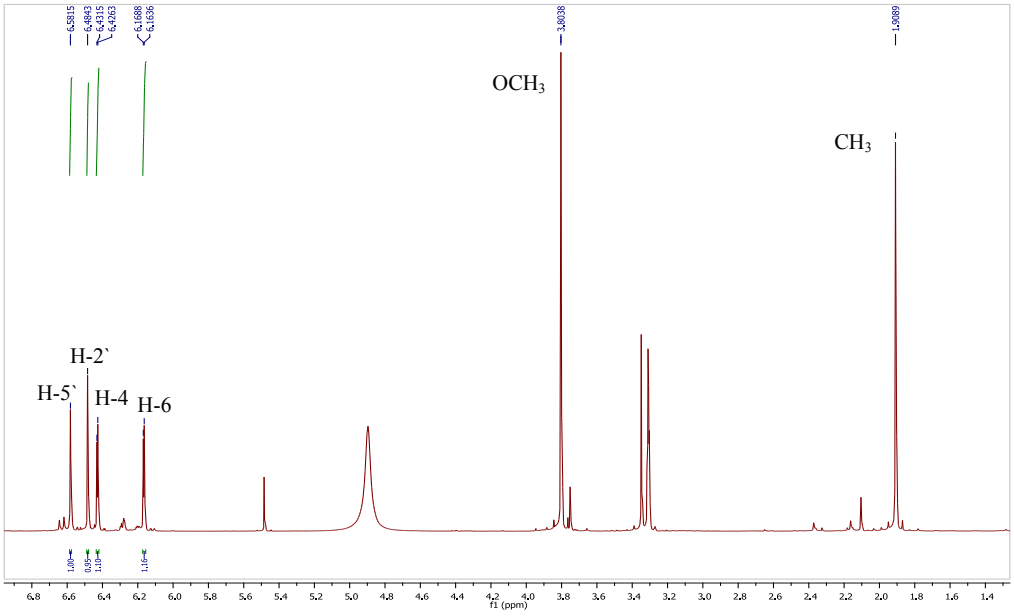


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Alternariol-5-*O*-methyl ether (**7**).

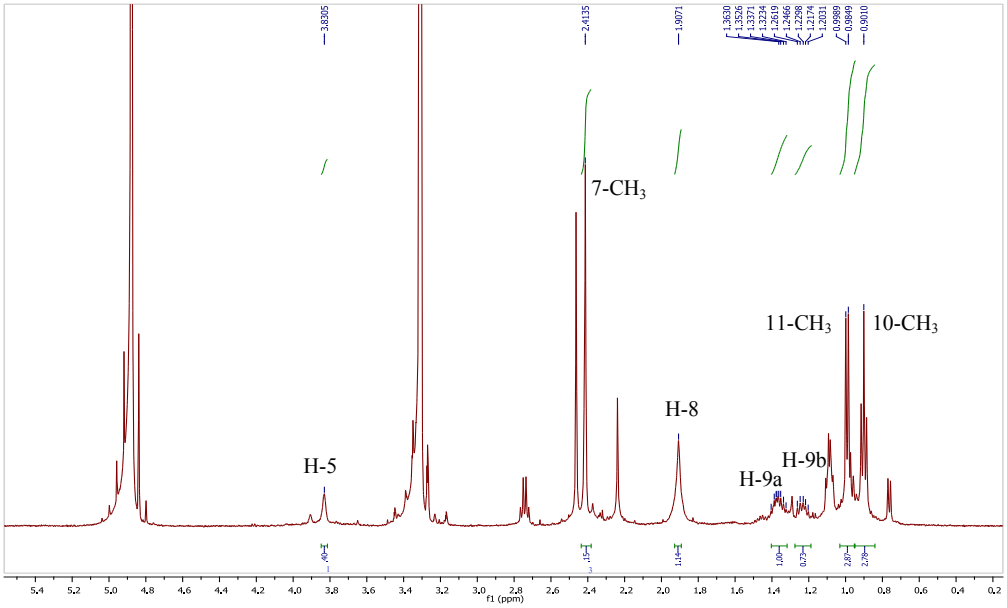


Attachment: The ^{1}H NMR spectrum of Altenusin (**8**).

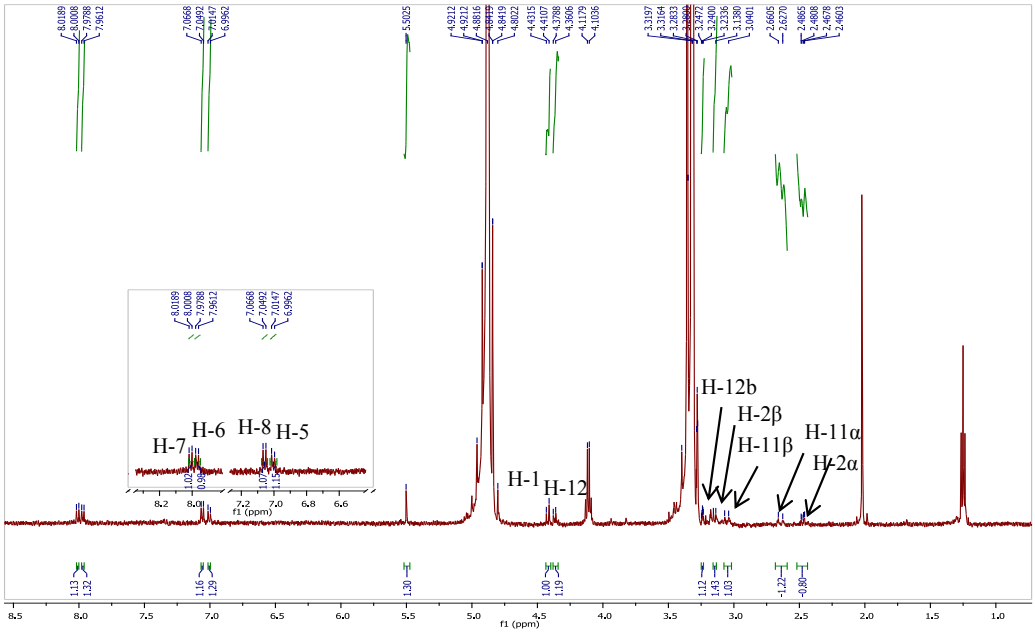


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Tenuazonic acid (**9**).

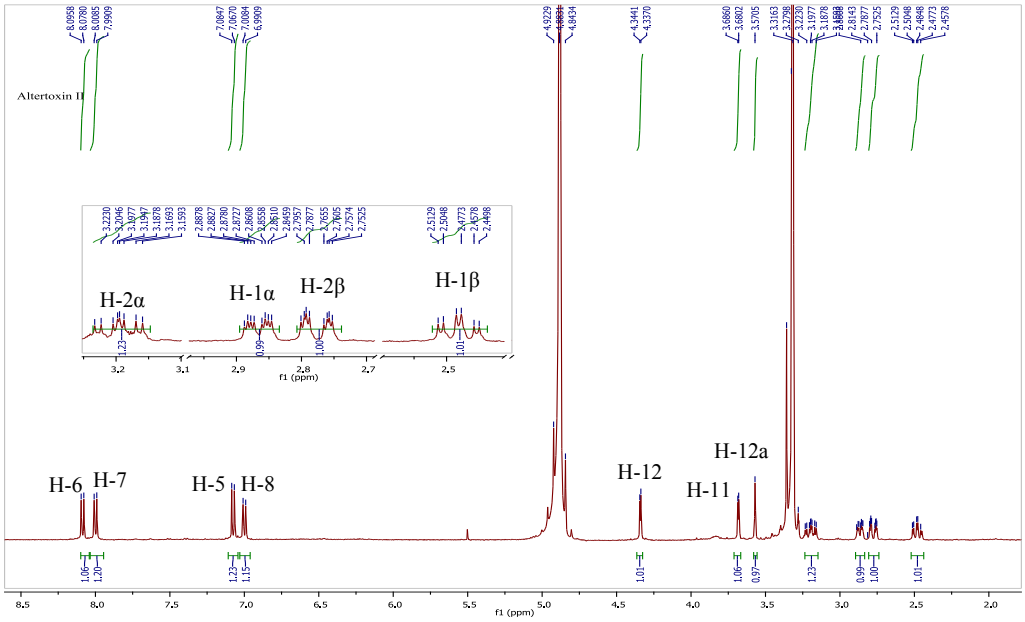


Attachment: The ^{1}H NMR spectrum of Stemphytriol (**10**).

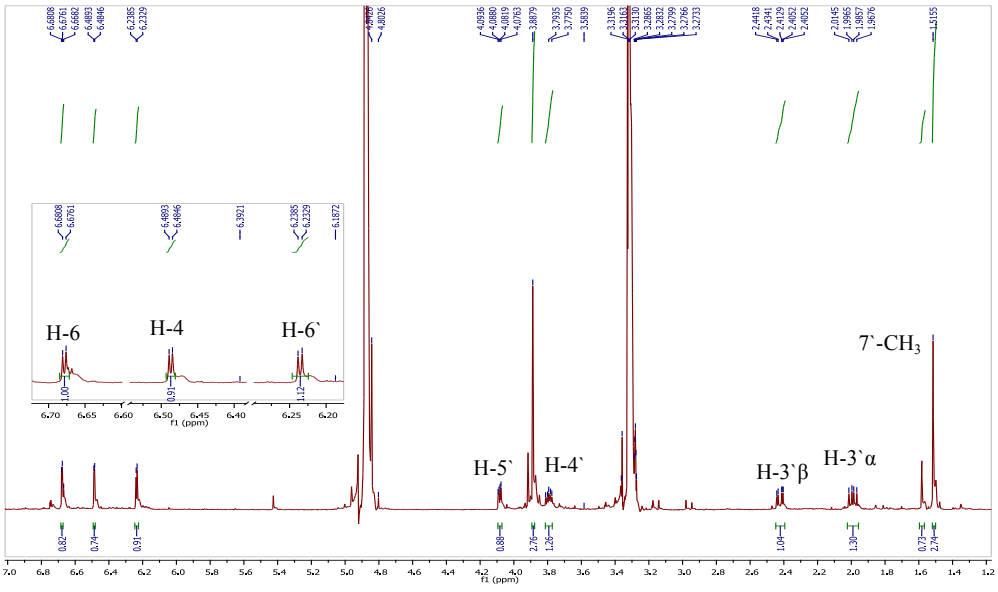


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Altertoxin II (**11**).

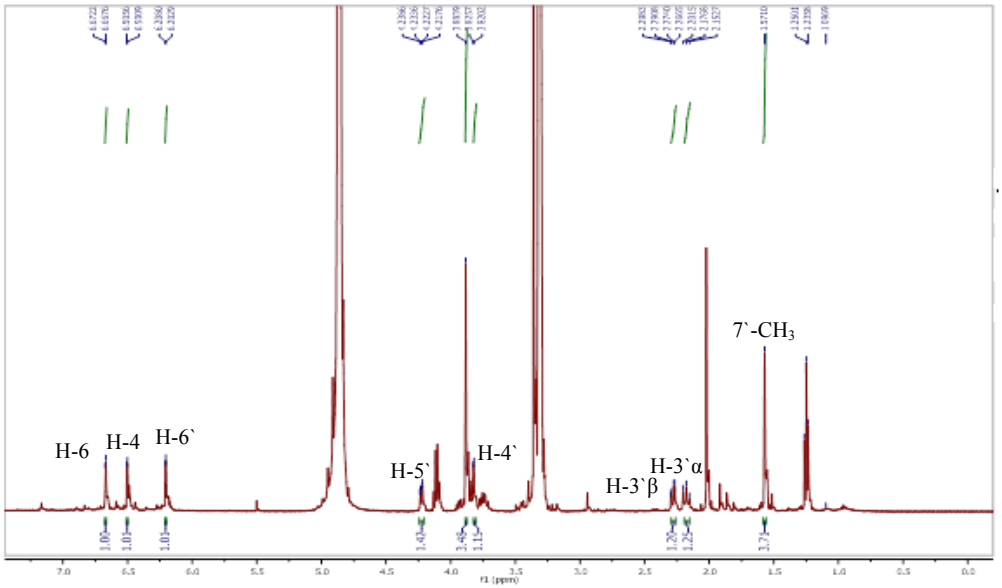


Attachment: The ^{1}H NMR spectrum of Altenuene (**12**).

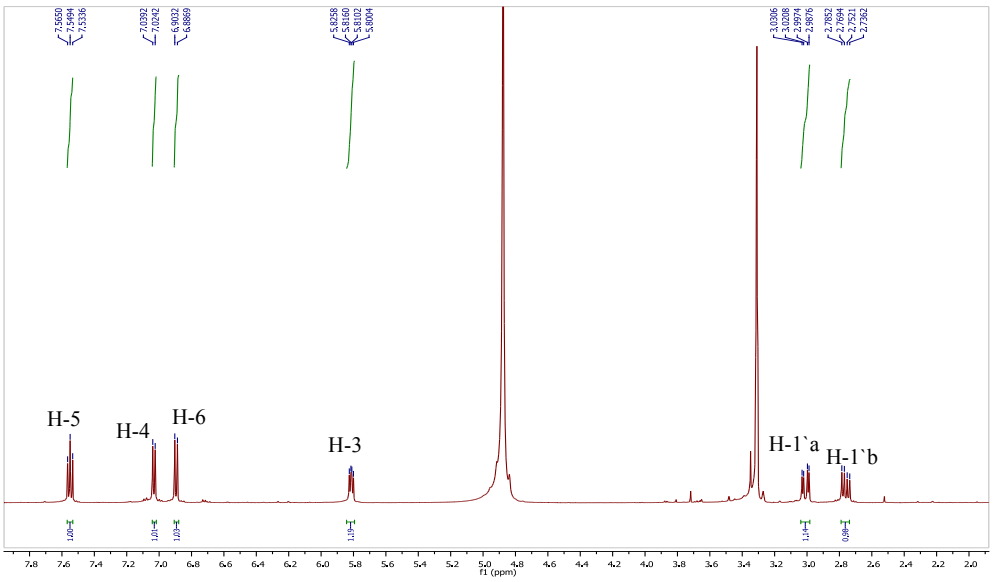


| *Attachments*

Attachment: The ^{1}H NMR spectrum of 4` -Epialtenuene (**13**).

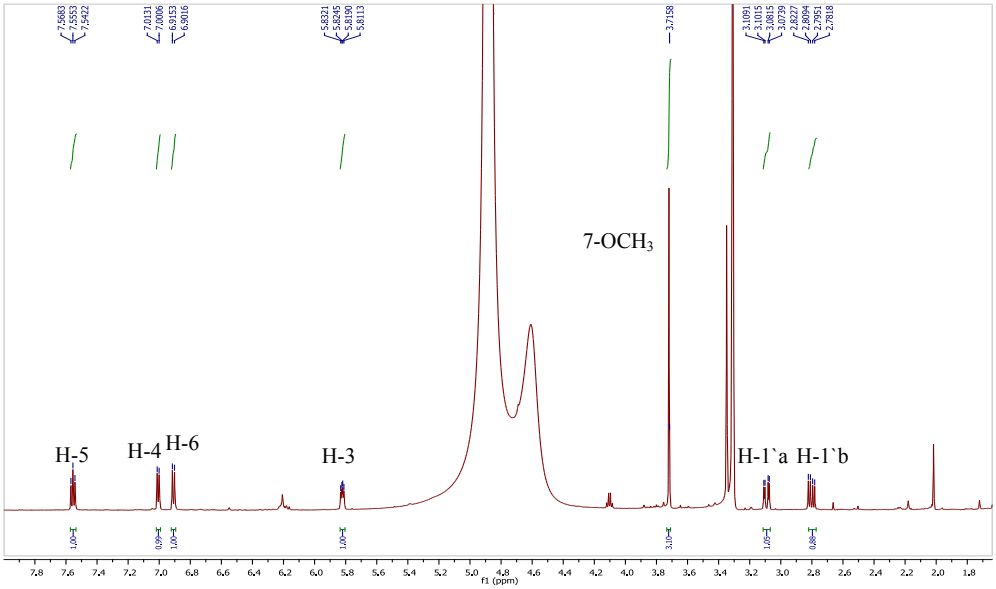


Attachment: The ^{1}H NMR spectrum of Isoochracinic acid (**14**).

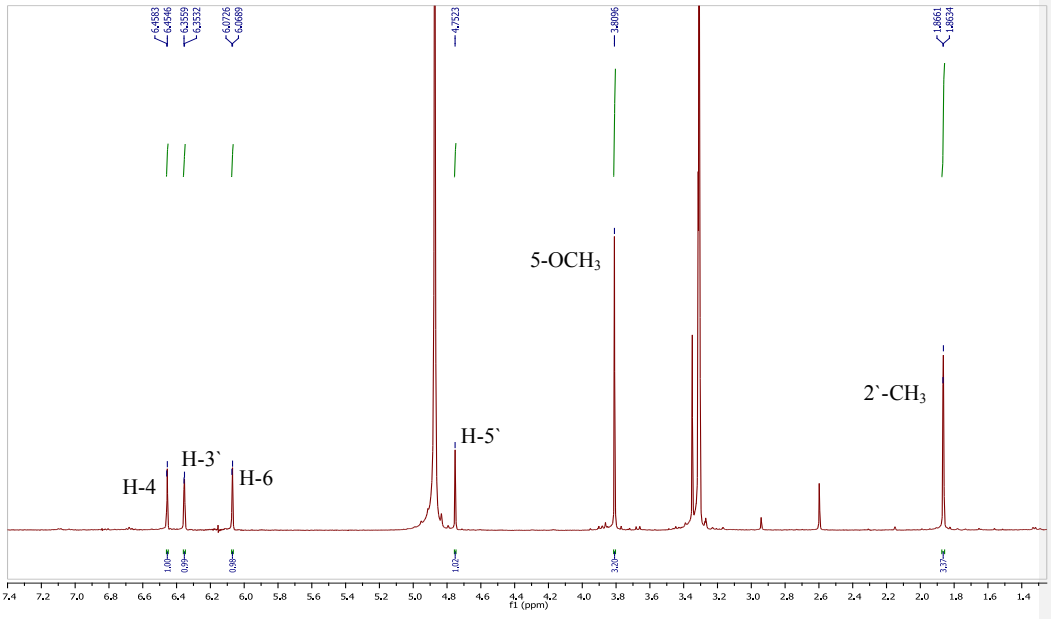


| *Attachments*

Attachment: The ^{1}H NMR spectrum of 7-Methoxyphthalide-3-acetic Acid (**15**).

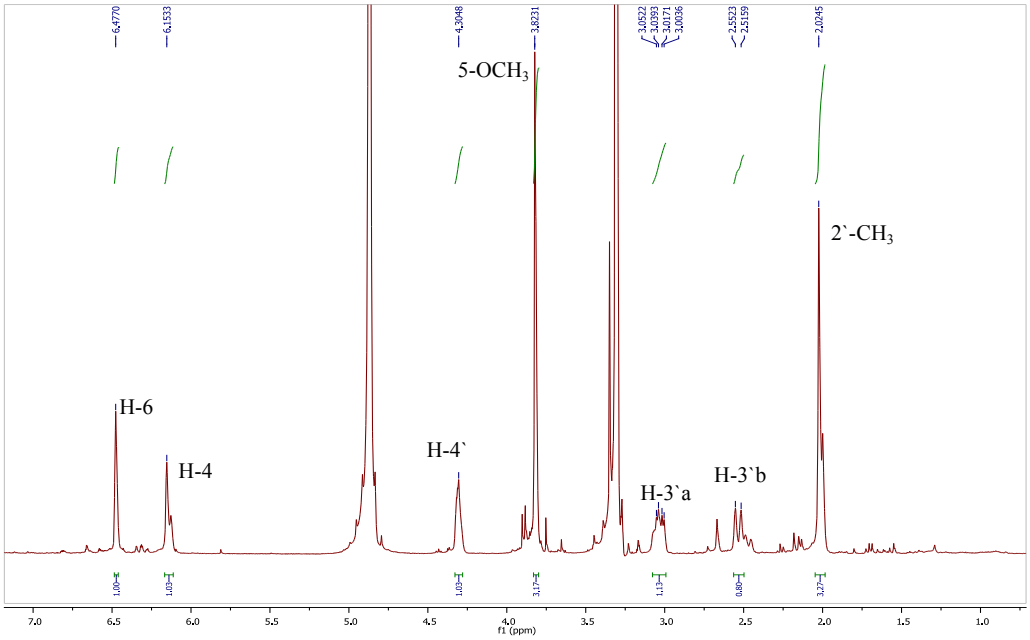


Attachment: The ^{1}H NMR spectrum of Talaroflavone (**16**).

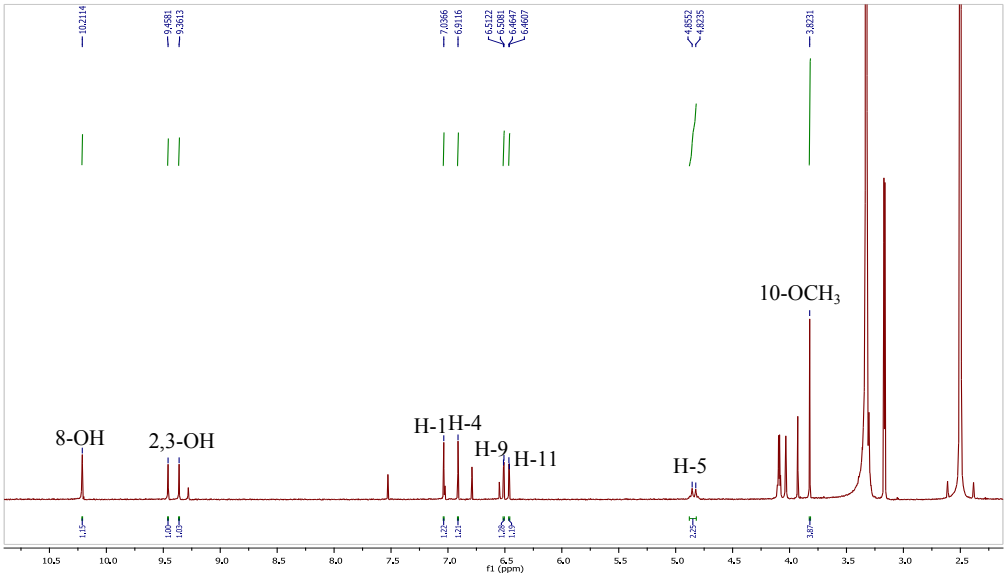


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Alternaric acid (**17**).

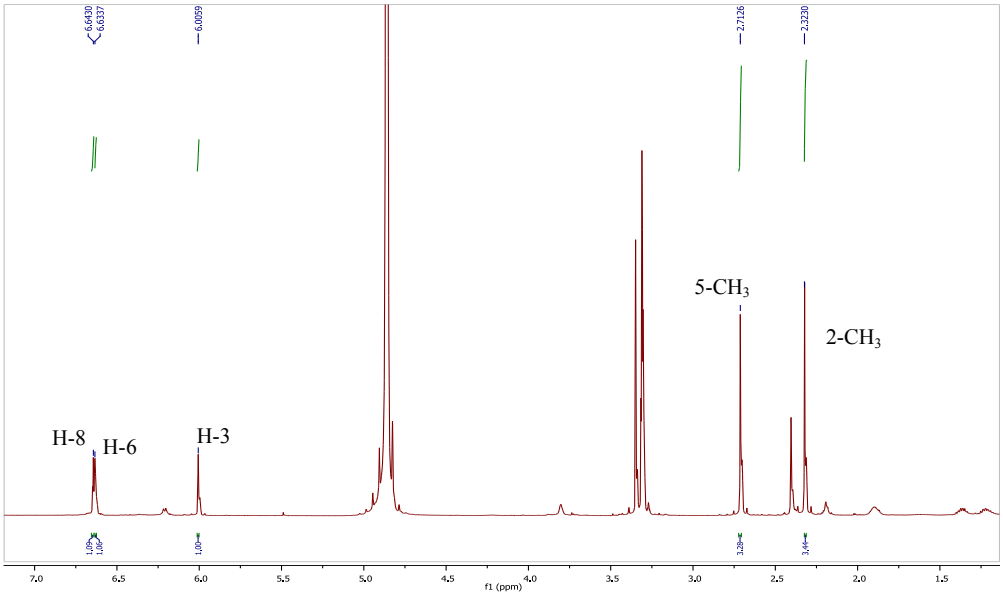


Attachment: The ^{1}H NMR spectrum of Alterlactone (**18**).

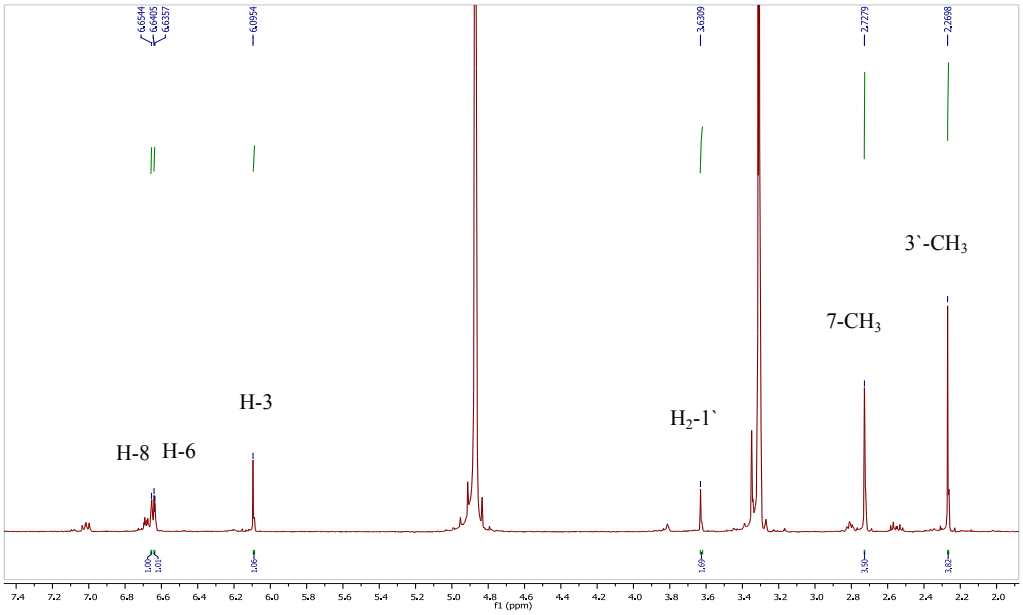


| *Attachments*

Attachment: The ^{1}H NMR spectrum of 2, 5-Dimethyl-7-hydroxychromone (**19**).

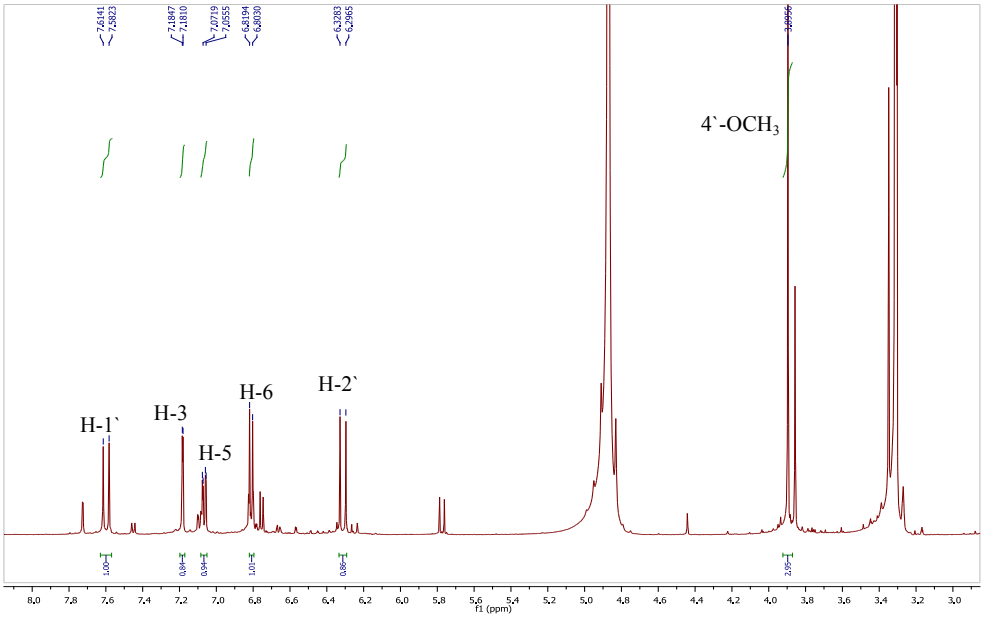


Attachment: The ^{1}H NMR spectrum of Altechromone B (**20**)

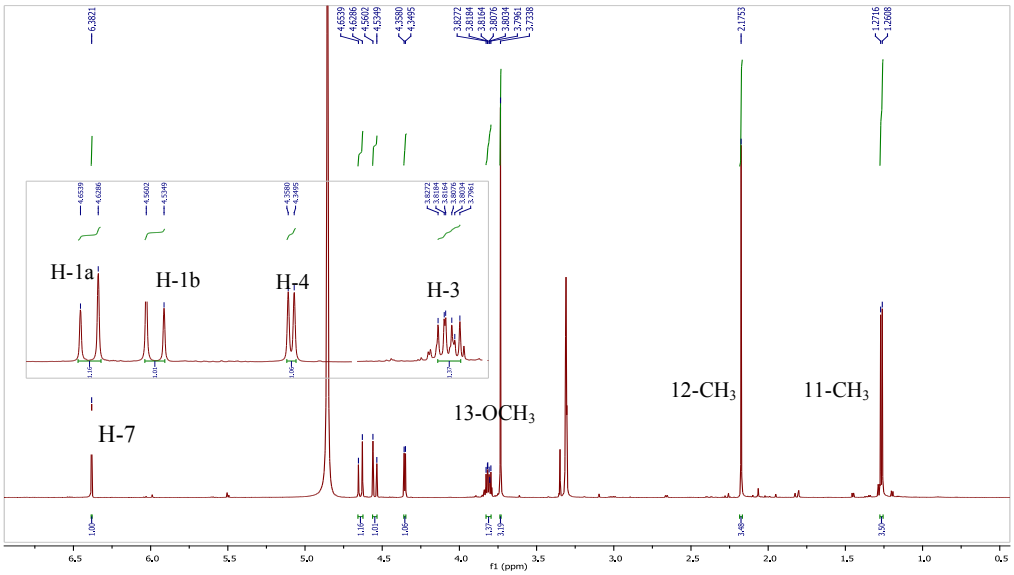


| *Attachments*

Attachment: The ^{1}H NMR spectrum of Ferulic Acid (**21**)

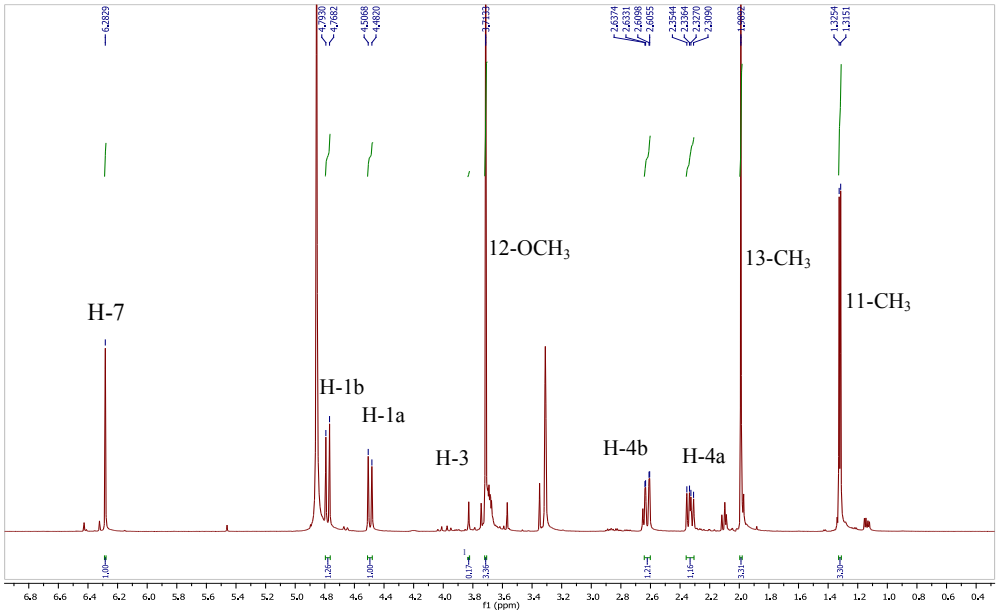


Attachment: The ^{1}H NMR spectrum of 6-Hydroxy-8-methoxy-3,5dimethylisochromanol (**22**).



| *Attachments*

Attachment: The ^{1}H NMR spectrum of 6-Hydroxy-8-methoxy-3, 5-dimethylisochroman (**23**).



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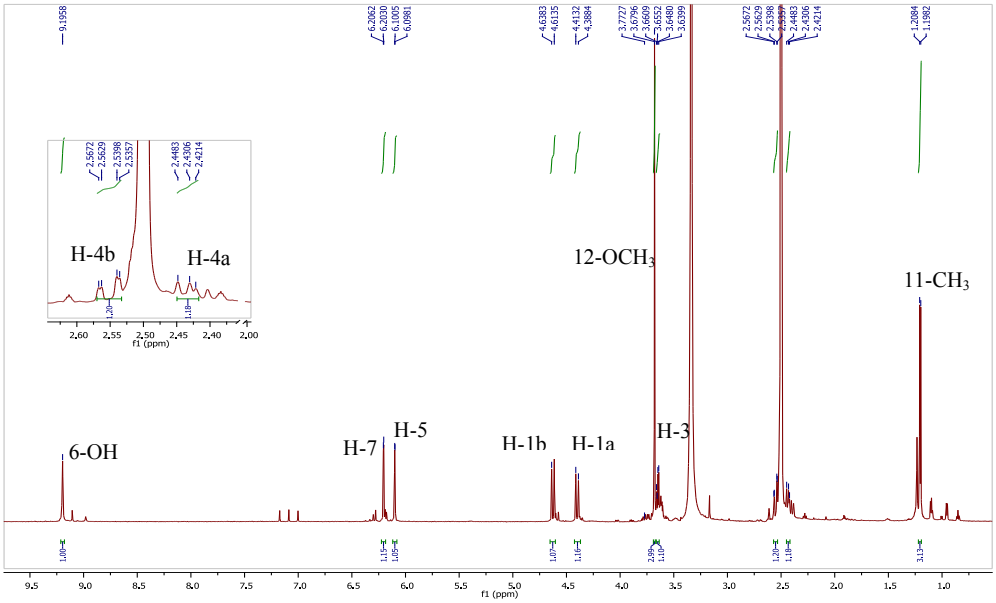
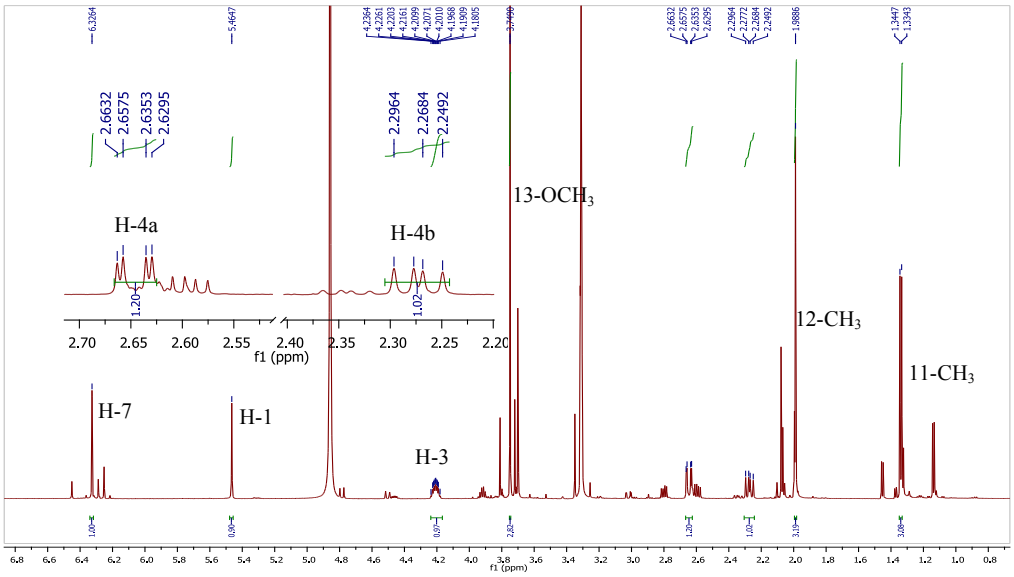
Attachment: The ^{1}H NMR spectrum of Arohynapene D (**24**).

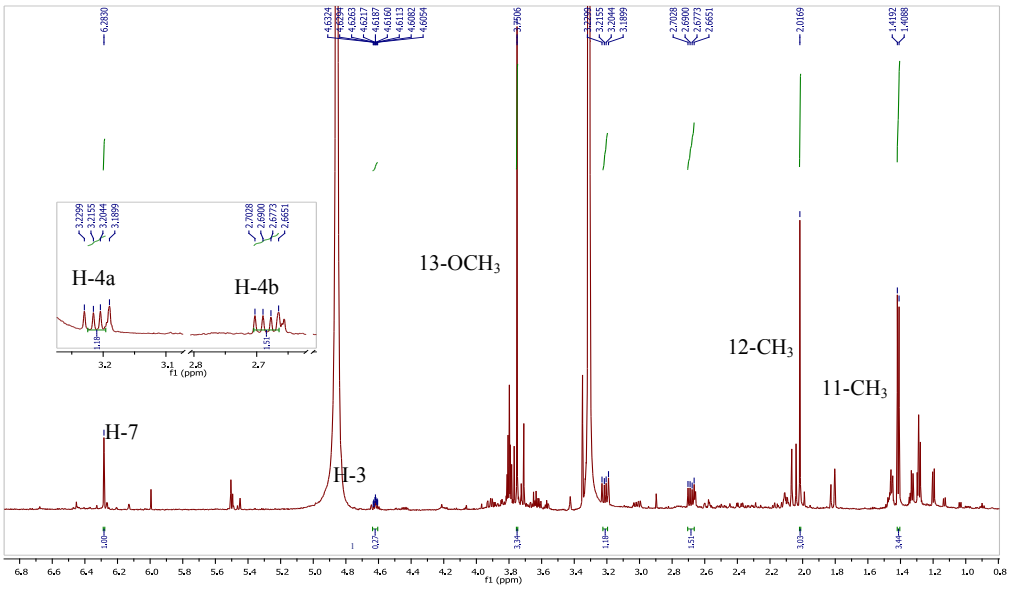


| *Attachments*

Attachment: The ^{1}H NMR spectrum of 1, 6-Dihydroxy-3, 5-dimethyl -8-methoxyisochroman (**25**).

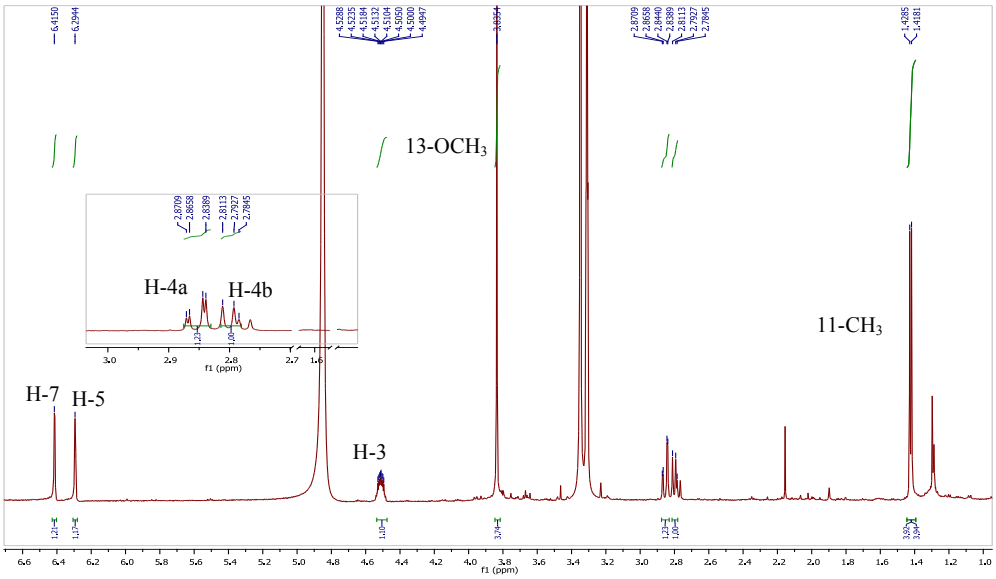


Attachment: The ^{1}H NMR spectrum of 6-Hydroxy-8-methoxy-3, 5-dimethyl-3, 4-dihydroisocoumarin (**26**).

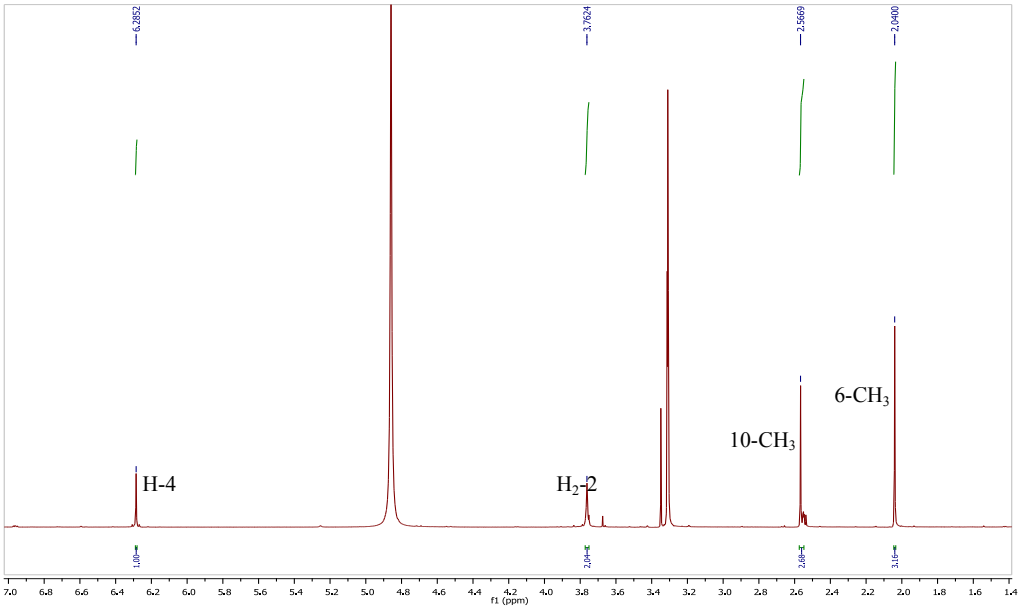


| *Attachments*

Attachment: The ^{1}H NMR spectrum of 6-Hydroxy-8-methoxy-3-methyl-3,4-dihydroisocoumarin (**27**).

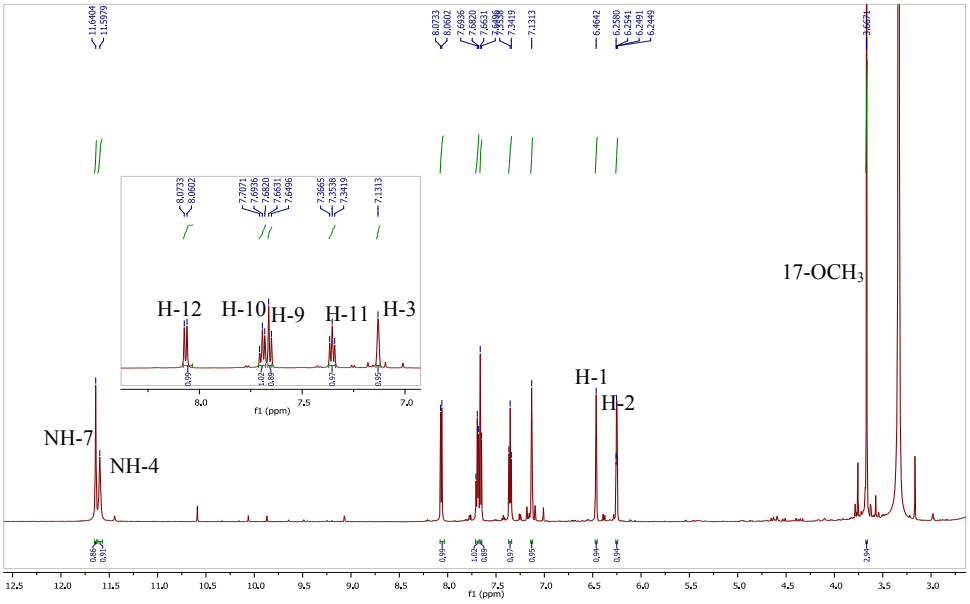


Attachment: The ^{1}H NMR spectrum of 6-Methylcurvulinic acid (**28**)

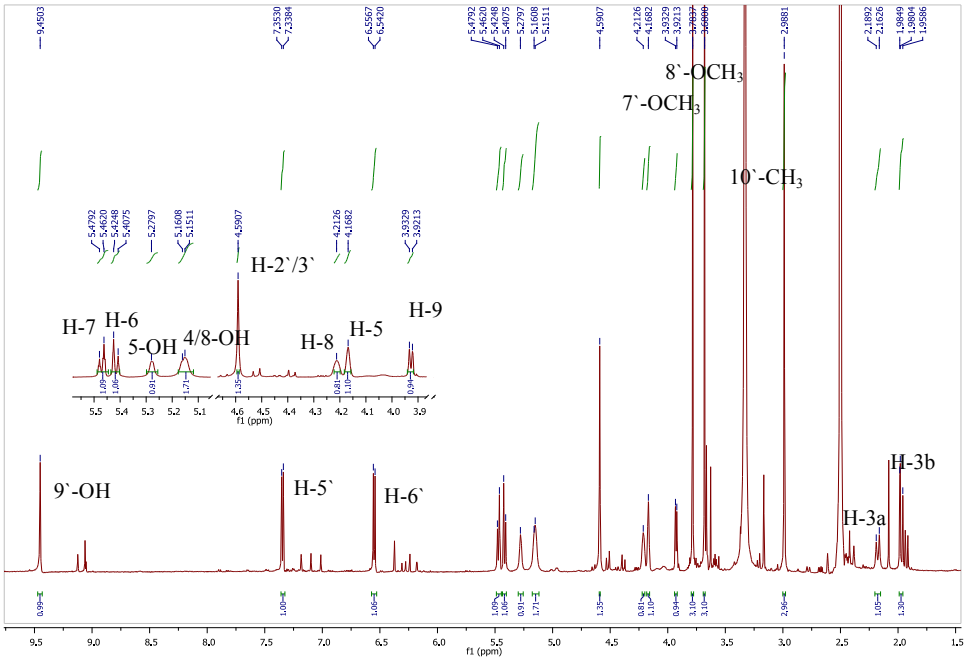


| *Attachments*

Attachment: The ^{1}H NMR spectrum of 2-Methyl-Penicinoline (**29**).

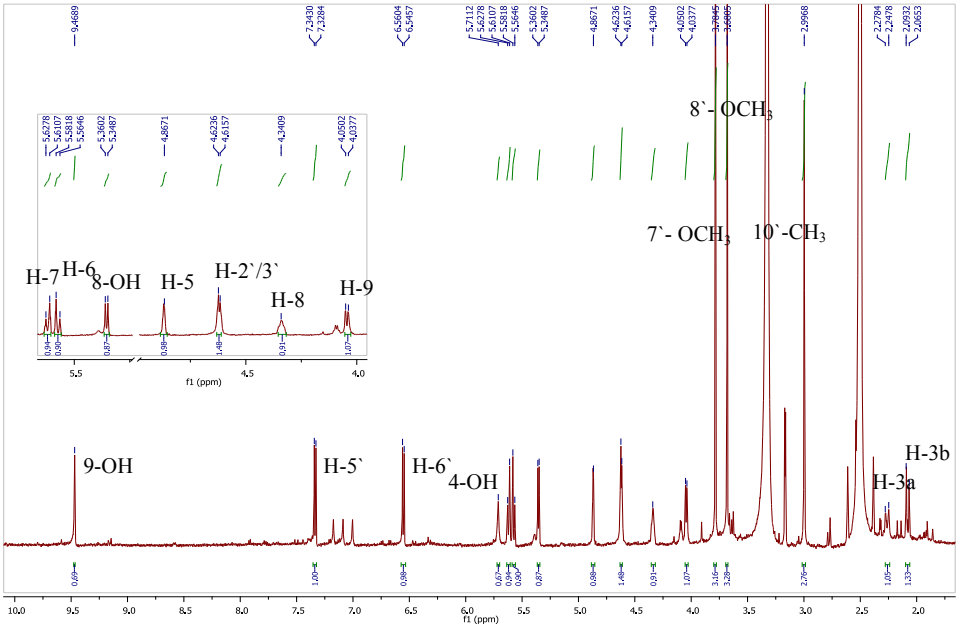


Attachment: The ^{1}H NMR spectrum of Pretichodermamide C (**30**).

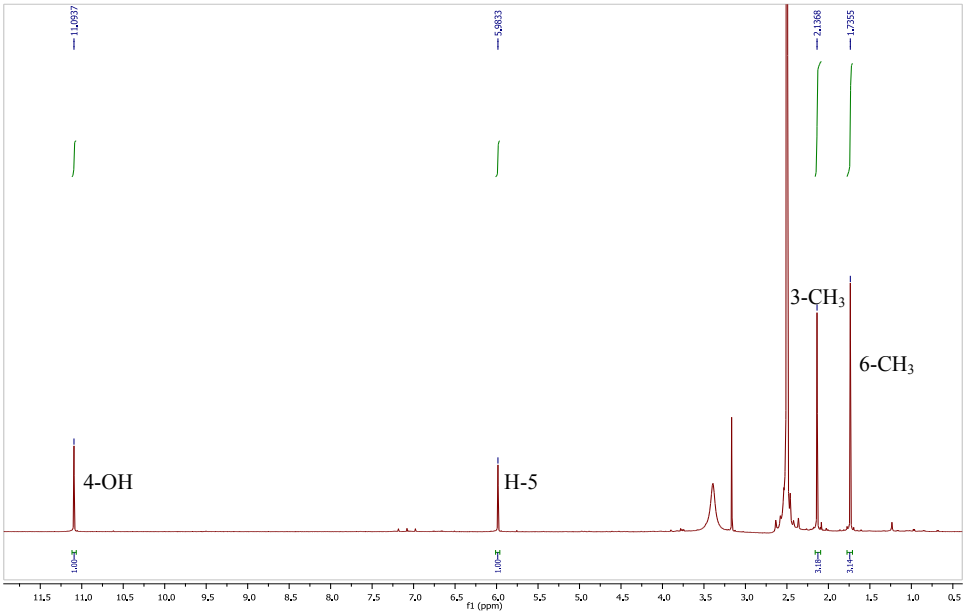


| *Attachments*

Attachment: The ^{1}H NMR spectrum of N-Methylpretrichodermamide B (**31**).



Attachment: The ^{1}H NMR spectrum of Methyltriacetic lactone (**32**).



9. Resume:

Name: Raha Saud Mohammad Mahmmoud Orfali
Day of birth: 21st of December 1981 in Madinah, Saudi Arabia
Nationality: Saudi

Course of Education

- Postgraduate studies at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich Heine-Universität Düsseldorf, Germany, since 2010.
- Receiving the M. Sc. degree in Pharmaceutical Sciences (Pharmacognosy) from the Faculty of Pharmacy, King Saud University, Riyadh, in 2009. The research project was entitled "Phytochemical and biological study of *Tamarix nilotica* growing in Saudi Arabia".
- Postgraduate studies at the Department of Pharmacognosy, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, 2007-2009.
-General and special preparatory courses of the M. Sc. in Pharmaceutical Sciences (Pharmacognosy) at Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, in 2007.
- Graduation with B. Sc. in Pharmacy, grade distinction, from the Faculty of Pharmacy, King Saud University Riyadh, Saudi Arabia in 2005.
- Ideal Student award, King Saud University, Riyadh, Saudi Arabia, for the year 2004.
- Graduate study in Pharmaceutical Sciences at Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, since 2000.
- Graduation with secondary school certificate, in 2000.

Scientific Experience

- Ph.D. student at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Germany, since 2010.
-A visitor student researcher at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Germany, 2008.
- Assistant lecturer at Department of Pharmacognosy, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, from 2006–2009.

-Supervising and teaching the graduate practical courses of pharmacognosy and Phytochemistry for 4th semester students at Department of Pharmacognosy, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, 2006.

-Supervising and teaching the graduate practical courses of pharmacognosy and Phytochemistry for 6rd–9th semester students at Department of Pharmacognosy, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, from 2007–2009.

- Demonstrator at Department of Pharmacognosy, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia, since 2006.

- The head of student`s activities committee, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia 2006-2008.

Publications

1) Orfali, R. S., Ebada, S. S., El-Shafae, A. M., Al-Taweel, A. M., Lin, W. H., Wray, V., and Proksch, P. (**2009**). 3-*O*-*trans*-Caffeoylisomyricadiol: A new triterpenoid from *Tamarix nilotica* growing in Saudi Arabia. *Z. Naturforsch.*, ***64c***, 637–643.

2) Orfali, R. S., Hassan, A. A., Ebramim, W., Müller, W. E. G., Lin, W., Daletos, G., and Proksch P (**2014**). Pretrichodermamide C and N-Methylpretrichodermamide, two new cytotoxic epidithiodiketopiperazine from hyper saline lake derived *Penicillium* sp. *Phytochemistry Lett.* (Accepted).

Conferences

- The 2nd Riyadh International Dental & Pharmacy Meeting, 2007.
- The 7th international Saudi pharmaceutical conference, 2007.
- The 9th international pharmaceutical sciences conference and exhibition, college of pharmacy, 2005.
- The 6th international Saudi pharmaceutical conference, 2003.
- 3rd symposium of psychiatric pharmacy, 2004.