ISOLATION, OPTIMIZATION AND CHARACTERIZATION OF SECONDARY METABOLITES FROM SOIL BORNE FUNGI *SCRELOTIUM* AND *ASPERGILLUS* SPECIES

Muhammad Rizwan

CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY
UNIVERSITY OF PESHAWAR
2016

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Muhammad Rizwan

A thesis submitted to the University of Peshawar in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology

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This dissertation is submitted by Muhammad Rizwan as partial Fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology

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2016

AUTHOR'S DECLARATION

I solemnly declare that the research work presented in this thesis was carried out in accordance with the requirement of the University of Peshawar's regulation for research Degree programs. The author has not been submitted for any other academic award. The work is original and of author's own data. While work done in collaboration with, or with assistance of, other is indicated as such. The views expressed in the thesis, belongs to the authors.

Date: 09/09/2016 Signature of Scholar 

DEDICATED TO

MY SWEET MOTHER

&

MY LOVING FATHER

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	ABBREVIATIONS
ACP	Acyl Carrier Protein
AIDS	Acquired Immuno-deficiency Syndrome
BSL	Brine Shrimps Lethality
^{0}C	Centigrade
cc	column chromatography
CDH	Cellobiose dehydrogenase
CoA	acetyl-coenzyme A
DCM	Dichloromethane
DMAPP	dimethylallyl diphosphate
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
FAR	Fluorescence Activity Ratio
FSC	Forward Scatter Count
GGPP	geranylgeranyl PP
HMBC	*Heteronuclear Multiple Bond Correlation*
IPP	isopentenyl diphosphate
IR	Infrared
MDR	Multi-drug resistance
MCG	Mycelial compatibility group
MHA	Muller Hinton Agar
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Bank
P-gp	P-glycoprotein
PKS	Polyketide Synthase

RPM	revolution per minute
SOM	Soil organic matter
SSC	Side Scatter Count
TLC	Thin-layer chromatography
UV	Ultraviolet
µg	Micro gram
VRI	Veterinary Research Institute

ACKNOWLEDGEMENT

First of all, I bow my head to the Omnipresent, Omnipotent and Omniscient, Al-Mighty **Allah** and i bow my head in His gratitude for enabling me to achieve one of the goal of my life. Million and million times peace (Darood- o- salam) upon the holy Prophet Hazrat **Muhammad** (Peace of Allah be upon him), Who is blessing for humanity as a whole and for whole of the universe and Whose life and teachings is a source of eternal.

First and foremost I wish to thank my Supervisor, **Dr. Bashir Ahmad, Meritorious Professor,** Centre of Biotechnology and Microbiology. He has been supportive since the days I began working as an undergraduate. His personal interest, faithfulness, efficient contribution, sincere advices, marvellous and ongoing support during the period of this research work. I would always consider myself to be fortunate and privileged to have worked under his guidance.

I feel great honour to express my deep sense of gratitude to my kind and scholarly Co-supervisor **Dr. Sadiq Azam**, Assistant professor at Centre of Biotechnology and Microbiology, for his inspiring guidance, constant encouragement, cool, calm and cooperative behaviour during the course of work which made it feasible to carry out this research work with success.

I am also thankful to **Prof Dr. Ghosia Lutullah** Director of the Centre of Biotechnology and Microbiology, University of Peshawar for her valuable guidance and cooperation during my student life at COBAM.

The acknowledge will be incomplete without thanking of **Dr. Abdur Rauf**, Assistant Professor, Department of Geology, University of Swabi for his cooperating and relaxing behaviour at every tough time that I faced during my research work. I appreciate the valuable suggestion, personal interest, extended cooperation and continuous help through the entire period.

I would also like to extend my sincere thanks to **Miftah udin**, **Zahid lala** and **Jamal kaka** for their personal interest in my PhD

I am blessed by having a friends; **Dr. Muhammad Bilal**, **Said Hassan and Muhammad (UoS)** who always provided support and motivation to me. My appreciation also goes to all my lab fellow especially **Zobia Afsheen**, **Saifullah Khan, Aminuulah, Sadia, Aamir Aziz, Kashif, Aishma Khatak, Nabya Hafeez, Kishwar Sultan, Humaira Naz, Idrees, Saira jamil and Seema Nawaz.**

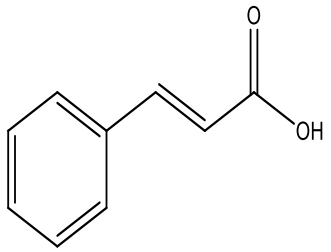
Finally my warmest appreciation and deepest sense of gratitude is for my Father and Mother, who enabled me to find best in myself and for their constant encouragement and limitless prayers. It gives me great pleasure to thank my brothers; **Muhammad Unwan** and **Muhammad Mehran** and sisters for their constant support, care and prayers and making the most difficult times of my life trouble free and uncomplicated. May i fulfill their expectations (Ameen)

MUHAMMAD RIZWAN

Abstract

In the present dissertation, our research findings on the production of bioactive secondary metabolites by soil borne fungi are discussed. The application of microorganisms for the welfare of human beings is the main goal of biotechnology. In this study, two phytopathogenic fungi, *Sclerotium rolfsii* and *Aspergillus flavus* were isolated from soil samples collected from Malakand Division, Khyber Pakhtunkhwa, Pakistan. Growth parameters (nutrient media, temperature, pH, incubation period, and static/shaking intervals) were optimized for achieving maximum production of bioactive secondary metabolites. Five nutrient media were used for fungal growth. *S. rolfsii* produced maximum amounts of metabolites in Czapek yeast broth media (CYB), whereas maximum formation of metabolites by *A. flavus* was found in potato dextrose media (PDB). Furthermore, the crude secondary metabolites in ethyl acetate (EtOAc) and *n*-hexane extract obtained from each medium were screened for their activities against different pathogenic bacteria. The EtOAc and *n*-hexane fractions obtained from the CYB medium were more active against pathogenic bacteria as compared to the crude metabolites obtained from the other media in the case with *S. rolfsii*, whereas PDB was the media, in which *A. flavus* yielded the largest quantities of secondary metabolites, showed significant results against pathogenic bacteria. EtOAc and *n*-hexane fractions of both fungi were screened for their *in vitro* effects, including antifungal, phytotoxic, and insecticidal properties and brine shrimp lethality. The *in vivo* activities of the metabolites, such as acute toxicity, analgesic, and sedative action, were also studied. The results showed that the ethyl acetate (EtOAc) fraction was more active as compared to the *n*-hexane fraction due to the presence of polar constituents. The results obtained from the examinations of the biological activities indicated that both fungi produced bioactive secondary metabolites which have antimicrobial, phytotoxic, insecticidal, cytotoxic, analgesic, and sedative effects. The EtOAc fraction of *S. rolfsii* and *A. flavus* was significantly more active against carbonic anhydrase with IC_{50} values of 45.40 ±0.75 and 59.89

±1.65, respectively. Similarly, the *n*-hexane fraction of both fungi also showed significant results against carbonic anhydrase (62.5 and 63% at 0.2 mg/mL, respectively) with IC_{50} values (45.40 ±0.75 and 61.3 ±1.75, correspondingly). The EtOAc and *n*-hexane fraction of both fungi exerted insignificant influence against urease. In short, the isolated crude metabolites exhibited remarkable inhibition activity against carbonic anhydrase. The bioactive EtOAc fraction of both fungi were further subjected to column chromatography (cc), which yielded one new and five known compounds. Their purity was confirmed by thin-layer chromatography. The structures of the isolated compounds were elucidated by using various modern spectroscopic analyses, including ^{1}H-NMR, ^{13}C-NMR, HMBC, and EI-MS spectra. One new and four known secondary metabolites were isolated from *S. rolfsii* and one known compound was isolated from *A. flavus*. The bioactive compounds isolated from *S. rolfsii* were: cinnamic acid (**1**), chlorogenic acid (**2**), Screlotiumol (**3**), *o*-coumaric acid (**4**), and gallic acid (**5**), whereas kojic acid (**6**) was isolated from *A. flavus.* Two compounds were obtained in high quantity, chlorogenic acid (**2**) and screlotiumol (**3**), and assessed for their effects on the reversion of multidrug resistant (MDR) mediated by P-glycoprotein (P-gp). In cancer cell lines, the multidrug resistant P-glycoprotein is a target for chemotherapeutic drugs. Both tested compounds showed an excellent MDR reversing impact against the mouse T-lymphoma cell line in a dose-dependent manner. Furthermore, compounds (**2** and **3**) were subject to molecular docking. Optimal effects of molecular docking were obtained by compounds (**2** and **3**) as compared to the standard treatment. Therefore, the preliminary results obtained in the present investigation indicate that these compounds could be used in the selection of potential targets for the treatment of cancer.



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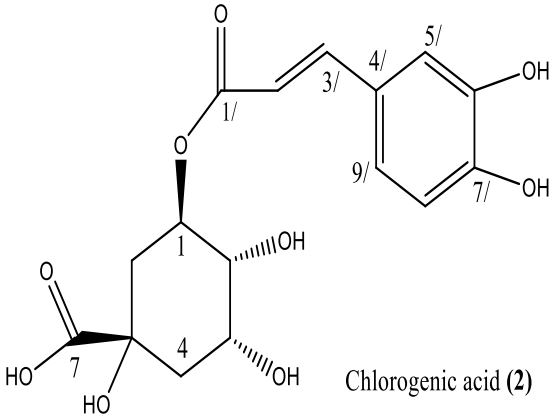

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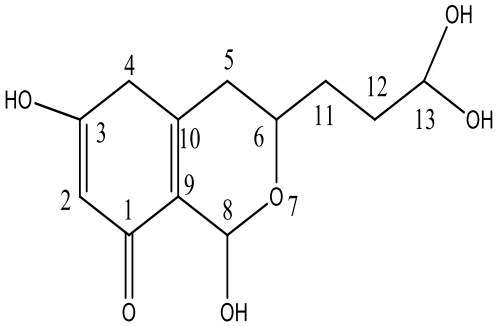
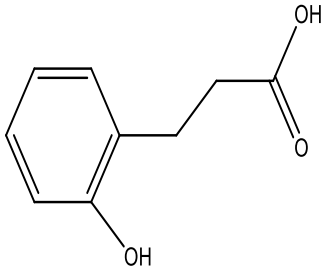
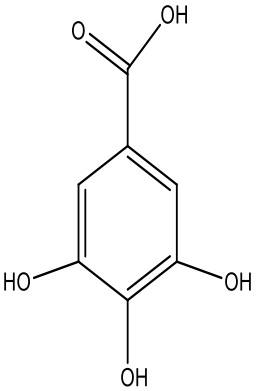


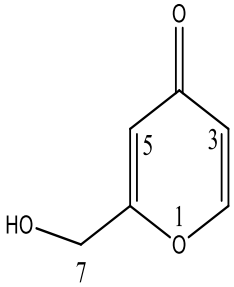
screlotiumol (**3**)



O-Cumaric acid (**4**)



Gallic acid (**5**)



Kojic acid (**6**)

1. INTRODUCTION & LITERATURE REVIVEW

1.1. Soil as a microhabitat

Soil organic matter (SOM) is usually considered to comprise two main agents: the "living" (roots and microbial populations) and the "non-living" (nutrients of different origins) pool [1]. The decomposition of organic matter provides energy for microbial growth and carbon for the synthesis of new cell material [2]. Carbon is the main constituent, typically accounting for around 58% of its total weight. Except carbon, the soil also contains oxygen, hydrogen, nitrogen, phosphorus, sulfur, proteins, carbohydrates, lignins, enzymes, and nucleic acids [3]. Plant material is usually considered the largest component of the soil organic matter, containing both recalcitrant and labile compounds. The labile substances, such as amino acids and sugar, are generally soluble and easy to degrade; the more recalcitrant are typically natural polymers, such as cellulose and lignin. Nevertheless, soil is generally poor in nutrients and energy sources (estimated to be only approximately 5% of its solid material content), when compared with the conditions used for microbial growth under laboratory conditions [4, 5]. Thus, microorganisms are in constant competition for nutrient sources [6], consisting mainly of carbohydrates, nitrogen, phosphorus, and ions, such as iron (Fe^{3+}) and calcium (Ca^{2+}) [3].

Nutrients are not homogeneously distributed throughout the bulk of the soil but rather heterogeneously dispersed, creating discrete microhabitats or "hot spots" [7, 5], where the microorganisms live and interact. The chemical, physical, and biological characteristics of these zones of high biological activity are thus different from one microhabitat to the next, and also vary with time and as influenced by environmental factors. In addition, the characteristics of organic matter in any soil are in a state of flux that is influenced by climate, vegetation, and agricultural practices [8, 5].

1.1.1. Characterization of soil microbial communities

Microbial communities inhabiting the soil environment are large and diverse. There is a close relationship between soil functioning and microbial activity; the microorganisms are sensitive and respond quickly to the changes in soil conditions. Therefore, the characteristics of microbial populations have been widely used as a tool for monitoring soil quality [9, 10]. Three main aspects of microbial populations are usually considered for assessing their relationship with soil functioning: population size, activity, and diversity. Characterizing soil microbial populations may have various applications in investigations on the impact of environmental factors, nutrient addition, or soil properties on microbial populations, as well as in the monitoring of soil health and the bioremediation of soil contaminants [11-15].

1.1.2. Microbial biomass and population size

Microbial biomass is known as the living component of SOM, which includes all living organisms smaller than 5–10 μm^{-3}. The diameter of the bacterial cell is usually less than 2 μm, bacterial population densities are approximately 10^9 cells/g in an agricultural soil, and their biomass is within the range of 50–500 Kg C ha^{-1}. On the other hand, the diameters of fungal hyphae range from 2 to 10 μm, easily reaching total lengths of 10–1000 m g^{-1} soil and a biomass of 1–500 Kg C ha^{-1} [16]. Actinomycetes, particularly from the genus *Streptomyces,* are also well-adapted and highly competitive soil inhabitants [17]. They are mainly known for their ability to degrade the more complex and recalcitrant fraction of soil organic matter and to produce a wide range of antibiotics (streptomycin) and geosmin-like compounds [17].

The use of both traditional (electron microscopy with staining procedures) and more advanced (gene- and antibody-based) experimental techniques has revealed that 80–90% of soil microorganisms live attached to solid surfaces, such as mineral particles and organic matter

[18]. The remaining 10–20% microorganisms have been found to live inside soil aggregates and in water films surrounding solid particles [19].

Although microbial biomass represents ≤ 5% of soil weight, it is critical for soil functioning [20. 21]. Among other roles, microorganisms are both a source and a sink for nutrients and exert key activities in organic matter decomposition and nutrient cycling, nitrogen fixation [16], and biodegradation and bioaccumulation of xenobiotic compounds (pesticides, synthetic polymers, and heavy metals) [22]. The information concerning the size of viable microbial populations is usually an adequate indicator of soil status [23] and may be collected using a wide range of methods. It is important, however, to note that in the literature some of these techniques provide an estimation of microbial population size, whereas others measure microbial biomass. The difference between both types of information is that biomass is usually expressed in carbon units [16].

1.1.3. Total soil microbial populations

Soil microbial populations are among the most complex, wide spread, and important components of the biosphere. Soil microbes are difficult to characterize genetically and phenotypically due to their high level of diversity [24]. Microbial populations are the keystone of the soil functions and structure [23]. Microorganisms, such as fungi, algae, bacteria, and nematodes are important to soil nutrition because of their substantial role in the degradation of plant residues and other organic matter in the soil [25]. The characteristics of the microbial populations thriving in contaminated soils can supply valuable information regarding soil quality and microbial activities, and can also contribute to determining the extent to which the microbial population has acclimatized to the soil site conditions [26]. Furthermore, the evaluation of the potential of microbial populations to degrade pollutants is facilitated, and the

possible necessity for provision of supplementary starter cultures that enhance the biodegradation process can also be easily assessed [27].

1.2. Introduction to Fungi

The kingdom of fungi contains some of the most important microorganisms in the biosphere [28]. Fungi are heterotrophic single-celled, multi-nucleated, or multi-cellular organisms, which include mushrooms, molds, and yeasts. Fungi live as saprobes, symbionts, or parasites. Initially, fungi were classified in the plant kingdom, because, similarly to plants, they are immobile. However, fungal organisms lack the vascular tissues (xylem and phloem) that form leaves, stems, and roots of plants. Most multi-cellular fungi are composed of multiple filaments, called *hyphae*. Molecular data revealed that fungi originated one billion years ago [29]. Fungi is the second-largest group after that of insects, and its species are widespread in nature. Fungi are located in the Antarctic ice, as well as in tropical and temperate regions. They can survive in soils, sea water, and the surface of mountain tops [30]. Fungi attack mammals, plants, fish, protozoa, and insects, causing diseases. Generally accepted estimates indicate that approximate number of fungal species on earth is 1.5 million [31]. However, no precise information exist on the exact number of the known fungal species, but a number within the range of 72,000–100,000 can be reasonable. This data show that the part of the fungi known today does not exceed 5% of all estimated existing species. A huge number of fungi exist in the world that have not described yet, which implies that these organisms represent an important source of natural compounds with diverse chemical structures and functions [32]. Until now, ninety nine thousands species of fungi have been discovered, and approximately twelve hundred new species per year are described [33, 34]. Fungi perform a wide range of functions: some of them are parasites, pathogens, or decomposers, and others are beneficial partners in symbiosis with plants, animals, and algae. The identified species of fungi are commonly categorized into four phyla or divisions: Basidiomycota (toadstools, puffballs, and

mushrooms), Zygomycota (common bread mold), Ascomycota (Yeasts), and Deuteromycota (the genera *Verticillium, Pythium*) [29].

Fungi represent an essential part of the soil microbial population, usually, depending on nutrient condition and soil depth, constituting more of the soil biomass than bacteria [35]. Many important plant pathogens, such as rusts and smuts, and plant growth-enhancing microorganisms are fungi. The largest numbers of fungal species in the soil are saprobic, and they exert exceedingly important functions in the degradation of plant structural complex polymers: lignin, cellulose, and hemicellulose, and thus they are involved in the maintenance of the global carbon cycle. The catabolic activities of fungi provide them with the ability to grow and survive on cheap substrates under laboratory conditions. This characteristic, combined with their ability to produce commercially significant secondary metabolites and enzymes, explains their valuable contribution in the field of biotechnology.

1.3. Fungi and Bacteria interaction

The communities of bacteria and fungi in the ecosystems exert vital functions, as they separately and together degrade and mineralize organic compounds [36]. It was observed that their combined action leads to better results in comparison to the separate activity of either. Bacteria and fungi often share the same microhabitat [37] and interact with each other. Generally, such interactions may be antagonistic, neutral, or beneficial for the microbes. Antagonism may be expressed by the production of antifungal or antibacterial compounds or simply by the competition for nutrients that can lead to a great reduction of the fungal biomass in the presence of competing bacteria and vice versa [38]. Some bacteria may even obtain nutrients by mycophagy (feeding on living fungi) [39, 40].

A neutral interaction exists when there is neither positive nor negative effect caused by the presence of the other organism; these are the cases in which the organisms have different

niches. However, a beneficial interaction is desirable so that efficient degradation of pesticides can be achieved.

Beneficial interactions can either be commensal, with one of the organisms benefiting and the other remaining unaffected, or mutual, with both organisms benefiting from the interaction [41]. It has been shown that soil fungi can create niches where bacteria can thrive. For example, it has been found that the survival of the *Variovorax paradoxus*-like strain HB44 in sterilized soil was significantly improved by the presence of the fungus *Lyophyllum* sp. strain *Karsten*, and that the bacterial strain grew readily on compounds, in particular glycerol, released by the fungus [42]. In addition, Warmink et al. (2009) discovered that bacteria found in the mycosphere (fungiphiles) could utilize specific fungal exudates, but the free-living in the bulk soil bacteria could not [37]. The degradation of organic matter (i.e., nutrient cycling) is considered the most important function of soil organisms: soil fauna is valuable for mixing the litter and microorganisms, mainly bacteria and fungi; then, these materials degrade and release simple and complex nutrients, which become available for plant growth [43, 16].

1.4. Importance of Fungi

Fungi are the most important microorganism in the biosphere, attracting a great interest for biotechnological applications [44]. They act as a factory and are of significance for the production of a variety of chemicals of a high economic value, including antibiotics, commercial flavoring foods, and biochemicals, such as organic acids and enzymes [45].

1.4.1. Fungi as a factory for production of Biochemicals

A number of organic acids, such as itaconic, fumaric, citric, and lactic acid and gibberellins are produced industrially by fungi [46]. Some filamentous fungi cause the fermentation of cereal straw to ethanol, i.e., *Fusarium* spp. [47]. A wide range of enzymes have been produced by fungi that are used in different industries, e.g., amylase is used in the brewing

industry, and pectinase is utilized in the manufacturing of fruit juices [45]. Citric acid, produced by *Aspergillus* spp., is employed in the manufacturing of soft drinks, salts, and medicines. The gallic acid generated by *Aspergillus* and *Penicillin* spp. is utilized in the printing and leather tanning industries.

1.4.2. Fungi for flavoring food production

Fungi are also used for the production of commercially available flavoring food. Moreover, substances released by filamentous fungi are added to many food products (e.g., in some cheeses) to improve their odor, flavor, and color [48]. Some fungi are also used for an increase of the protein content of animal feed [49, 50]. The nutritional value of some product can also be improved by the addition of fungi or their products [51].

1.4.3. Use of fungi in environmental biotechnology

Fungi play a substantial environmental role, e.g., they participate in the treatment of hazardous wastes such as cyanide [52] and in the bioremediation of various chemical compounds, including those of soil pollutants [53-55] and the ones present in sewage [56]. White-rot fungi have the ability to break lignin, so they are used in the breakdown of variety of environmental pollutants [57]. Fungal biomass is also utilized to absorb metal ions from solutions; e.g., *Aspergillus* mycelium was used to remove zinc (Zn) from polluted water in Australia [58, 59].

1.4.4. Use of Fungi in Agriculture

Fungi are used as a biocontrol agent, preventing harm to humans and the environment caused by the application of pesticides that are conventionally used against insects and weeds [60]. In addition, fungal microorganisms act as a bio fertilizers and contribute to the improvement of crop yields [61]. Mycorrhizal fungi provide a linkage between the nutrient absorbing organs of plants and soil and optimize the uptake of soil phosphorus, which results

in an increase in the crop yield. Nematophagous fungi are also present in nature [62], which are natural enemies of nematodes and reduce their populations in the soil. When available in large quantities in the soil, they can attract, capture, and kill nematodes. A large number of nematophagous fungi have been discovered, most of which control plant parasitic nematodes [63]. The species *Paecilomyces lilacinus* has been extensively tested as a control agent against nematodes under field conditions [64] and is now commercially available in the Philippines under the trade name "Bioact".

1.4.5. Fungi and Pesticide degradation

Pesticides are the compounds used for different purposes; i.e., they are targeted to be toxic to certain groups of organisms. Insecticides target insects to protect crops from being eaten or damaged, fungicides target fungi to protect the crop from fungal attacks, and herbicides are aimed to protect the crops from the growth of specific weed plants. The global use of herbicides is extensive, and they account for the largest part of the overall pesticide utilization [64]. However, apart from their importance, pesticides have adverse effects on other non-target living organisms in the same ecosystems. Finally, some different pesticides may cross the level of human consumption through the groundwater contamination; therefore, the issue with pesticide pollution is extremely serious.

Saprotrophic fungi produce a wide range of extracellular enzymes which are essential for the degradation of plant materials [65], and these enzymes may also enable the fungal degradation of organic pollutants. For many years, white-rot fungi have been considered as top fungal candidates for bioremediation purposes due to their potent enzymatic activity [66, 67]. Some studies showed the effectiveness of fungal metabolites against pesticides. It was evidenced that a number of fungi have the ability to degrade phenyl urea herbicides, including

diuron. The species *Rhizoctonia solani* and *Bjerkandera adusta*, both belonging to the Basidiomycetes, were identified as the most efficient degraders of diuron [68-70].

1.4.6. Soil fungi and biodegradation

Mycodegradation is the biodegradation by fungi. Fungi are involved in biodegradation as well as bioremediation. Previous studies focused on the bioremediation potential of bacterial degraders. However, filamentous fungi possess characteristics which are advantageous in heterogeneous environments. It was considered that bacterial bioremediation was efficient, but filamentous fungi possess some qualities which are advantageous only in heterogeneous environment. Reportedly, the biodegradation and bioremediation realized by fungi was better than that by bacteria [71, 72]. Although fungi are non-motile, they can respond quickly to changing environmental conditions to survive or escape [73]. Abiotic factors that primarily affect the mycelial growth include temperature, water potential, pH, oxygen accessibility, and nutrient status [74]. However, filamentous fungi can escape unfavourable conditions, which in heterogeneous environments give them an advantage. Fungal hyphae are also able to infiltrate solid substances and reach microhabitats, the water-filled micropores in soil [75, 76]. Fungi may in this way achieve a much better contact to the nutrients and contaminants in environments where the compounds, trapped in microspores, are heterogeneously present and inaccessible to bacteria in other ways [77]. Communities of fungi and bacteria have vital functions in the environment, as they separately and together degrade and mineralize organic compounds [36].

1.5. Need of new Natural resources

The health problems experienced by people are increasing constantly. Different diseases are emerging, e.g., multi-drug resistance bacterial infections, cancer, acquired immunodeficiency syndrome (AIDS), and heart disease. The existing drugs are losing their effectiveness, so a

search for new and more effective drugs is underway, and fungi are often regarded as a novel source of bioactive compounds [78]. Curative agents for life-threatening diseases, such as acute respiratory syndromes, multidrug-resistant tuberculosis and *Staphylococcus aureus* nosocomial infections, life-threatening viral infections, and AIDS, are dramatically and urgently needed. Multi-drug resistant bacteria are the new emerging problem in the world; this emphasizes the critical significance of the search for new antibiotics [79]. A number of secondary metabolites produced by different fungi are used in the pharmaceutical and agricultural industries. Alkaloids are utilized in the treatment of migraine; cyclosporine is applied as an immunosuppressant, griseofulvin is employed in the antifungal therapy, and cytochalasins are widely administered as an anticancer agent [80-82].

1.6. Microbial activity of soil fungi

Microbial activity is used to indicate the wide range of activities carried out by microorganisms [83]. It is highly influenced by changes in the environmental and soil conditions [16]. Generally, microbial activity and growth are optimal when soils are near field capacity (c.a. 0.03–0.1 MPa), with near-neutral soil pH (6–7), and soil temperatures between 20–30°C [84].

Since microbial activity involves a complex of microbial processes, it cannot be evaluated by measuring a single parameter [9]. Soil respiration and enzymatic activities are the most widely measured microbial indicators for a diverse number of applications since they relate to the whole (active) soil microbial community. Any of these parameters can be estimated in the absence (actual activity) or presence (potential activity) of added substrates [16]

Nature has made microorganisms to produce a wide diversity of secondary metabolites. Based on the observation, natural products were the first and only medicines available to human beings. According to the World Health Organization (WHO), 80% of the total population

depends on natural products for their health care [85, 86]. A wide range of pharmaceutically significant compounds, belonging to all structural classes, are produced by fungi [87]. Mycophenolic acid was the first natural product obtained from *Penicillium glaucoma* in 1896, as described in the well-known story of the discovery of penicillin by Alexander Fleming [88]. The discovery of penicillin opened the door for the discovery of other drugs; and it enabled and empowered the research on the action of antibiotics [89].

1.7. Microbial metabolism

The metabolism is one of the most important qualities of a living cell. These processes allow the cells to reproduce and maintain their structure. Metabolic processes are divided into two main categories: catabolism and anabolism. Catabolism is the destructive process. The breakdown of the food material by which energy is released. Conversely, anabolism is the constructive process, in which the obtained energy is used to construct new macromolecules (**Fig 1.1**). There are two major types of metabolism: primary and secondary. The anabolic and catabolic processes refer to the primary metabolism. In the secondary metabolism, the compounds produced are not required for the cell growth of organisms [90]. Secondary metabolites have great importance for ecological adaptation; i.e., toxins produced by different fungi contribute to mutualism and ecological adaptation. Both the phytotoxic compounds, produced by phytopathogenic fungi against the host plant and the antibiotics, synthesized by saprophytic fungi against other organisms, are the parts of fungal defence mechanism. These are highly complex structures, and specific enzymatic reactions are involved in their synthesis. The secondary metabolites are a gift of nature to perform important biological activities. Other biological activities of these chemicals are not known [91]. A study revealed that the production of secondary metabolites occurs in stress conditions. Under batch culture condition, secondary metabolites are produced at the end of the exponential growth phase, when nutrient deficiency has started [92]. While secondary metabolites were produced throughout the exponential phase

under continuous culture conditions. The production of secondary metabolites depends on the composition of medium. It was noted that these metabolites are often generated from the intermediate products accumulated in the media or cell during the primary growth. The types of the secondary metabolites are specific for each particular group of fungi [93].

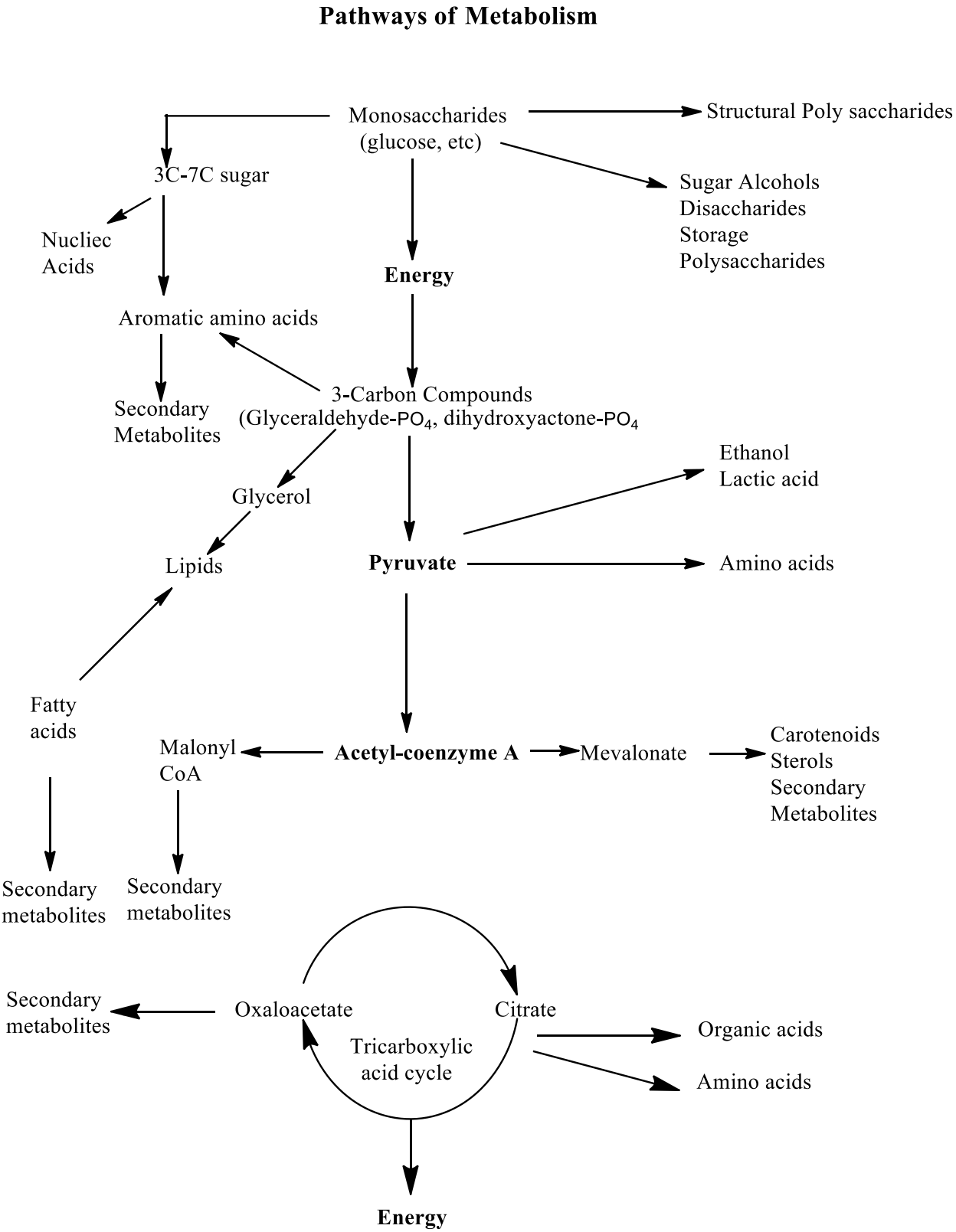


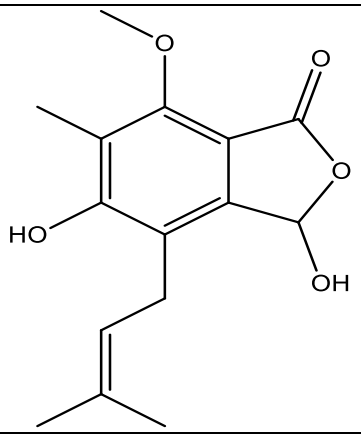
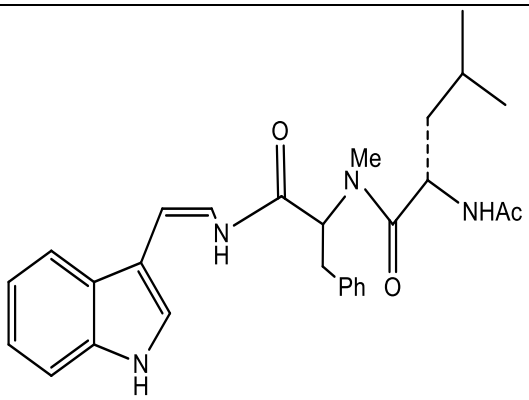
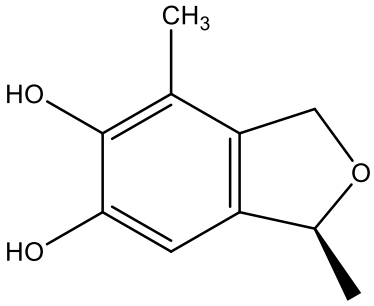
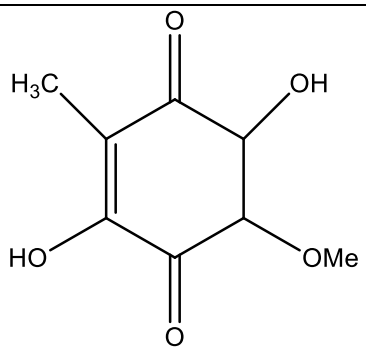
Figure 1.1: Pathways of metabolism

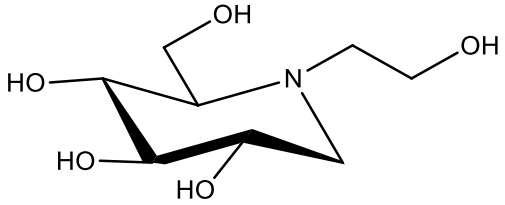
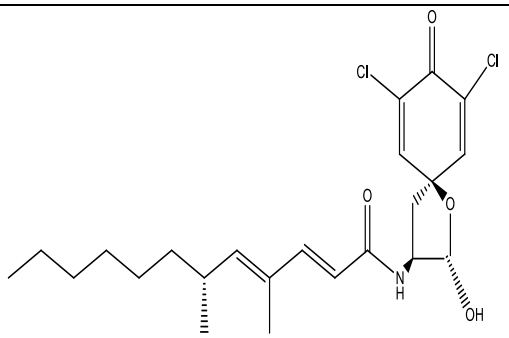
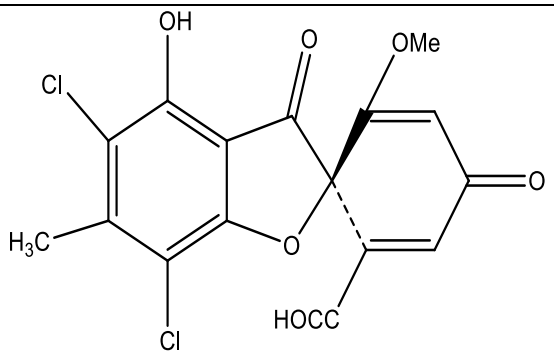
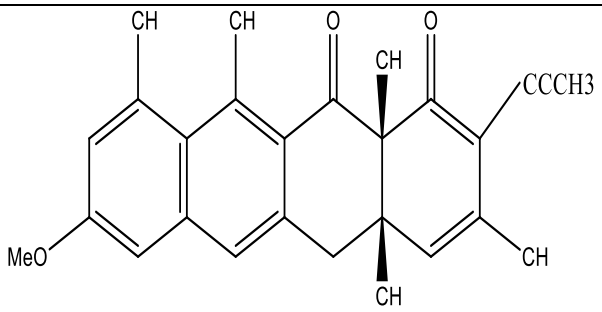
1.8. Secondary metabolites

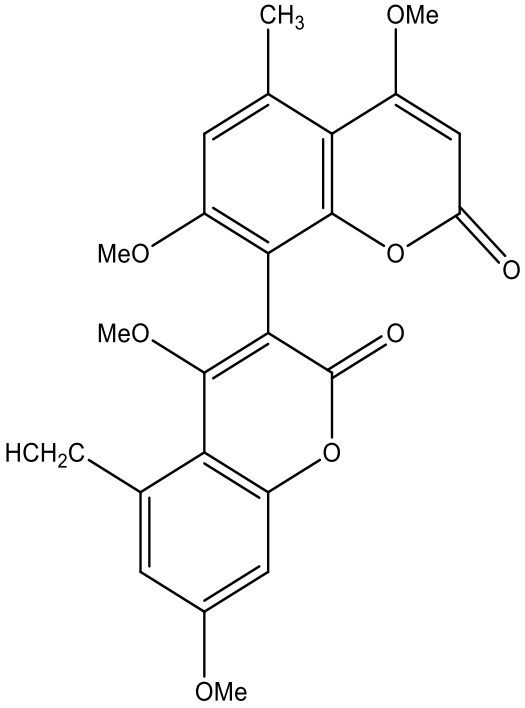
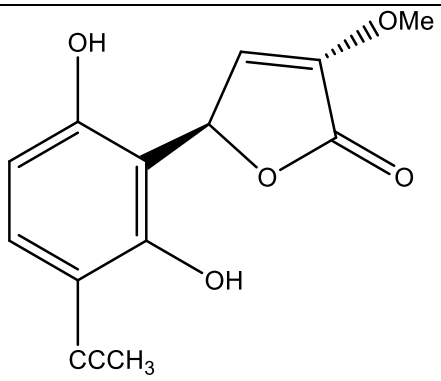
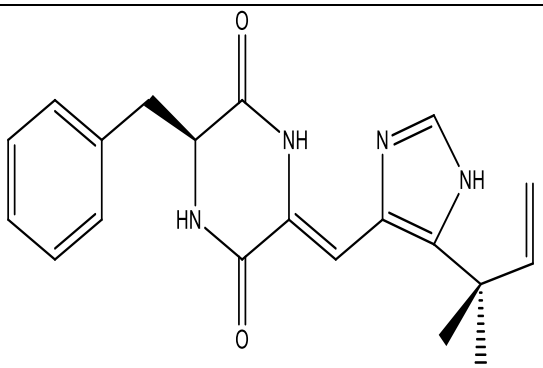
Fungi have the ability to produce bioactive compounds called secondary metabolites. Secondary metabolites are not necessary for growth or developmental process of the producing organism. Due to their bioactive nature, these metabolites have been used by humans in different industries, especially the pharmaceutical industry [94]. These substances act as a chemical shield for the fungus [95]. Secondary metabolites are low-molecular-weight compounds (generally with molecular masses < 3000 Da) [96, 97]. Different hypotheses have been presented in the past in an attempt to speculate on the reason for the production of secondary metabolites. According to one view, these chemicals are waste or detoxification products. Fungi produce a wide range of pharmaceutically active compounds. Secondary metabolites derived by fungi have been proven to be valuable sources of novel drugs and leading compounds for new pharmaceuticals [98]. Chemically new compounds with various biological activities have been isolated from fungi (**Table 1.1**). Many of these secondary metabolites have been proven to inhibit fungal and bacterial growth as well as the growth of parasites, viruses, and cancerous cells [99]. For this reason, search for new bioactive secondary metabolites and new applications of the previously discovered ones may provide development of novel pharmaceuticals compounds. Phytopathogenic fungi, which cause a variety of diseases in plants, are relatively unexplored and have the ability to produce novel natural products for pharmaceutical industry. It is noted that about 300,000 plant species are present on earth, and each of them may support the growth and survival of at least one or more species of phytopathogenic fungi. These fungi attack fruits, leaves, flowers, and stems before and after harvest. Some of the most common plant pathogenic fungi belong to the genera *Cochliobolus, Fusarium, Alternaria, Botrytis, Sclerotinia, Geotrichum,* and *Penicillium* [100]. The discovery of new antimicrobial secondary metabolites yielded by phytopathogenic fungi is an important alternative to curb the tendency of the overwhelmingly increasing levels of drug resistance of

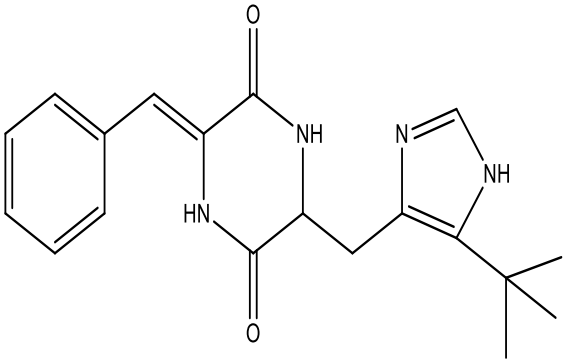
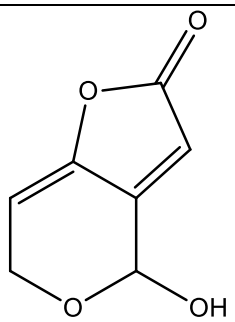
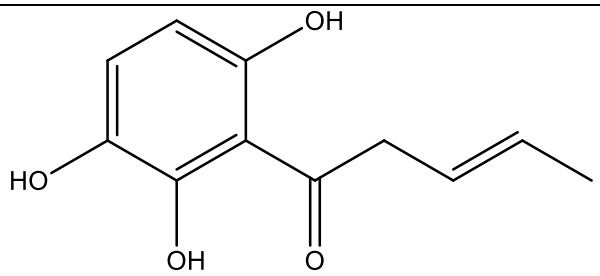
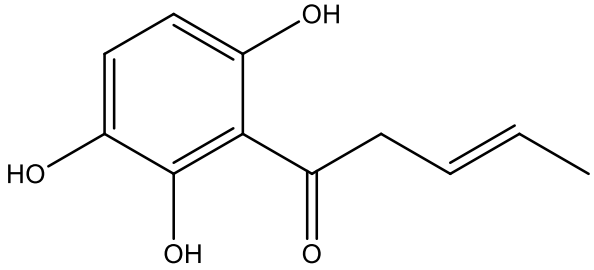
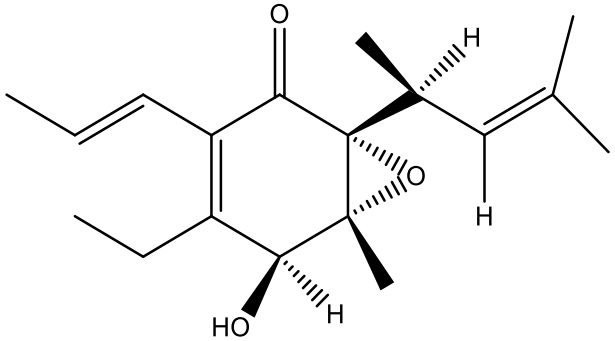
plant and human pathogens and the lack of the effective antibiotics against various bacterial species. The resistance among key microbial pathogens has increased at an alarming rate worldwide [101]. Many virulent pathogens, viz., *Staphylococcus aureus, Mycobacterium tuberculosis, Enterococcus* spp., *Streptococcus pneumoniae, Candida* spp., and *Pseudomonas aeruginosa* that are responsible for a wide range of infectious diseases have developed resistance to most classes of antibiotics [102]. The potential of phytopathogenic fungi to inhibit or kill other microorganisms is well known, and few of them have also been exploited for the production of antimicrobial metabolites. Secondary metabolites from the phytopathogenic fungi *Sclerotium rolfsii* and *Diplodia maydis* showed strong antibiotic activity against many multidrug resistant bacteria, including *Acinetobacter baumannii, Enterococcus cloacae, Klebsiella pneumoniae, Proteus mirabilis,* and *Staphylococcus aureus*. *Curvularia* and *Fusarium* have also been reported to act as important inhibitors of bacterial growth through the production of *Curvularin* and *Ascoclorin* [103, 104]. Similarly, production of antimicrobial naphthoquinone pigments from the phytopathogenic filamentous fungus *Fusarium verticillioides* has also been confirmed [105]. In spite of the so many potential implementation aspects of plant pathogenic fungi, they are still publicly regarded as organisms causing disease and destruction. They are considered to be number one enemies of mankind and higher animals. It is quite surprising that plant pathologists have covered only the destructive facet of these microorganisms and have completely neglected their usefulness which is sometimes much more important than the destructiveness alone. The potential of phytopathogenic fungi to inhibit or kill other microorganisms is well known, but only few have been screened for useful products. The exploration and screening of plant pathogenic fungi may lead to the discovery of some novel drugs of significance for the welfare of human beings.

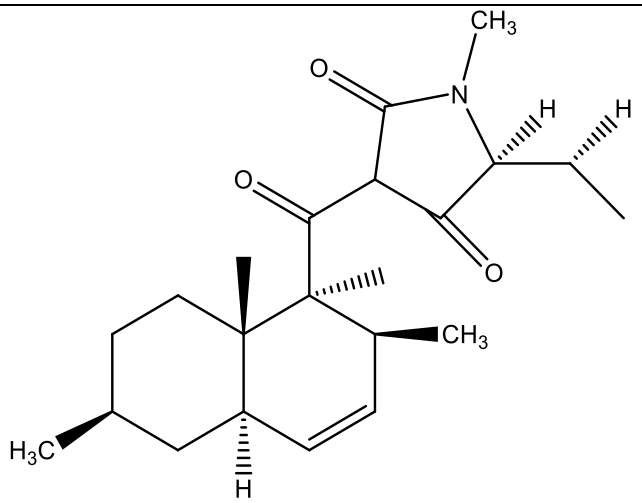
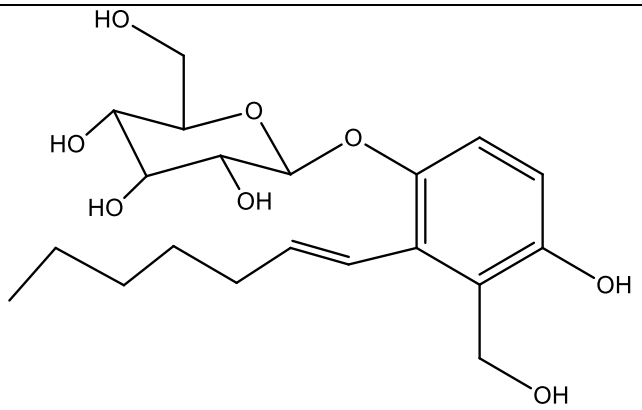
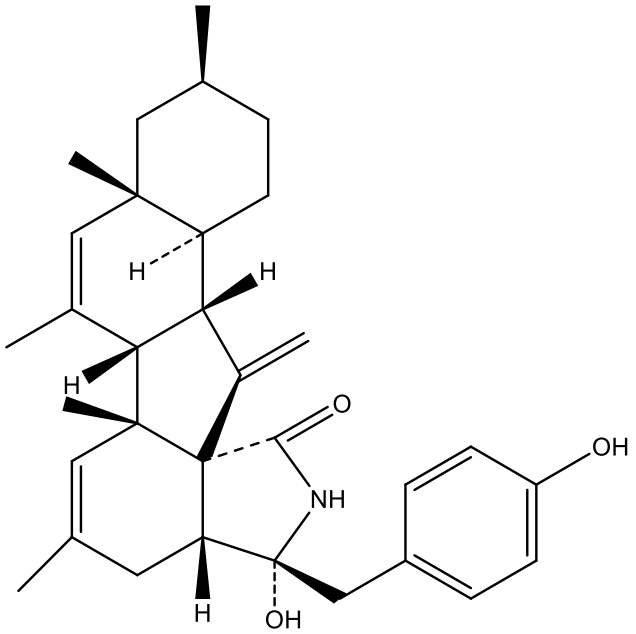
Table 1.1: List of some important secondary metabolites isolated from fungi and their function

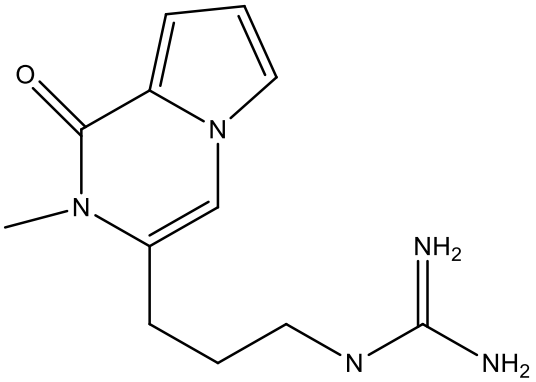
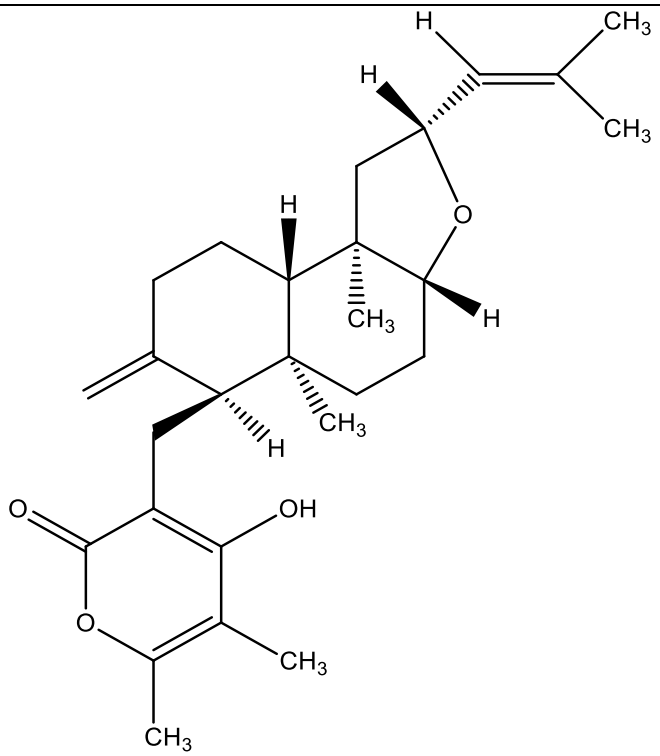
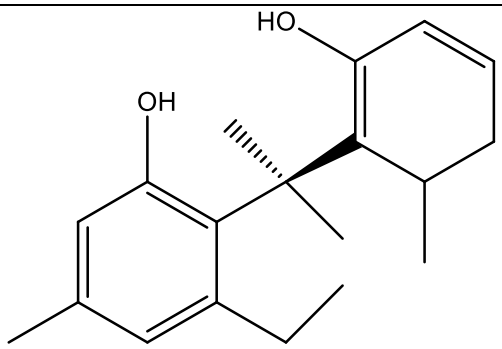
Name	**Structure**	**Biological activity**	**Ref**
Asperdurin		Antifungal	[106]
Aspergillamide A		Cytotoxic	[107]
1,3 Dihydro-4-methyl-1,5,6,7-isobenzofuranetrol		Anti-influenza	[108]
Spinulosim		Cosmetic product	[109]

Name	**Structure**	**Biological activity**	**Ref**
Migilitol		α glucosidase inhibitor	[110]
Gymnastatin A		Cytotoxic	[111]
Edrin		Antifungal	[112]
Antibiotic TAN 1612		Cytotoxic	[113]

Name	**Structure**	**Biological activity**	**Ref**
Aflavarin		Insect antifeedat	[110]
Antafumicin A		Antifungal	[114]
Halimide		Cytotoxic	[115]

Name	**Structure**	**Biological activity**	**Ref**
NPI-2358		Cytotoxic	[116]
Patulin		Mycotoxin	[117]
Ochratoxin A		Cytotoxic	[118]
Maltoryzine		Antibacterial	[119]
Jesterone (Cyclohexenone Epoxide)		Antifungal	[120]

Name	**Structure**	**Biological activity**	**Ref**
Cryptoein (Tetramic acids)		Antifungal	[121]
Pestaloside (Aromaric β-glucoside)		Antifungal	[122]
Phomopsichalasin		Antimicrobial	[123]

Name	**Structure**	**Biological activity**	**Ref**
Peramine		Insecticidal	[124]
Subglutinol A		**Immunomodulating agent**	[122]
Pestacin		Antioxidant	[125]

1.8.1. Classes of secondary metabolites on the basis of structure

Secondary metabolites are grouped into different classes on the basis of their biosynthesis. As some compounds are involved in more than one biosynthetic pathway, this classification has some limitations. These compounds are produced as a result from the primary and secondary metabolism. Primary metabolites (carbohydrates acetyl-coenzyme A (CoA) and amino acids) act as building blocks in secondary metabolites.

1.8.1.1. Polyketides

Polyketides are a group of structurally diverse compounds that include certain cholesterol-lowering drugs, antimicrobial, and antitumor potential. They are produced through the acetate pathway and are constructed by units of malonyl-CoA and acetyl-CoA. The biosynthesis mechanisms of polyketides are identical to of fatty acids biosynthesis. Polyketide synthases (PKS), which are variable in function, architecture and size in different types of organisms, are involved in the synthesis of polyketides. The general biosynthesis of polyketides starts when the acetyl group of acetyl-CoA is shifted to the unit of the PKS (β-ketoacyl synthase (KS)). In a similar transacylation, an extender unit, commonly malonyl-CoA, is shifted to an acyl carrier protein (ACP). This is followed by a Claisen-type reaction between the malonyl-ACP and the acetyl-KS during which a decarboxylation of the malonyl-ACP takes place. As a result, synthesis of β-ketoacyl-ACP occurs, which takes part in either of the two pathways depending on the type of PKS. Pathway A shifts the β-ketoacyl component to another KS, and then the generated β-ketoacyl-KS enters again into the cycle. In such a way, after a number of cycles, a poly β-keto thioester is produced. On the other hand, in pathway B, the β-ketoacyl part passes through one, two, or three optional reductive steps before entering the cycle again. Usually, aromatic polyketides such as anthraquinones biosynthesis are due to pathway A, whereas pathway B is directed towards the production of macrolides and polyethers (**Fig1.2)**. When the

backbone of the polyketide is finished completely, it is released from the PKS through lactonization or hydrolysis. After the release from the PKS, the polyketide can undergo some processes, such as rearrangements, cyclizations, and further tailoring reactions (alkylations, glycosylations, or methylations). Similarly, some PKSs utilize other starter and extender units apart from acetyl-CoA and malonyl-CoA, leading to the synthesis of structurally diverse compounds [97, 126]

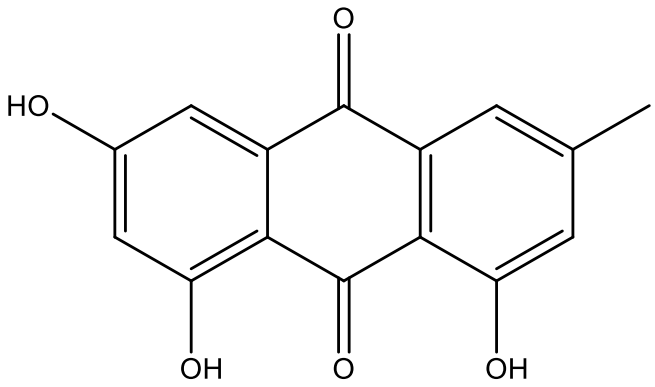


Figure 1.2: Example of polyketide

1.8.1.2. Terpenoids

Terpenoids include a class of secondary metabolites with >3500 identified compounds, including many substances with analgesic, anxiolytic, antitubercular, mutagenic [127], and anticancer activities [97]. Many of the more volatile terpenoids provide fragrance and flavours to essential oils and herbal teas. Terpenoids are sometimes stated as isoprenoids, because they are derived from isoprene units having five carbons, although isoprene (**Fig 1.3**) itself is not involved in the biosynthesis pathways. On the basis of the number of isoprene units, terpenoids are divided into different classes. These are: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), and tetraterpenes (C40). Meroterpenoids is the term used in the cases, when the isoprene units are attached to compounds from other structural classes (prenylation). A carbon skeleton that does not contain multiple of five carbons are known as modified terpenoids such as the steroids, which are the example of modified triterpenes.

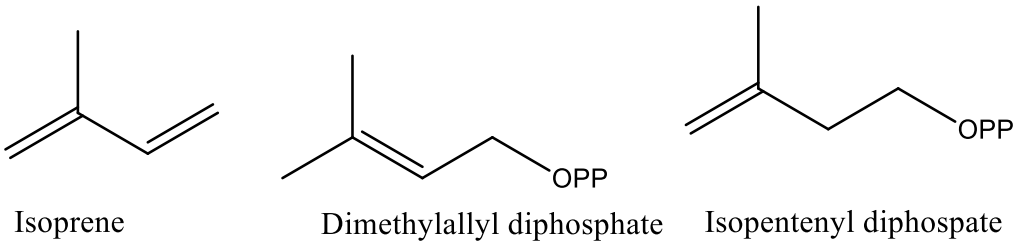
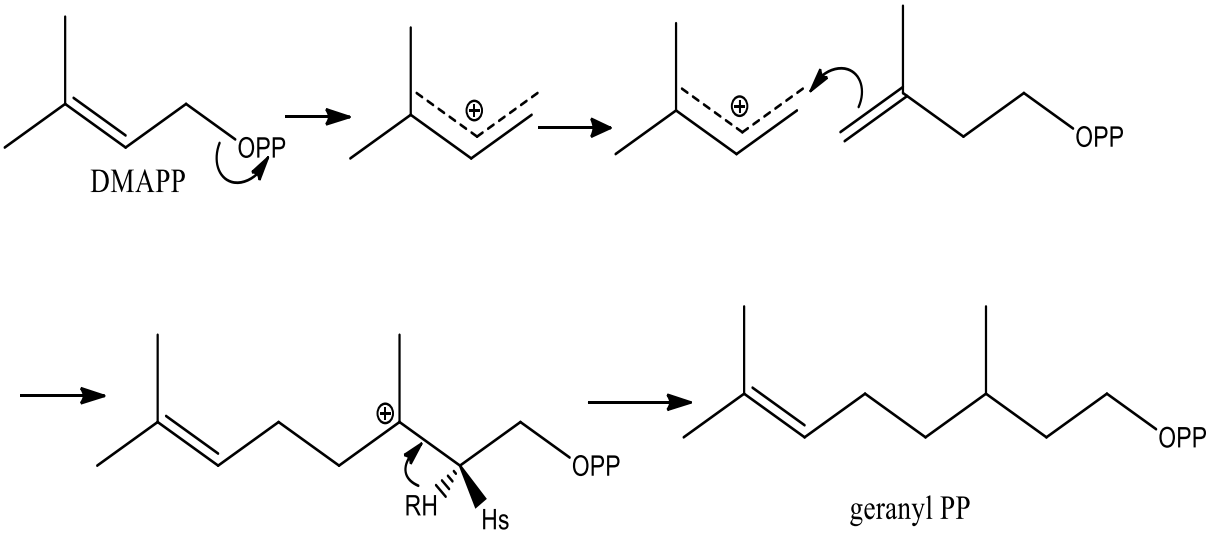


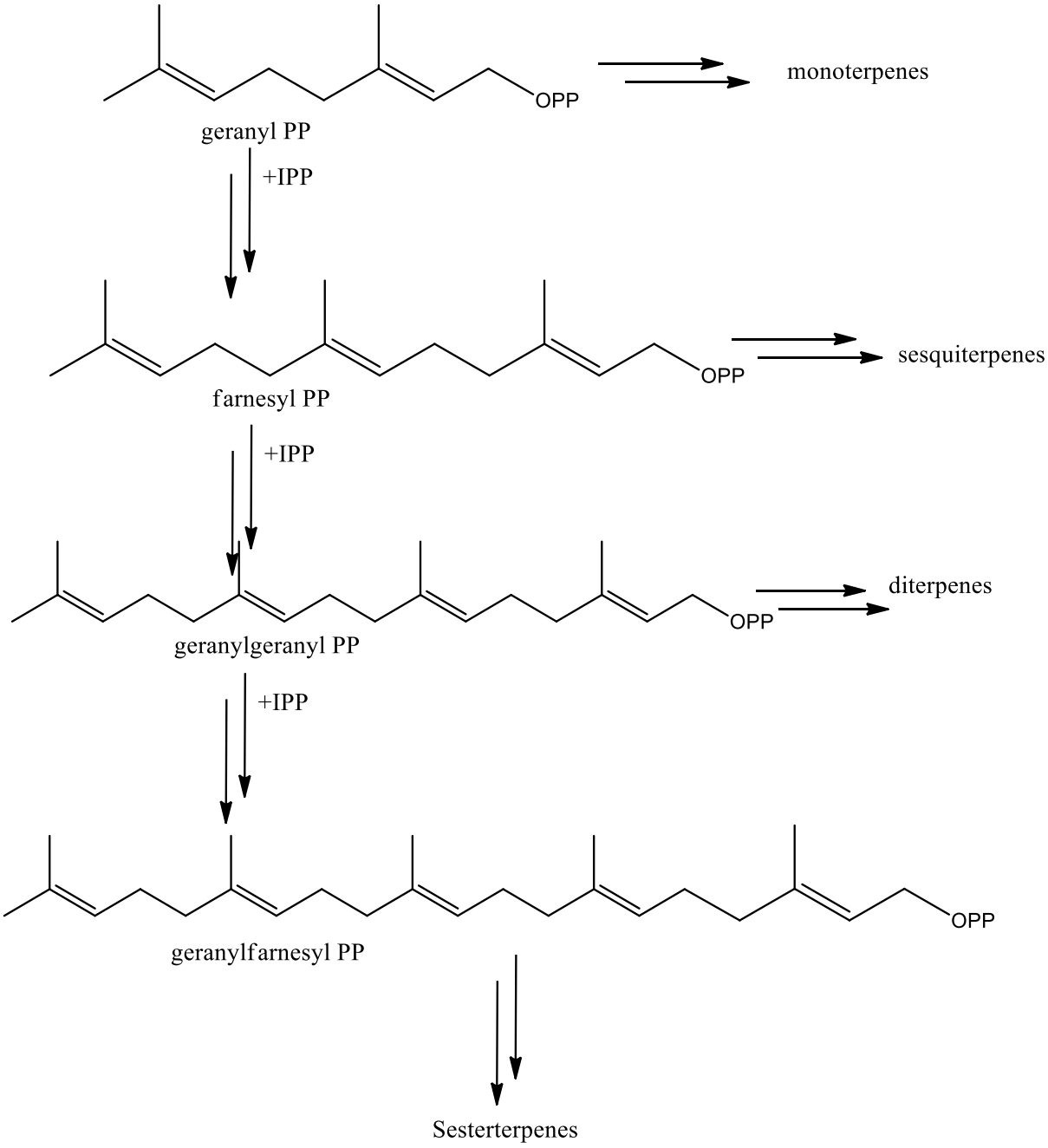
Figure 1.3: Structure of isoprene, dimethylallyl diphosphate and isopentenyl diphosphate

1.8.1.3. Diphosphate

The two fundamental building blocks to terpenoids are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP1). These can be produced by either the mevalonate and methylerythritol phosphate pathways, depending on the compounds and the type of producing organism. In the methylerythritol phosphate pathway, IPP and DMAPP are derived from pyruvic acid and glyceraldehyde 3-phosphate, whereas in the mevalonate pathway they are derived from acetyl-CoA. Hemiterpenes are synthesized from one DMAPP, whereas the precursor of monoterpenes, geranyl diphosphate, is formed from one IPP and DMAPP, both are joined in a head-to-tail manners. The DMAPP forms a resonance-stabilized dimethylallylic cation, which becomes electrophilic by the leaving of OPP. An electrophilic addition of the dimethylallyl cation to the double bond of IPP produces a tertiary cation, from which a proton is stereospecifically lost, forming geranyl PP (**Scheme 1.1**). The geranyl PP can either continue the process and generate monoterpenes or, by leaving of OPP and addition to another IPP, generate another compound known as farnesyl PP (FPP), which are the precursor of sesquiterpenes. The formations of geranylgeranyl PP (GGPP) and geranyl farnesyl PP, which are the precursors of diterpenes and sesterterpenes, respectively, are synthesized by the addition of one or two IPP to farnesyl PP (**Scheme 1.2**). The precursors of the different terpenoids pass from a sequence of cyclizations, rearrangements, oxidations and glycosylations. There are different manners of rearrangements and cyclization in one and the same precursor. As a result, structural diversity of this group is observed. Lanosterol is a precursor of many steroids in fungi and animals. Three sequential electrophilic cyclizations occur after the formation of squalene oxide from squalene. Rearrangement of formed five-membered ring occurs to form a six-membered ring. As a result of another electrophilic cyclization, a protosteryl cation is formed. Lanosterol is then produced after a sequence of hydride and methyl shifts and a final double-bond formation [97, 128].



Scheme 1.1: Schematic view of the formation of geranyl PP from DMAPP



Scheme 1.2: The chemical mechanism of the formation of the precursors of mono-, sesqui- , di- and sesterterpenes

1.8.1.4. Phenyl propanoids

Shikimate pathway is only used by microorganisms and plants. Phenylpropanoids are biosynthesized through the same pathway. In general, the shikimate pathway produces the tyrosine and phenylalanine which are aromatic amino acids. In order to obtain these amino acids, animals, in general, ingest plants or perhaps microorganisms.

Through cinnamic acid and/or 4-coumaric acid, an array of biosynthetic routes can be followed to synthesize biomolecules such as phenylpropenes, lignans, lignin, flavonoids, stilbenes and coumarins (**Fig 1.4**). In plants, phenylpropanoids primarily contribute to the production of smell, color, and flavor that are involved in the interaction of plants with animals and pathogens. These flavors are also used by humans as in the case of some spices such as cloves and cinnamon that contain volatile phenylpropenes. The pharmacological properties of phenylpropanoids include hypertensive, anti-viral, anti-tumorigenic, and anxiolytic activities [129, 130].

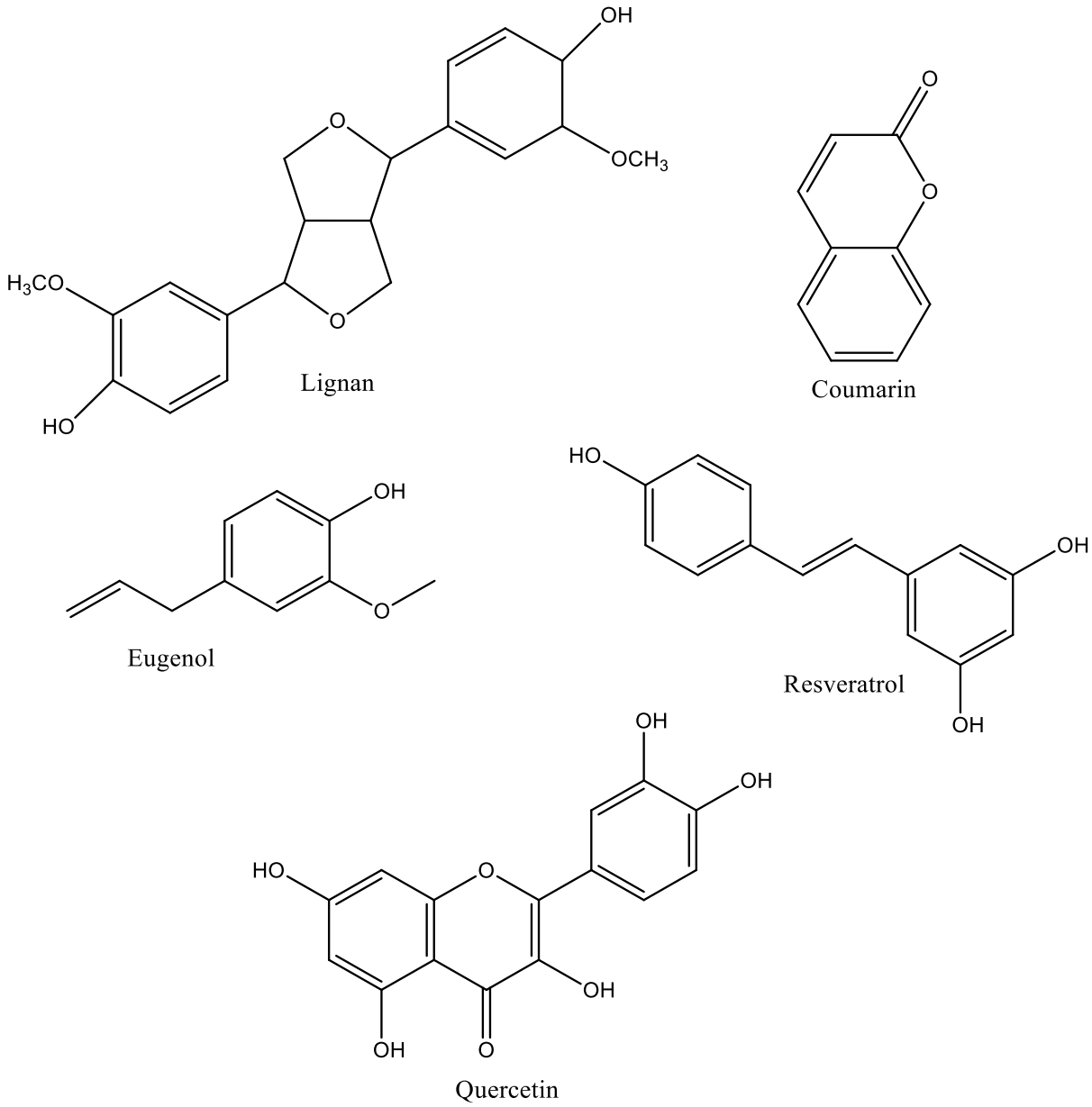


Figure 1.4: Examples of phenylpropanoids.

The name of the shikimate pathway is derived from the compound, shikimic acid, which is produced involving a number of steps, from phosphoenolpyruvate (PEP) and erythrose-4-phosphate. Therefore, shikimic acid pathway, in general, is linked to primary metabolism, because these two compounds serve as intermediates in the pentose phosphate pathway (PPP)

and glycolysis, respectively. Shikimic acid, after its synthesis, is 3-phosphorylated, and the 5-hydroxy group of the resulting shikimic acid 3-phosphate makes a nucleophilic attack on the phosphorylated carbon of a protonated PEP. The phosphate (P) group originating from the recently attached PEP is *β*-eliminated, and the 3-*P* group is removed by a 1, 4-elimination forming chorismic acid.

Due to Claisen rearrangement, chorismic acid turns into prephenic acid. Subsequently, prephenic acid transforms into phenylalanine; this process can be carried out using more than one possible ways, however only one is being chosen to be discussed here. Following a transamination reaction, involving pyridoxal 5'-phosphate (PLP) and an amino acid that provides the amino group (possibly glutamic acid), prephenic acid is turned into L-aerogenic acid. L-phenylalanine can then be formed by a decarboxylation, while tyrosine is formed by a decarboxylative reaction, which retains the hydroxy group (-OH), for which there is no proposed mechanism [131, 97]. Using the E2 mechanisms, L-tyrosine and L-phenylalanine are converted into *p*-coumaric acid and cinnamic acid, respectively. As mentioned above, these are the precursors to synthesize numerous phenylpropanoids, which can often be noted for the characteristic C_6C_3 scaffold present in most phenylpropanoids.

1.8.1.5. Amino acids, peptides

Biomolecules (enzymes, proteins and peptides) are synthesized in the ribosome and in general, are not listed as secondary metabolites. However, ribosomal peptides are perceived as secondary metabolites although their relative molecular masses in some cases exceed the size of 3000 Daltons. These biomolecules include snake venoms, endorphins, and mushroom toxins. However, this piece of work would only focus on non-ribosomal peptides and small compounds derived from amino acids such as penicillin and cephalosporin. Non-ribosomal peptides are biosynthesized using non-ribosomal peptide synthetase (NRPS), which act like

polyketides (PKs). The assembly of these peptides using amino acids is brought about in a similar manner to the one involved in the assembly of polyketides using polyketide synthases (PKSs). In general, the first of the modules loads the first amino acid to a peptidyl carrier protein (PCP) domain, whereas the last module releases the peptide either by hydrolysis or cyclization. The range of the NRPSs is much greater than this, however, as the description discussed above only leads to formation of cyclic and linear peptides. Some non-ribosomal peptide synthetase also add unusual amino acids or hydroxylated acids, resulting in ester linkages instead of some of the amide bonds, forming the so called depsipeptides [132, 97]. Penicillins and cyclosporins are β-lactam antibiotics which contains β-lactam ring in their molecular structure. They are produced by the assembly of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine by different NRPSs, based on their production from different species. Formation of isopenicillin N occur after a number of transformation reactions. After the formation of isopenicillin N, the biosynthetic pathways diverge, as the penicillin scaffold is ready, while for the production of the cephalosporin scaffold, the thiazolidine ring of penicillin N has to be transformed to a dihydrothiazine ring. Due to the replacement of α-aminoadipic acid with a phenylacetic acid, benzylpenicillin is produced. Addition of carboxylic acid to the culture medium, some of the therapeutic penicillins are generated by replacing this acyl side-chain. This process can change the range of activity as well as stability towards acid of the specific drug.

1.8.1.6. Alkaloids

The alkaloids are cyclic organic compounds having nitrogen. These compounds have limited distributions in nature [133]. Meissner (1814) proposed the collective term alkaloids for this set of molecules due to their basic properties. They were successfully extracted by an acidic aqueous extraction due to their increased solubility in water upon protonation of the nitrogen. These alkaloids were classified on the basis of the basicity criterion that exists today;

however, the compounds containing only amide nitrogen can still be classified as alkaloids. The alkaloids often exert biological effects on the CNS and might act as analgesic, anxiolytic, or hallucinogenic [98]. Apparently, it is probably the group of compounds, which also includes certain controversial compounds such as nicotine, morphine, cocaine, and caffeine (**Fig 1.5**). However, recent research on these alkaloids shed light on some of the vital physiological processes of the CNS. Furthermore, almost 50% of the plant-derived pharmaceuticals fall under the category of alkaloids [134]. Endogenous alkaloids act as a neurotransmitters in animals such as melatonin play a role in sleep regulation. In plants, alkaloids serve as protective role against animals, because many of them are toxic and affect the neurotransmission pathway [135].

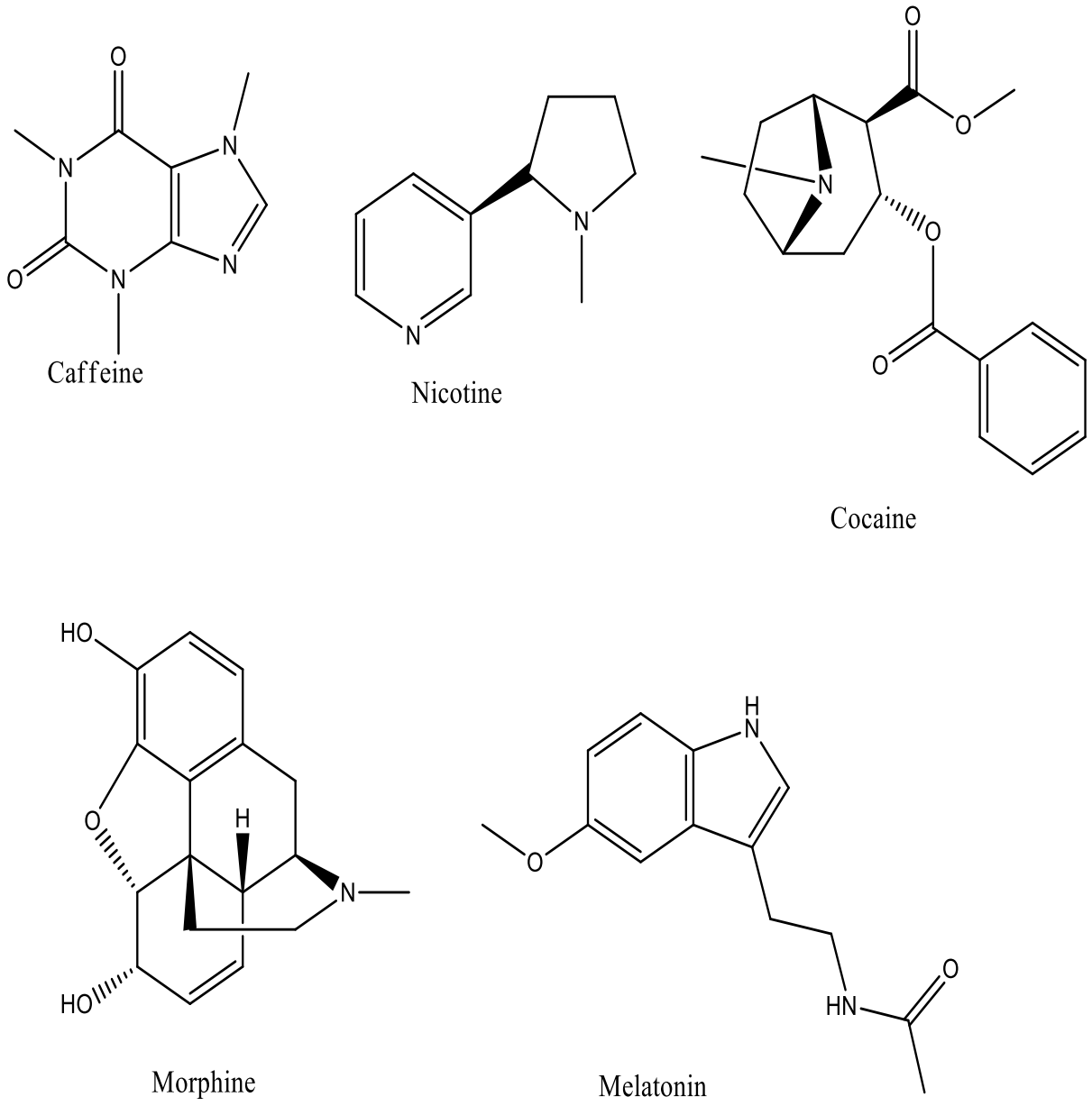
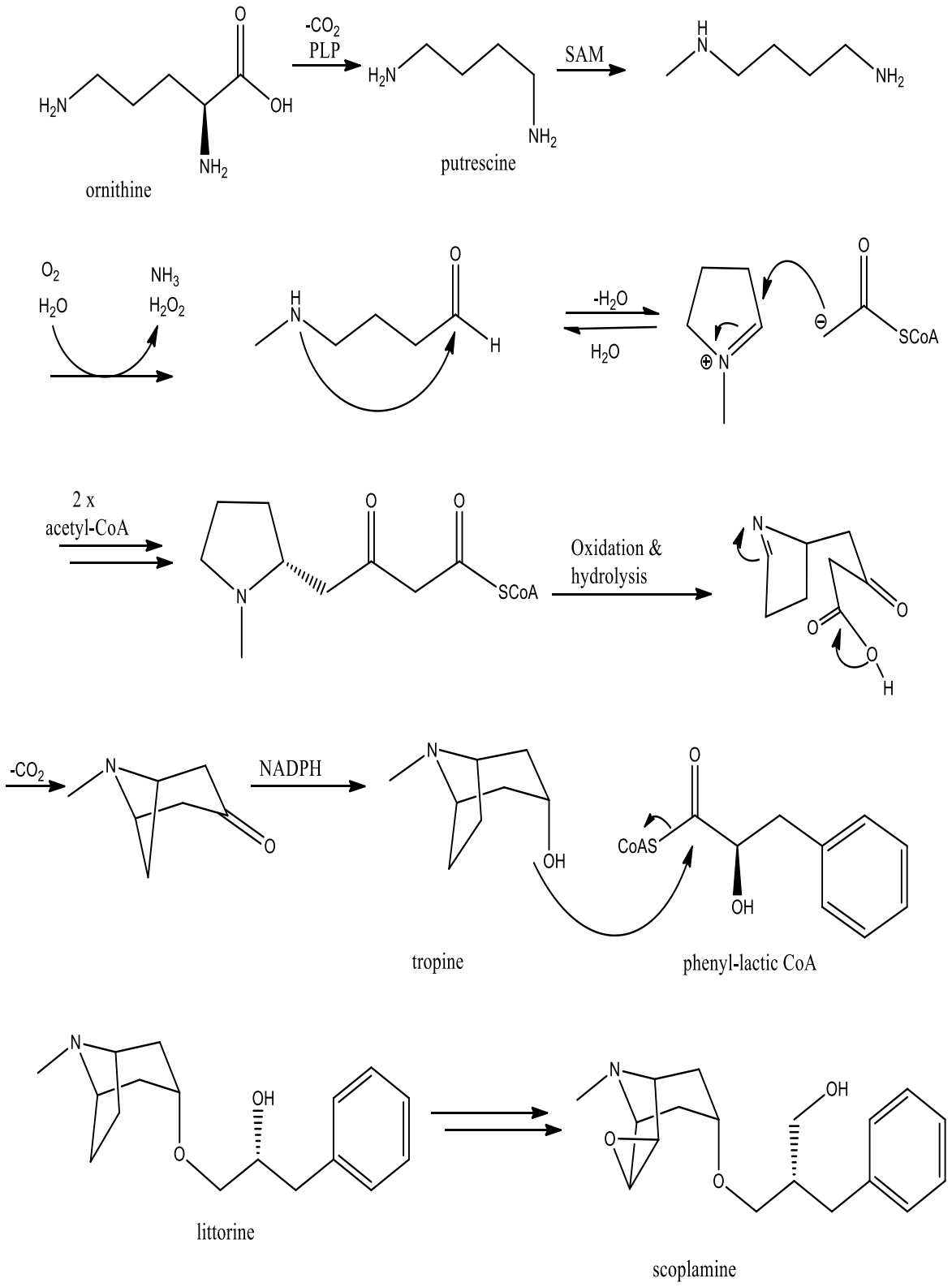


Figure 1.5: Structure of some widely used alkaloids**.**

The biosynthesis of alkaloids involves the use of building blocks from other structural classes, such as phenylpropanoids and terpenes that combine with a nitrogen containing cyclic moiety derived from an amino acid (tryptophan, lysine, tyrosine or ornithine). An example of an alkaloid derived from phenylalanine and ornithine is the scopolamine, hallucinogen. The

pathway involved in the formation of scopolamine is presented in **Scheme 1.3**. The formation of putrescine is brought about by the decarboxylation of ornithine, wherein one of the amino groups is methylated by S-adenosyl methionine. The remaining amino groups are transformed to aldehyde by the action of diamine oxidase. The attack of the methylated amino group on the aldehyde and a subsequent loss of water lead to the formation of an iminium cation. To the electrophilic carbon so formed, two consecutive additions of acetyl-CoA are achieved via Claisen and Mannich reactions. Electrophilic iminium cation form, when oxidation of the pyrrolidin in the compound occur and the thioester function is hydrolyzed to an acid function. Through a possible decarboxylative generation of an enolate, a tricyclic compound is formed, which after a stereospecific reduction of the remaining ketone forms tropine. Littorine is formed by esterification of tropine by phenyllactic CoA (derived from phenylalanine). Formation of scopolamine is occur as a result of unusual rearrangement of the phenyllactate moiety and a two-step epoxidation of the tropinyl moiety.



Scheme 1.3: Biosynthesis of scopolamine, SAM stands for Sadenosyl methionine.

1.9. *Screlotium Rolfsii*

The plant pathogen, Basidiomycetes *Sclerotium rolfsii*, causes southern blight in a wide range of plants [136, 137]. *S. rolfsii* is found on peanut in all parts of the world [138]. This plant pathogen has a broad host range of more than 500 plant species [139]. The formation of sclerotia is one of the key characteristics of *S. rolfsii* which are tan to black in color and are uniform in size measuring 1.5 mm [140]. Sclerotia are composed of a hard external husk, middle cortex, and an innermost medulla composed of loosely arranged hyphae [141, 142]. This pathogen grows at temperatures in the range of 27-30°C. The germination of sclerotia occurs myceliogenically in warm and moist conditions, and spread rapidly on both, the soil and the stem of plants [140]. The early stages of the disease are characterized by yellowing and wilting of plant tissue, followed by necrosis of leaves and stems [143]. Oxalic acid, a necrotizing agent, produced by *S. rolfsii* is primarily involved in the degradation or breakdown of the cell walls of plants [144, 145]. After tissue degradation and depletion of nutrients, sclerotia are formed, and dispersed on the soil. *S. rolfsii* exists in tropical and subtropical regions of the world and causes serious economic losses in the form of large yield losses in crops of high economic importance. The pathogen attacks a number of cultivated and non-cultivated plants but rarely the cereals. *S. rolfsii* infection starts either directly from soil-borne sclerotia which propagate to form fine cottony hyphae infecting the host plants or sclerotia penetrating through the lower/upper surfaces of the leaves through rain splashes where they germinate and cause leaf spots [146]. A soil moisture content of 90% and soil temperature of 25–30°C play significant role in the development of the disease [147]. In addition, development of sclerotia also affected by biotic and abiotic factors directly or indirectly [148, 149].

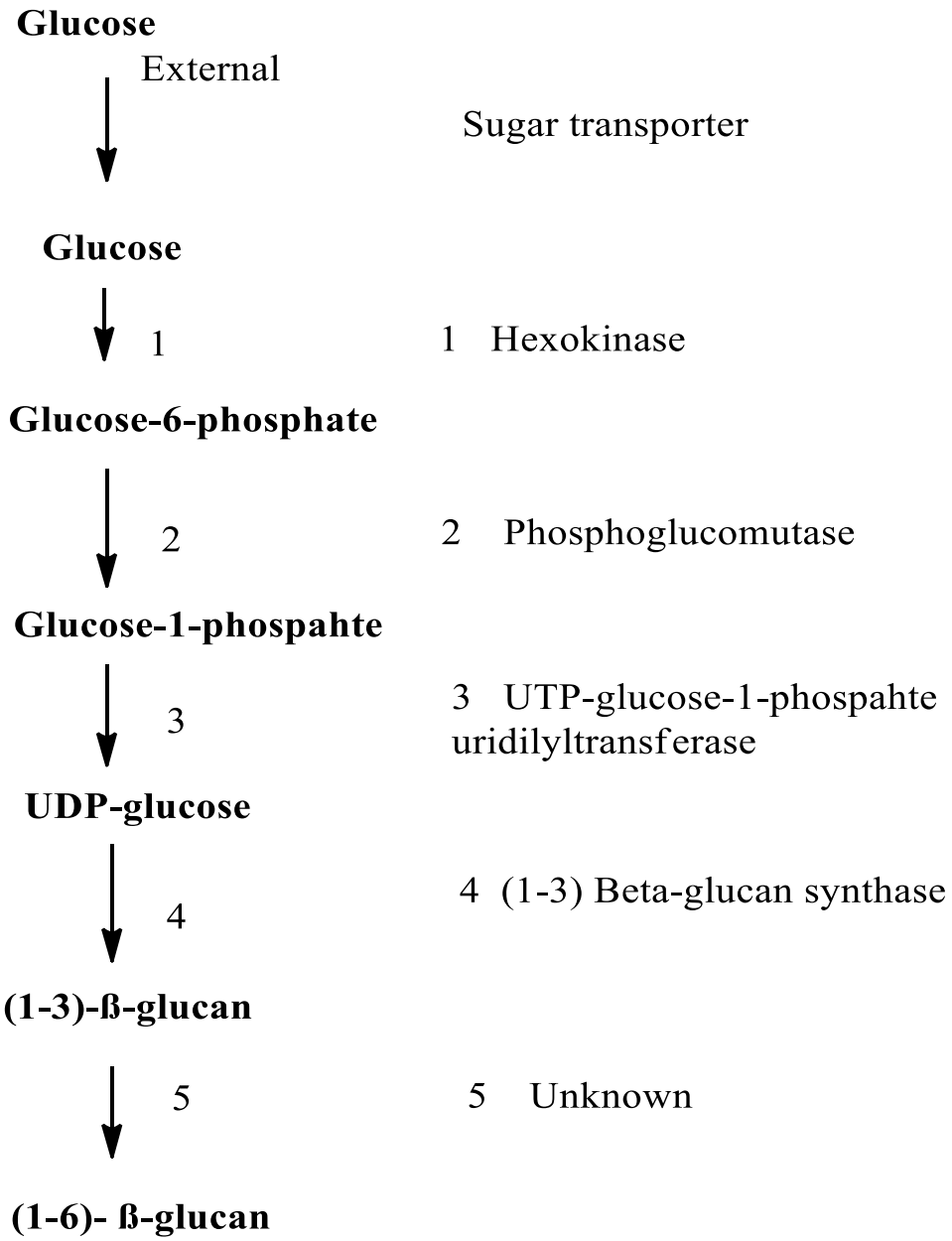
1.9.1. Mycelial Incompatibility

S. rolfsii, a soil borne phytopathogenic fungus, causes disease in a wide range of plant species. Mycelial incompatibility was observed in different isolates of *S. rolfsii*. *Sclerotium* having same mycelial compatibility group (MCG) share greater genetic similarity as compared to isolates from different MCG. Twenty-five groups among the 72 isolates have been investigated so far. The spasmolytic killing of hyphae were also observed during the growth of incompatible isolates on the same plates. It was noted that when hyphae of different mycelial compatibility group isolates confront one another, a distinct zone of separation was observed. The molecular data show that MCG isolates exhibit identical banding pattern. Different MCG groups present different colony morphology, size of sclerotia, color, and mycelial growth rate [150].

1.9.2. Biopolymer production

Scleroglucan is an exopolysaccharide of biotechnological importance. *S. rolfsii* is known for the production of scleroglucan [151]. Scleroglucan shows interesting viscous properties. Scleroglucan is known as a multifunctional metabolite having many industrial applications, which include cosmetics, food industry, oil recovery, and medical applications. However, only little information is available on the biosynthesis of scleroglucan by *S. rolfsii*, and most steps of the pathway are hypothetical [152] (Schematic overview presented in **Scheme 1.4**). The different species of *Sclerotium*, such as *S. glucanicum*, *S. delphini*, and *S. rolfsii*, produce varying amounts of biopolymer under different conditions of cultivation. The differences in properties of the biopolymer depend on several factors, viz., the composition of growth media, sources of nitrogen and carbon, temperature, pH, oxygen supply, etc. An experiment conducted on the biopolymer production by *S. rolfsii* noted that the amount of scleroglucan depend on the concentrations of carbon and nitrogen. The production of EPS was

higher in media containing $NaNO_3$ and $(NH_4)_2SO_4$ as the nitrogen source. It was also observed that high concentrations of EPS were obtained with yeast extract [153]. Another study observed that the biomass and EPS were higher when nitrate was used as a source of nitrogen as compared to NH_4 [154]. The pH of the culture also influences the production of polysaccharides by fungi. The pathway for the biosynthesis of scleroglucan is closely related to the production of oxalate, a strong acid and reducing agent. Therefore, the oxalate prevents the use of scleroglucan in many industries such as cosmetics and food industry. Selection of C-source and their concentration distinctly affect the production of scleroglucan [153]. The nitrogen source is the second important factor for scleroglucan production; $NaNO_3$ shows significant effect on the production of scleroglucan whereas $(NH_4)_2SO_4$ decreases the scleroglucan production up to 70% [155]. Other factors such as initial pH, P-source and addition of various substances also influence production of scleroglucan. It was also observed that the yield of scleroglucan increases due to higher phosphate concentrations, while the addition of L-threonine or ascorbic acid was noted to decrease the production. High concentrations of sucrose or glucose supplemented with $NaNO_3$ as N-source are the best conditions for production of scleroglucan [153].



Scheme 1.4: Hypothetical screloglucan synthesis by *S. rolfsii*

Scleroglucan is a homopolymer polysaccharide which is soluble in water. The molecular weight is about 540,000 kDa. The structure of scleroglucan is determined by using nuclear magnetic resonance (NMR) **(Fig 1.6)** [156].

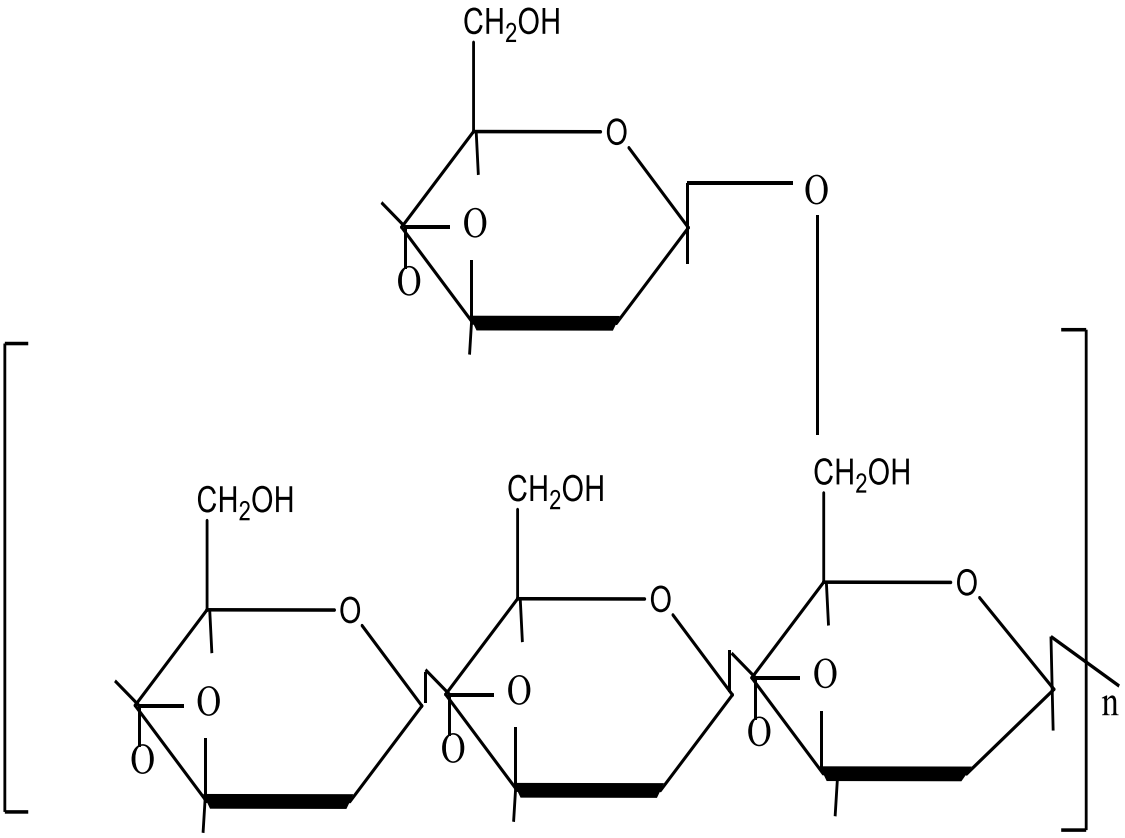


Figure 1.6: Structure of scleroglucan presented by NMR

1.9.2.1. Industrial applications of scleroglucan

Scleroglucan has a wide range of industrial applications, such as in oil recovery (for drilling mud, thickening and for better oil recovery) [157], printing inks, preparation of adhesives, water colors, and as components of liquid animal feeds [158]. Scleroglucan is also used in cosmetics as it can be used in various protective lotions and skin care creams [159]. Scleroglucan may be used as a laxative in pharmaceuticals industry or for stabilizing suspensions or as tablet coatings. It is well-known that scleroglucan also possess immune stimulatory effects as compared with other biopolymers. The use of scleroglucan as an antimicrobial, anti-tumorigenic, and antiviral agent has also been reported [160, 161].

1.9.3. Biocontrol agents against weeds

A weed is an undesired plant imposing adverse effect on economically important crop and forest plants. A variety of different physical and chemical methods have been developed to overcome the adverse effect of weeds, but no single method has been proven to produce satisfactory results just because each method has its own limitations. A method that exploits the pathogenicity of fungi could be developed as eco-friendly and effective alternative to the conventional methods for weed control [162]. Among the pathogenic fungi, *Sclerotium* is a unique mycoherbicidal fungus. Beside its pathogenicity, it can also be used in weed control. Few studies have shown that *S. rolfsii* is effective in the controlling of the cosmopolitan weed, *Parthenium* (*Parthenium hysterophorus L*) [163, 164].

1.9.4. Important Enzyme Secreted by *S. rofsii*

1.9.4.1. *β*- Mannanase

S. rolfsii secretes a wide range of cellulolytic and hemicellulolytic enzymes. *β*-1,4-Mannans are substituted heteropolysaccharides that are commonly found in plant tubers, seeds, and wood. *β*-1,4-Mannanase is the enzyme that hydrolyses *β*-1, 4-mannans. This enzyme is

produced by algae, bacteria, and fungi. The production of this industrially important enzyme, *β*-1,4-mannanase by *S. rolfsii* in a glucose based medium and under stressed condition have been investigated. *S. rolfsii* produces a multi-enzyme that could bring about the degradation of *α*-mannans and *β*-mannans. Mannan is a polysaccharide mainly composed of mannopyranose units and is a common component in the cell walls of plants and microorganisms. These multi enzymes systems include *β*-mannanase, glucosidases, mannosidases, galactosidases, and acetyl mannan esterases. Galactosidases and Mannosidases have lots of applications in the pharmaceutical industry in the production of biologically interesting oligosaccharides. *β*-l-Mannanases are also used in the coffee extraction and fruit-juices industry [165, 166].

1.9.4.2. Laccases

It is reported that *S. rolfsii* produces two laccases; SRL1 and SRL2 having different molecular weights (55 and 86 kDa), respectively [168]. The laccase production was found to be triggered by the addition of 2, 5-xylidine to the nutrient media. Upon treatment of sclerotia with a combination of chitinase and 1,3-glucanas, two different laccases (SRL1 and SRL2) are produced. The most important laccase, SRL1 has been isolated and evaluated in the decolorization of industrially important wool azo dye, Diamond Black PV200 without the addition of redox mediators. For maximum activity, 62 °C was determined as the optimum temperature. Studies on the stability of the enzyme showed that SRL1 was remarkably stable at 18°C and pH 4.5 without losing the activity even after seven days. SRL1 was strongly suppressed by sodium azide and fluoride. The dye solutions decolorized with immobilized laccase could be conveniently used for re-dyeing.

1.9.4.2. Cellobiose dehydrogenase

S. rolfsii grows and survives on dead plant material present in the soil by the formation of sclerotia, which later germinate and attack the cell wall of young plants causing necrosis.

Cellulose, Pectin and hemicellulose could be degraded completely by using various enzyme complexes. *S. rolfsii* also secretes oxalic acid, which acts synergistically with enzymes causing injury to plant tissues [168]. Cellobiose dehydrogenase (CDH) is synthesized and released extracellularly, when wood- and cellulose-degrading fungi including *S. rolfsi* grow on cellulose. It oxidizes the reducing end of cello-oligosaccharides and cellobiose to their corresponding 1, 5-lactones, which are then hydrolyzed to -COOH in an aqueous environment. To date, cellobiose dehydrogenase has been isolated and characterized from soft-rot fungi, white-rot fungi, and brown-rot fungi [169-172].

1.9.4.4. β-D-xylosidase

Xylans, the components of plant hemi-cellulose, are potential feedstocks for producing fuel and food. D-xylose is the primary sugar obtained through the acid or enzymatic hydrolysis of xylans. It is fermented to ethanol by specific yeast strains. Organic chemicals, acetic acid, and xylitol are also derived from xylans. Xylan degrading enzymes have been reported from fungi, bacteria, and yeast [173]. *S. rolfsii* secretes a wide spectrum of xylan and mannan-degrading enzymes, such as production of extracellular *β*-D-xylosidase in shake flasks by *S. rolfsii* have been described [174].

1.10. *Aspergillus* Species

Aspergillus is one of the oldest and common genera of fungi. *Aspergillus* received its name due to microscopic spore-bearing structure, which resembles the Aspergillum, the instrument used to sprinkle holy water in the Roman Catholic churches [175, 176]. This asexual spore producing structure is the defining microscopic marker distinguishing different species of the genus [177]. During vegetative growth, the mycelium can differentiate and enlarge forming a "T" or "L" shape cell called the foot cell. The conidiophore, a stalk-like structure, develops from the foot cell and culminates as the spherical vesicle. Primary and secondary sterigmata extend from the vesicle, with the latter producing the asexual conidiospore [178]. In addition to morphological similarities, phylogenetic analysis offers further support to the fact that all *Aspergillus* species form a monophyletic group [179]. Despite these similarities, the genus as a whole is extremely diverse and show evolutionary distances comparable to that of humans and fishes [180]. Until now, more than 250 *Aspergillus* species have been identified [181]; and roughly one third of them can also produce meiotic ascospores via sexual reproduction [182]. *Aspergillus* spores spread commonly as bioaerosol wherein they are transported by air currents and spread over depending on the prevailing environmental conditions. When these spores come in contact with a liquid or solid suource, they get deposited and if conditions are favorable (moisture), germination occur [183]. *Aspergillus* possesses characteristic features that are both beneficial and detrimental to the human society. Being on the positive side, several species of *Aspergillus* are used in making traditional Asian foods and beverages; others are utilized as "cell factories" in the production of industrially important compounds, e.g., citric acid. Furthermore, the cholesterol-lowering drug lovastatin is produced by *A. terreus* [184]. On the contrary, some species of *Aspergillus* produce toxic chemicals that can contaminate crop stocks [185], and other species can cause infections in humans and other animals [186-189]. Owing to their importance, the representative genomes of several species

have been sequenced **(Fig 1.7**), providing a rich resource of understanding the pathogenicity, specialization, and evolutionary history of the *Aspergillus*

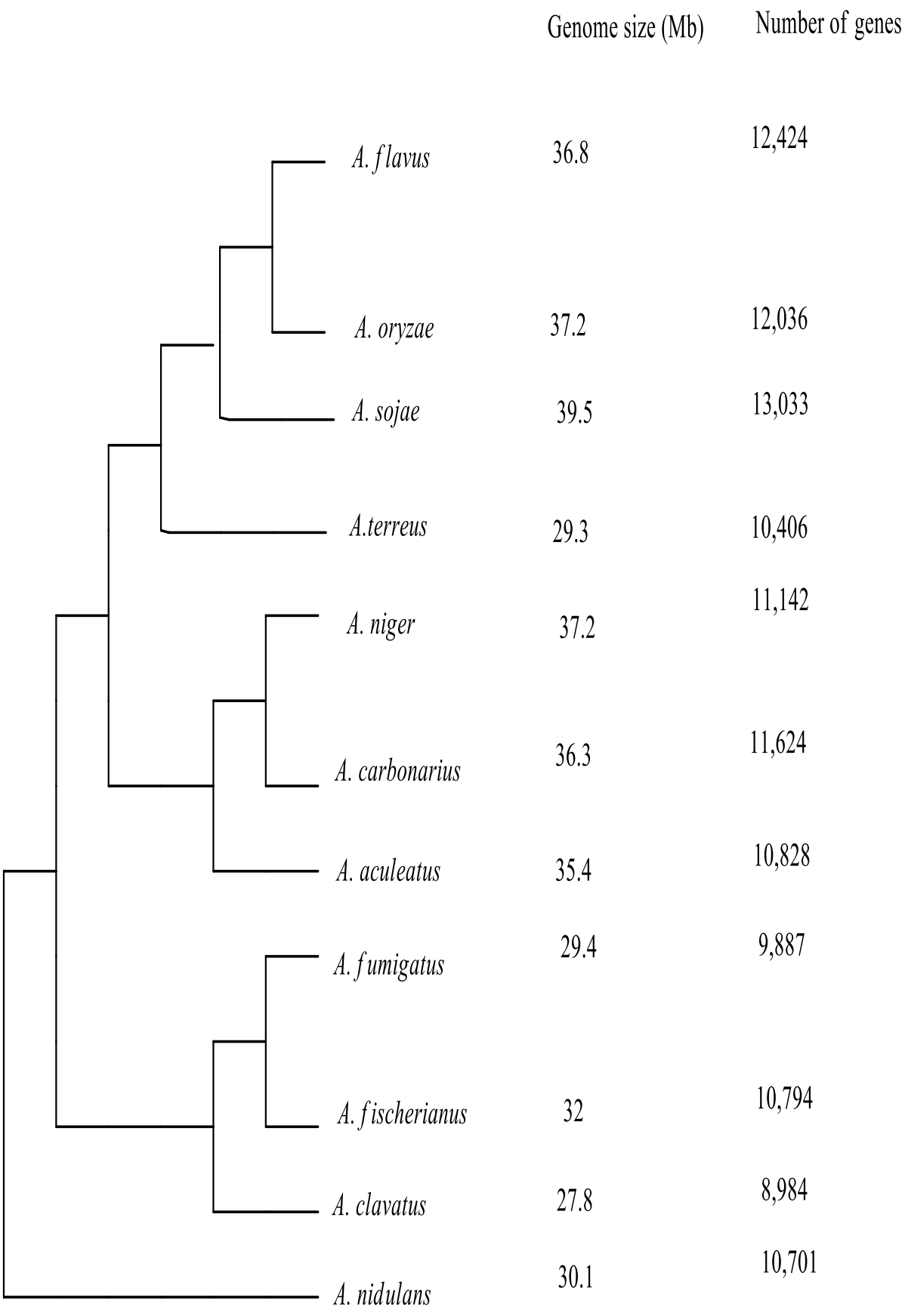


Figure 1.7: Representative genome of several *Aspergillus* species

1.10.1. Aspergillus Ecology

Aspergillus species are commonly saprophytic and frequently found in soil and decaying plant material. They secrete digestive enzymes to externally break down organic matter into more simple nutrients which they then utilize. These fungi play a very important role in the ecosystem, where they recycle nitrogen and carbon. *Aspergillus* spores are microscopic typically ranging in size between 2 and 3 μm and are thus easily dispersed through wind. As such, most *Aspergillus* species are ubiquitously distributed, and their conidia have been isolated at extreme environments such as high altitudes, Tibetan glaciers, Antarctica, and the Saharan Desert [190-192]. Approximately 20 *Aspergillus* species are also capable of causing opportunistic infections in humans [193, 194]. In particular, *A. fumigatus* causes the majority of human diseases, while *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* are involved to a lesser extent [195]. Although the vast majority of *Aspergillus* infections are caused by *A. fumigatus*, surveys of hospital air have not identified an over abundance of *A. fumigatus* conidia [196, 197].

1.10.2. *Aspergillus Flavus*

Aspergillus flavus has garnered worldwide importance due its industrial use and production of toxin. The Section Flavi is categorized in two classes of species; one includes the aflatoxigenic species, e.g., *A. parasiticus*, *A. flavus*, and *A. nomius*, that are responsible for serious complications reported worldwide in agricultural products and the other includes the non aflatoxigenic species, e.g., *A. sojae*, *A. tamarii*, and *A. oryzae.* These non-aflatoxigenic species are traditionally used in the production of fermented foods in Asia [198]. *A. flavus* is the most important fungal species that can be found in soil and other substrates. Among the genus *Aspergillus*, *A. flavus* is the most important economically as well as famous due to its potential to produce aflatoxins. *A. flavus*, one of the most abundant soil-borne mold is a saprobe

that is capable of surviving on many organic nutrient sources, plant debris, compost piles, cotton, animal fodder, dead insects, animal carcasses, outdoor and indoor environments, stored grains, and even immune compromised human and animals [199]. It has the ability to survive on a wide range of temperatures ranging from 12-48°C, but the optimum temperature ranges from 28-37 °C [200]. The life cycle of this fungus is divided into two stages: (1) colonization on plant waste in soil and (2) the attack on seeds and grain in growing crop plants causing different diseases [201]. The soil serves as a good reservoir for the primary inoculum of *A. parasiticus* and *A. flavus* [202, 203]. Under unfavorable conditions like dryness and poor nutrition, the mycelium form resistant structures known as sclerotia. Sclerotia are compact and pigmented hyphae which have the capability to survive under unfavorable condition for a long period of time [204]. The sclerotia germinate and produce new colonies when conditions become favorable**.** *A. flavus* produces the Lovastatin (cholesterol lowering drug) [205]. *A. flavus*, is an opportunistic plant pathogen, causing diseases many economical important crops; cotton, maize, groundnuts, and also tree nuts such as pecans, walnuts, pistachio nuts, and brazil nuts. Since *A. flavus* possesses no host specificity [206], it is capable of attacking seeds of both, dicots and monocots as well as seeds produced above ground (corn) and those below the ground (peanuts). Under favorable conditions, *A. flavus* can cause ear rot disease on maize causing significant economic losses [207]. The toxin of *A. flavus* was first described as the cause of a severe animal poisoning incident in England in 1960 called the Turkey X disease [208]. *A. flavus* has been implicated in many severe human diseases such as invasive aspergillosis. It can also cause diseases in crops as well as insects [209]. The production of mycotoxins is species specific; so proper identification and characterization of the specific fungi is of key importance to adapt preventive strategies [210]. A range of different mycotoxins contaminate the poultry feed, and the most important of which are aflatoxins, such as ochratoxin A (OTA), B1, B2, G1, and G2 [211]

1.10.2.1. Aflatoxin

Aflatoxins are the one of the most studied and important group of mycotoxins which produce clinical toxicosis. They reduce the resistance of the organisms against diseases and interfere with the vaccine induced immunity, especially in poultry birds [212]. Aflatoxin B1 is the most studied and toxic as it possesses potential hepatocarcinogenic properties [213, 214]. Every year there is an extensive quantity of livestock and crops are lost due to contamination by toxigenic fungi. Feeding poultry and livestock with aflatoxin contaminated feed can lead to immune suppression and death as well as reduction of growth. It was noted that lower yields of crops and animals might also result due to aflatoxin contamination [215]. Aflatoxins are synthesized by few members of the *Aspergillus* species; among which *A. parasiticus* and *A. flavus* are the most serious and problematic species. The diseases caused by aflatoxin depend on different factors, such as species, nutrition, age and sex. In mammal, the target organ affected by aflatoxins is the liver, so aflatoxicosis is classified as a hepatic disease [216, 217]. *A. parasiticus* and *A. flavus* are commonly known as weedy moulds and under high moisture, it can grow on a wide range of substrates. Aflatoxins have been characterized from all major cereal crops as well as from sources such as marijuana and peanut. The transformation products of Aflatoxin are sometimes found in milk products, meat and eggs, when these animals are nourished with contaminated grains [218]. The prevention of exposure of human beings to aflatoxins is challenging because *A. flavus* grows aggressively on many food items at all levels of the food chain, such as in storage, in the field, etc. [219]. Acute human aflatoxicosis has been investigated in many underdeveloped countries such as Thailand and India. The symptoms of severe aflatoxicosis include hemorrhagic necrosis of the liver, lethargy oedema [220]. It was also noted that among the aflatoxins, aflatoxin B is an effective carcinogen. The International Agency for Research on Cancer (IARC), World Health Organization (WHO) has classified aflatoxin B1 to be a human carcinogen in 1988 [221]. However, not all strains of A.

flavus produce aflatoxins; several strains are non-toxigenic [222]. Aflatoxins are secondary metabolites having carcinogenic, hepatotoxic, and teratogenic properties that can affect humans and/or animals [223]. The production of mycotoxin depends on several factors including substrate, fungal species, and temperature of the media, pH, humidity, and incubation time [224, 225]. It was observed that different strains of *A. flavus* produce aflatoxins at different rates when grown under identical conditions [226, 227]. The conditions which favor the formation of the aflatoxin include toxicity, their metabolism, DNA adduct formation, mutagenic, and carcinogenic activity [228]. The immunosuppressive ability of aflatoxin B1, mainly on cell-mediated immunity, has been described in various animal models [229]. It was reported that aflatoxin M1 is the major metabolic product of aflatoxin B. Aflatoxin M1 is usually excreted in the urine and milk of dairy cattle and other mammalian species that have nourished aflatoxin contaminated nutrients [230].

1.10.2.2. *A. flavus* as a Bioremediator

As a result of human activities, huge amounts of organic and inorganic complex compounds are release into the environment every year. The side effect of industrial activities is soil contamination. Among the modern technologies available to deal with contaminated soils, bioremediation based on the metabolic activity of microorganisms offer certain advantages [231]. As far as environmental pollution is concerned, petroleum and petrochemical products (complex mixtures of hydrocarbons) have been considered as the most serious problem. Hence, bioremediation is an alternative way of remediation of oil contaminated sites, by the addition of specific microorganisms (fungi, algae, bacteria, protozoa and cyanobacteria) or improvement of microorganisms already present in soil to enhance the biodegrading ability in both, *in-situ* and or *ex-situ* (in reactors) mechanism. The biodegradation of hydrocarbons in the soil is influenced by different physical, chemical, and biological factors [232]. Among these

microorganisms capable of bioremediation, *A. flavus* possess the potential to bioremedy soil of complex contaminants [233].

1.11. Aims and Objectives

Until now, very little work has been carried out on the exploration of phytopathogenic fungi. Besides, their pathogenicity, these fungi produced a wide range of beneficial compounds which can be used for the welfare of society. Therefore, realizing the need and importance of plant pathogenic fungi, the present investigation was undertaken. In the present research work, we explore the use of soil borne phytopathogenic fungi for the production of bioactive secondary metabolites.

The aims and objectives of the present study are:

a. **Isolation and Optimization of growth parameters of selected fungi:** To optimize the media for the growth and isolation of different fungi. To optimize the parameters pertaining to the maximum production of secondary metabolites such as culture media, pH, temperature, incubation period, and growth condition.

b. **Bioassay screening:** To evaluate the crude ethyl acetate and *n*-hexane fractions of *S. rolfsii* and *A. flavus* for antibacterial, antifungal, phytotoxic, insecticidal, cytotoxic, anti-cancerous, *in vivo* acute toxicity, analgesic and sedative properties

c. **Characterization of Secondary Metabolites:** To isolate the bioactive compounds from the EtOAc fraction of these fungi using column chromatography. To characterize the structure of the isolated and purified compounds (fair quantity) using different spectroscopic technique; ^{1}H-NMR, ^{13}C-NMR, UV, IR, EI-MS, HMBC, HMCQ, NOSY, COSY, and X-rays (where applicable).

d. **Molecular docking studies and the Reversal of Multidrug Resistance in Mouse Lymphoma cells.** To evaluate the isolated compounds (fair quantity) for their anticancer potential, and to determine their binding properties by computational docking.

2. MATERIALS AND METHODS

2.1. General Experimental Conditions

All the experiments were conducted at the Centre of Biotechnology and Microbiology (COBAM), University of Peshawar, International Centre for Chemical and Biological Studies (ICCBS), University of Karachi and Veterinary Research Institute (VRI), Peshawar. All the chemicals used in this study were of analytical grade.

2.1.1. Physical Constants

Optical rotations of the compounds to determine the melting points were performed on a Buchi 535 apparatus and JASCO DIP-360 digital Polari meter

2.1.2 Spectroscopy

UV spectra were recorded on a Hitachi UV 3200 spectrophotometer. The IR analysis was carried out using the JASCO A-302 IR spectrophotometer in $CHCl_3$. EI-MS was recorded on a double focusing mass spectrometer (Varian MAT 311 A) coupled with PDP 11/34 computer system. ^{1}H-NMR spectra were obtained using a Bruker AMX-400 and AMX-500 MHz instruments, while the ^{13}C-NMR spectra were recorded at 75, 100, 125 and 150 MHz. HMBC experiment were conducted for the determination of two and three-bond ^{1}H-^{13}C connectivities. The coupling constants (*J*) were measured in Hz from ^{1}H-NMR chemical shifts reported in δ (ppm).

2.1.3 Isolation and Purification of Compounds

Various chromatographic techniques were used for the isolation and purification of different chemical constituents of *S. rolfsii* and *A. flavus*.

2.1.3.1. Column Chromatography

Solvents of analytical quality were used for column chromatography and twice distillation were carried out before used. The size of the column and the granulometry of the silica gel were determined according to the amount of the sample and the degree of separation desired. First, slurry of the sample was prepared in a separate solvent and allowed to dry. In column chromatography, the stationary phase was silica gel-GF254, 60 from E. Merck (Art. 7734, 70-230 mesh) while different organic solvents viz. *n*-hexane, EtOAc, DCM, $CHCl_3$, and CH_3OH were used as mobile phase. Isocratic or stepwise gradient systems were applied, depending on the nature of the compounds and of their separation. The crude EtOAc extract obtained was further fractionated by open column silica chromatography using solvents of increasing polarity (*n*-hexane, EtOAc, DCM, $CHCl_3$, and CH_3OH) to obtain 15 to 20 fractions. Each fraction was analyzed using TLC, and then further purified by repeated column chromatography. Normal and reverse phase chromatography were carried out in order to purify the non-polar and polar compound.

2.1.3.2. Thin-layer Chromatography (TLC)

Thin-layer chromatography (TLC) was used for routine analyses. TLC was used for chemical screening of crude extracts, fractions and isolated pure compounds. Different solvent system (*n*- hexane, EtOAc, $CHCl_3$, and CH_3OH) in different ratios was used. 1000 µg of crude extract was dissolved in 1000 µL of EtOAc. Ceric sulfate, Vanillin-Phosphoric Acid, Iodine Solution and Dragendorff's reagents were used to visualize the spots in the TLC plates. The TLC plate was visualized under ultraviolet light at 254 nm and 366 nm. The TLC plates were labeled and preserved. The Rf values were calculated by using the formula.

$$R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent}}$$

2.1.4. Spot Locating Reagents

The spots on TLC plates were visualized by using locating reagents

2.1.4.1. Ceric Sulphate-Sulphuric Acid

Ceric sulphate was dissolved in sulfuric acid in order to prepare a saturated solution, and that solution used as spray on TLC plates [234]. Ceric sulfate gives pink color with steroid, bluish purple with terpene and yellow color with flavonoids.

2.1.4.2. Vanillin-Phosphoric Acid

The TLC plates were sprayed with a solution of vanillin (1 g) dissolved in 50% phosphoric acid and heated to 100-110ºC. The terpenes and steroids developed light pink or blue color and intense purple, respectively, while Terpenoidal and Steroidal glycosides developed pink color [234].

2.1.4.3. Iodine Solution

Spots appeared on the TLC plates when placed in a saturated atmosphere of Iodine vapors produced by warming Iodine crystals at 40-50 ºC [234].

2.1.4.4. Dragendorff's Reagent

(i) 8 gm of potassium iodide (KI) was dissolved in 20 mL of distilled water

(ii) 0.85 g of bismuth nitrate ($Bi(NO_3)_3.5H_2O$) was dissolved in a mixture of water and acetic acid (40:10mL) (iii) The stock solution was prepared by mixing (i) and (ii) in the ratio of 1:1

(iv) 5 mL of stock solution was diluted with a mixture made by mixing 90 mL of distilled water and 10 mL of acetic acid [234]. When the TLC plates were sprayed with this solution, the development of light (pink, brown) dark brown or blackish color indicated the presence of alkaloids, while light yellow to light pink indicated highly oxygenated terpenoids and steroids.

2.2. EXPLORATION OF FUNGI FOR BIOACTIVE METABOLITES

2.2.1. Soil samples collection

Soil samples were collected from different localities of Malakand Division in sterilized bags and were brought to the laboratory for further processing (**Fig 3.1**).

2.2.2. Culture media

A variety of different nutrient media were used for the growth and *in vitro* biological assays. Five different media were used for the growth of the microorganisms and the production of metabolites, while three different types of media were prepared for performing biological assays. The compositions of these media are presented in the **Table 3.1.**

Figure 2.1: Soil samples collected in sterilized polythene bags

Table 2.1: Composition of different types of media

S.No	Name	Media composition (g/L)			
1	**Czapek yeast broth**	S.No	ConstituentS		Concentration (g/L)
		1	K_2HPO_4		1.0
		2	**Czapek Concentrate (10.0 ml)**		
			1	$CuSO_4.5H_2O$	0.05
			2	KCl	5.0
			3	$ZnSO_4.7H_2O$	0.1
			4	$FeSO_4.7H_2O$	0.1
			5	MgSO4. 7H2O	5.0
			6	NaNO3	30.0
		3	Yeast extract		5.0
		4	Sucrose		30.0
2	**Potato dextrose broth**	1	Potato infusion		4.0
		2	Dextrose		20.0
3	**Meat extract Broth**	1	Peptic digest of animal tissue		10.0
		2	Sodium chloride		15.0
		3	Meat extract		3.0
4	**Sabouraud dextrose broth**	1	Dextrose		20.0
		2	Peptone, special		10.0
5	**Nutrient Broth**	1	Beef extract		1.0
		2	Yeast extract		2.0
		3	Peptone		5.0
		4	Sodium chloride		5.0
6	**Muller Hinton Agar**	1	Agar		17.0
		2	Starch		1.5
		3	Acid hydrolysate of casein		17.5
		4	Beef extract		2.0
7	**Muller Hinton broth**	1	Beef extract		2.0
		2	Acid hydrolysate of casein		17.5
		3	Starch		1.5

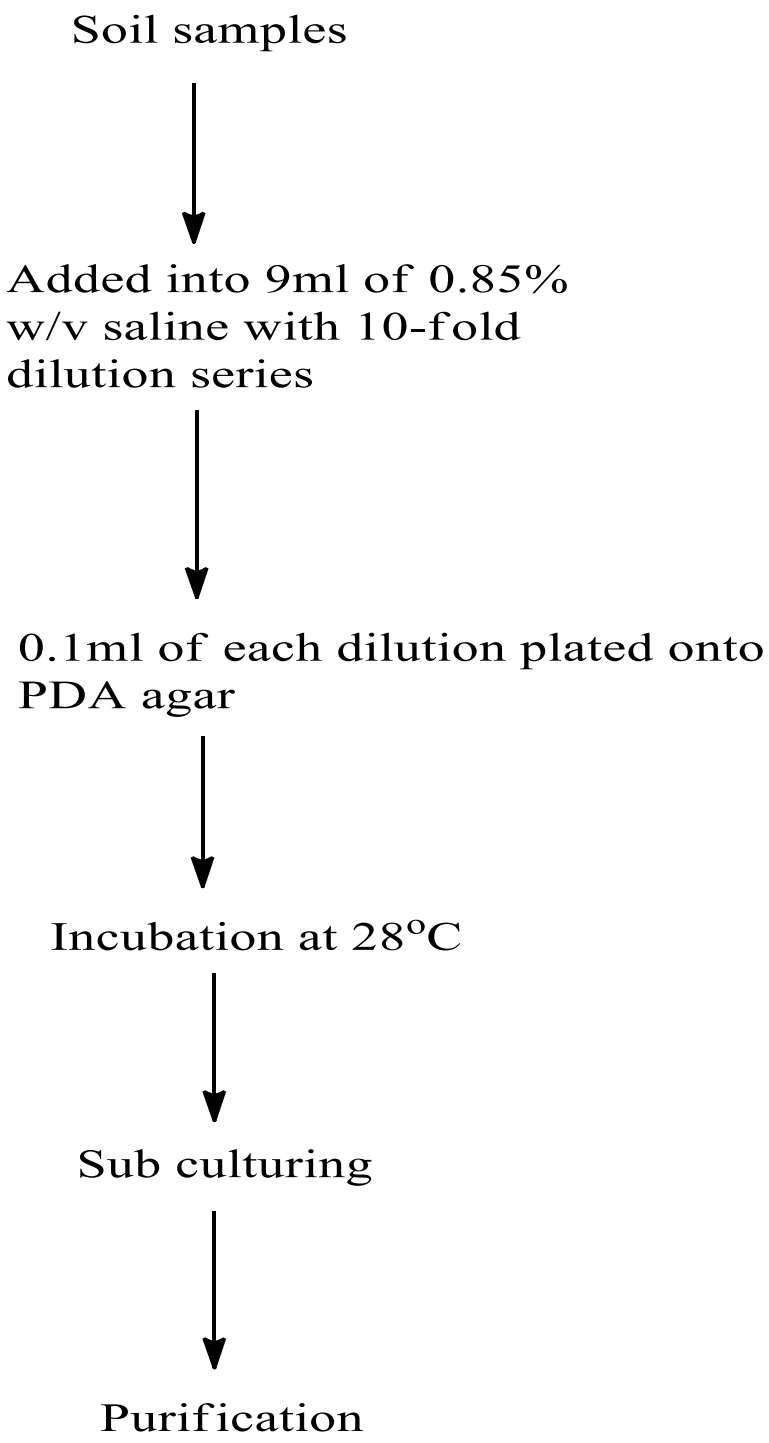
2.2.3. Isolation, identification and preservation of fungi

The samples were inoculated following the serial dilution technique in different media. The soil samples were screened for soil fungi using a serial dilution method [235]; 1 g of each soil sample was suspended in 9 mL of double distilled water with a 10-fold dilution series. Of the serial diluted samples, 1 mL of each was then plated onto PDA and incubated for one week at 28ºC. Each of the fungal species was purified by the sub culturing technique (**Scheme 2.1**). The fungi were identified morphologically and microscopically by a plant pathologist at the Department of Plant Pathology, The University of Agricultural Peshawar, KPK, Pakistan. The purified fungi were then preserved in Tween 80.

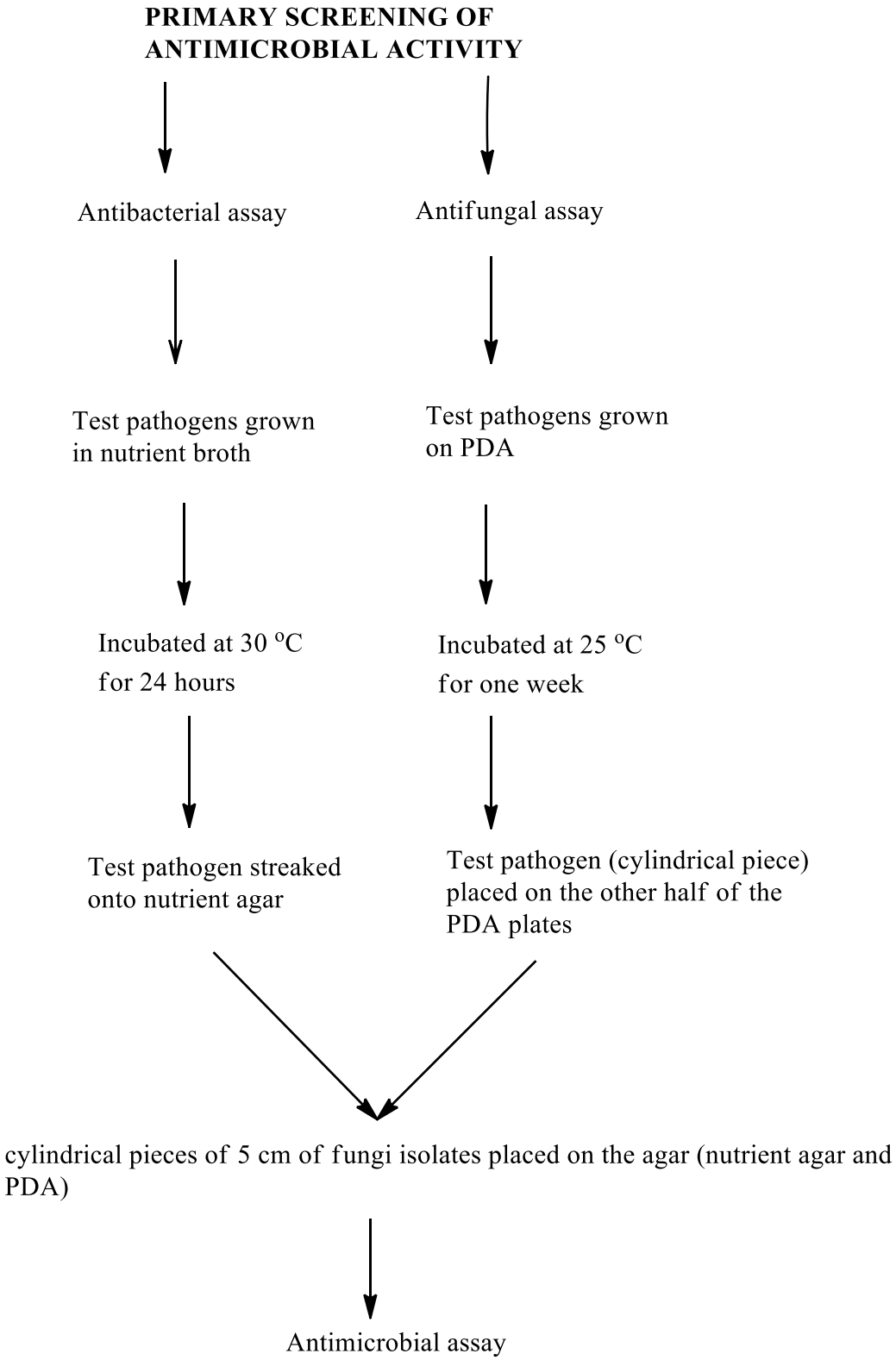
2.2.4. Dual Culture Assay

The bioactive fungi were selected by the dual culture technique. The dual culture assay was applied to determine antimicrobial activities of the isolated fungal culture. For the antibacterial activity, the test bacteria were inoculated in a NB and incubated at 30ºC for 24 hours. After incubation, the test pathogens were streaked on to the nutrient agar. Cylindrical pieces of 5 cm sized agar plugs were cut from seven days old fungal culture and placed on the agar previously streaked with test organisms. The plates were incubated for 24 hours at 30ºC and observed for clear inhibition zones [236].

For antifungal activity, *Aspergillus niger* was selected as the representative for fungi. Cylindrical piece of agar plugs of 5 cm were cut from seven days old fungal culture and placed against the test fungi and incubated for 5-7 days at 25ºC. Each plate contained one fungal isolate and one test fungus. The dimensions of the inhibition zones were measured after the incubation period [237]. (**Scheme 2.2)**



Scheme 2.1: Schematic overview of isolation and purification of soil fungi from soil samples



Scheme 2.2: Screening of bioactive fungi by dual culture method

2.2.5. Optimization of growth parameters

The various growth parameters, viz. media, temperature, incubation time, pH, and shaking/static condition for the maximum production of bioactive secondary metabolites were optimized as follows.

2.2.5.1. Standardization of Basal media for growth and production of secondary metabolites

Five different types of media, viz. Czapek yeast broth media (CYB), Potato dextrose media (PDB), Sabouraud dextrose broth (SDB), Meat extract broth (MEB), and Nutrient broth (NB) were used in this study. Five-day-old cultures of *S. rolfsii* and *A. flavus* were inoculated. The presence of biomass and bioactive crude secondary metabolites was determined after 13 days of incubation. For biomass production, the mycelium was harvested through centrifugation for 15 min at 8000 ×g. The mycelial pellets were washed repeatedly with distilled water and dried at 70ºC, and the dry weight was determined [238]. For crude metabolites, the supernatant was treated thrice with equal volume of EtOAc and concentrated with rotary evaporator under vacuum at 45ºC. The biomass and crude secondary metabolites were determined using the method of Kim *et al*., (2005) with some modifications [239]. The concentrated extracts were collected in sterilized and weighed vials. After drying the vials completely, the vials were weighed to determine the exact weight of crude metabolites expressed in µg/mL.

$$W = W_1 - W_2$$

where, W_1 - weight of vial +crude metabolites; W_2 - weight of vial

2.2.5.2. Optimization of Temperature

The fungi were inoculated in an optimized medium and grown in a range of temperature from 20-40ºC with intervals of 5ºC for 11 days. At the completion of incubation period, the weight of the biomass and the crude secondary metabolites were calculated. The procedure of

Agastian et al. (2013) was used with slight modifications [240]. The experiments were repeated thrice.

2.2.5.3. Optimization of pH

The fungi, *S. rolfsii* and *A. flavus,* were also grown in different pH range of 3-9 for 11 days. The weight of biomass and production of metabolites were calculated at each pH. The experiments were repeated thrice [240].

2.2.5.4. Optimization of incubation period

A five-day-old culture of fungi was inoculated in 13 conical flasks in the optimized media and incubated for 3-13 days at the optimized temperature. The biomass and production of crude metabolites were calculated at intervals of 24 hours for 13 days [240].

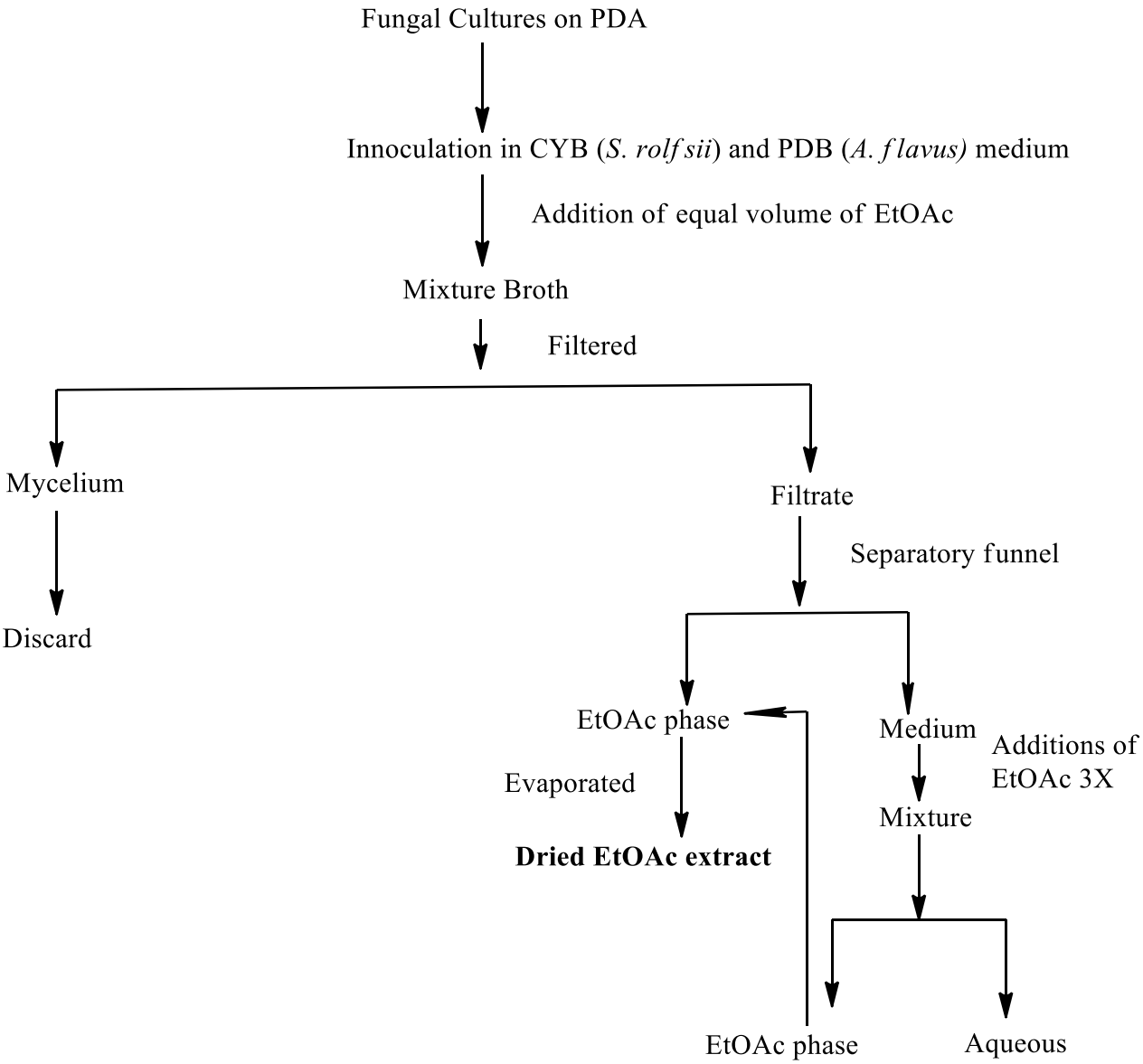
2.2.5.5. Static/shaking growth condition

The biomass and the production of metabolites of *S. rolfsii* and *A. flavus* were determined inoculating fungi in the optimized medium and grown as static culture as well as under shaking condition. The medium, temperature, pH, and incubation period were kept constant. The experiment was repeated thrice.

2.2.6. Extraction of crude metabolites

After the optimization of the growth parameters, the fresh fungal strains were cultured for the production of secondary metabolites. Czapek Yeast-extract Broth (CYB) for *S. rolfsii* and Potato dextrose broth (PDB) for *A. flavus* were prepared and sterilized at 121ºC for 20 min. A five-day-old culture was inoculated in each flask containing the media [241, 242]. The flasks were incubated at 25ºC at 150 revolutions per min (rpm). After the incubation period, 200 to 500 µL of 40% HCl was added to each flask enable the components of media to separate out. After vigorous mixing and grinding, the culture was filtered through a cheese cloth or filter paper.

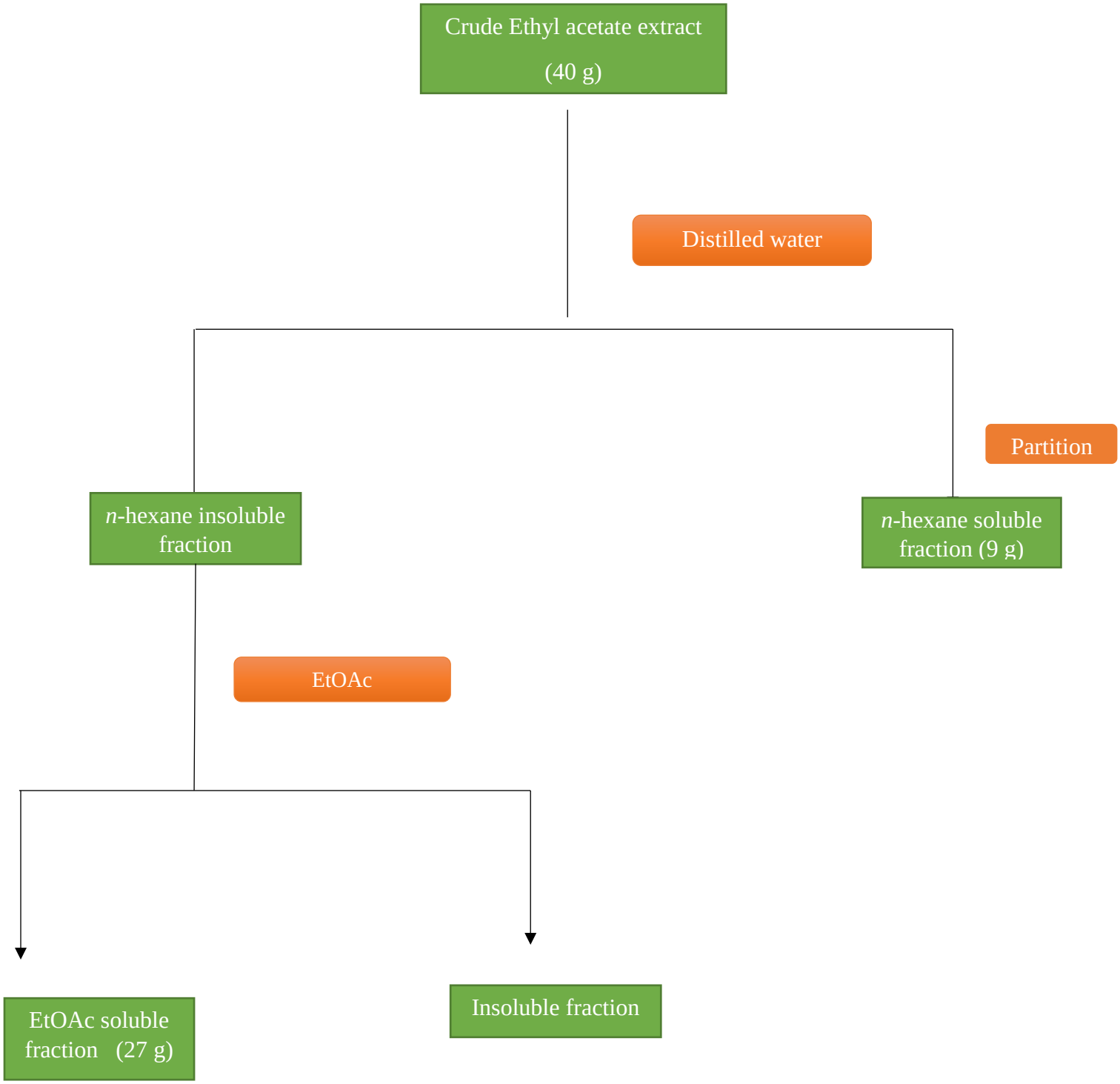
Equal volumes of ethyl acetate (EtOAc) was added to each flask containing media and mixed by shaking for 20 min. The mixture was left to stand for two hours. The process was repeated thrice. The mixture was shifted to a separating funnel in order to separate EtOAc portion. Anhydrous sodium sulfate was added to dehydrate the organics layer and filtered. EtOAc was filtered using the Whatman filter paper. A rotary evaporator was used to concentrate the extracts at 45ºC **(Scheme 2.3)**. The concentrated EtOAc extract was further dried in a fume hood to obtain solid residues.



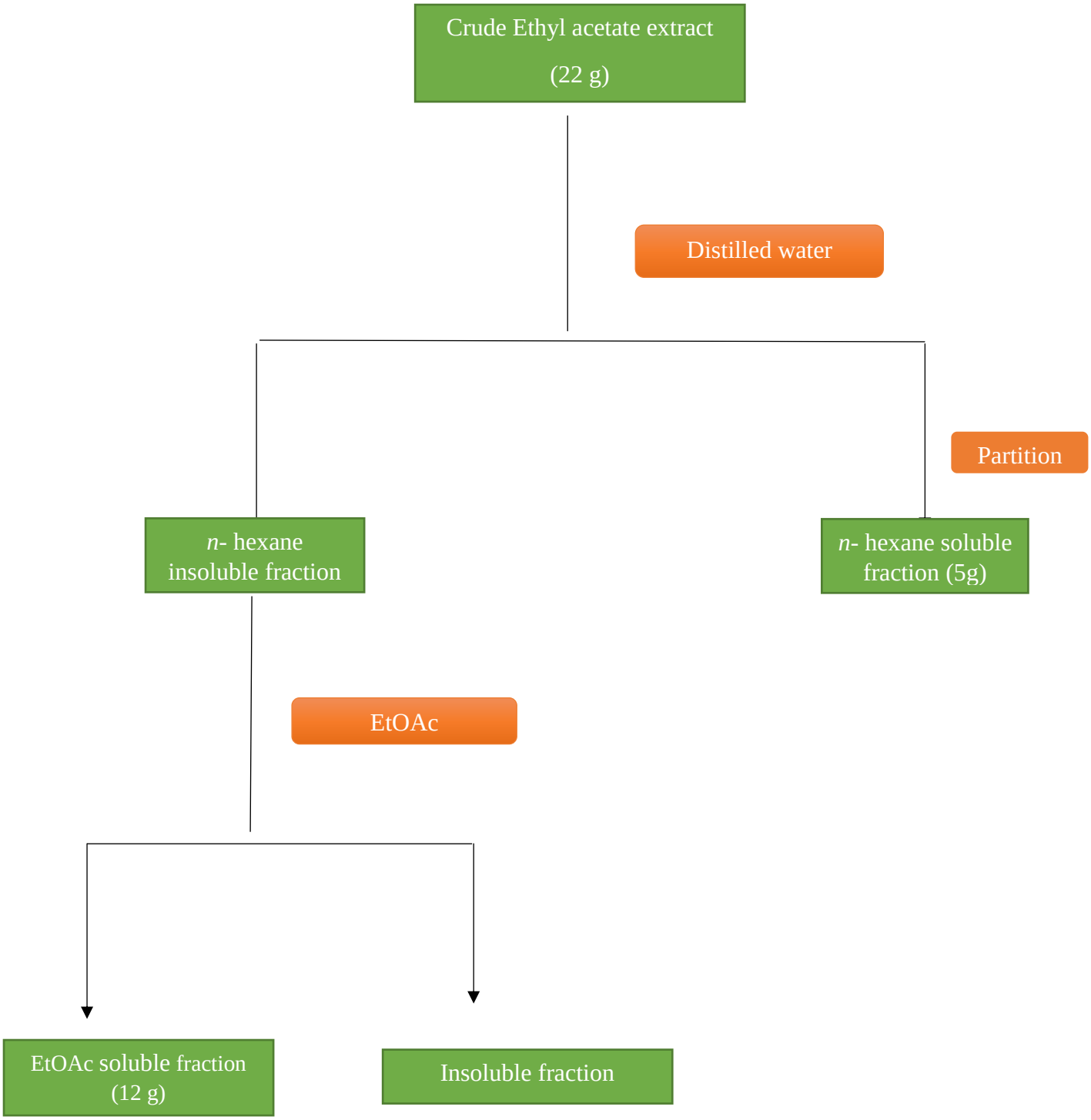
Scheme 2.3: Extraction of bioactive metabolites using EtOAc

2.2.7. Fractionation

The crude EtOAc extract of *S. rolfsii* and *A. flavus* was suspended in distilled water and partitioned with *n*-hexane (**Scheme 2.4** and **2.5**). The *n*-hexane soluble portion was recovered. This procedure was repeated for the EtOAc fraction.



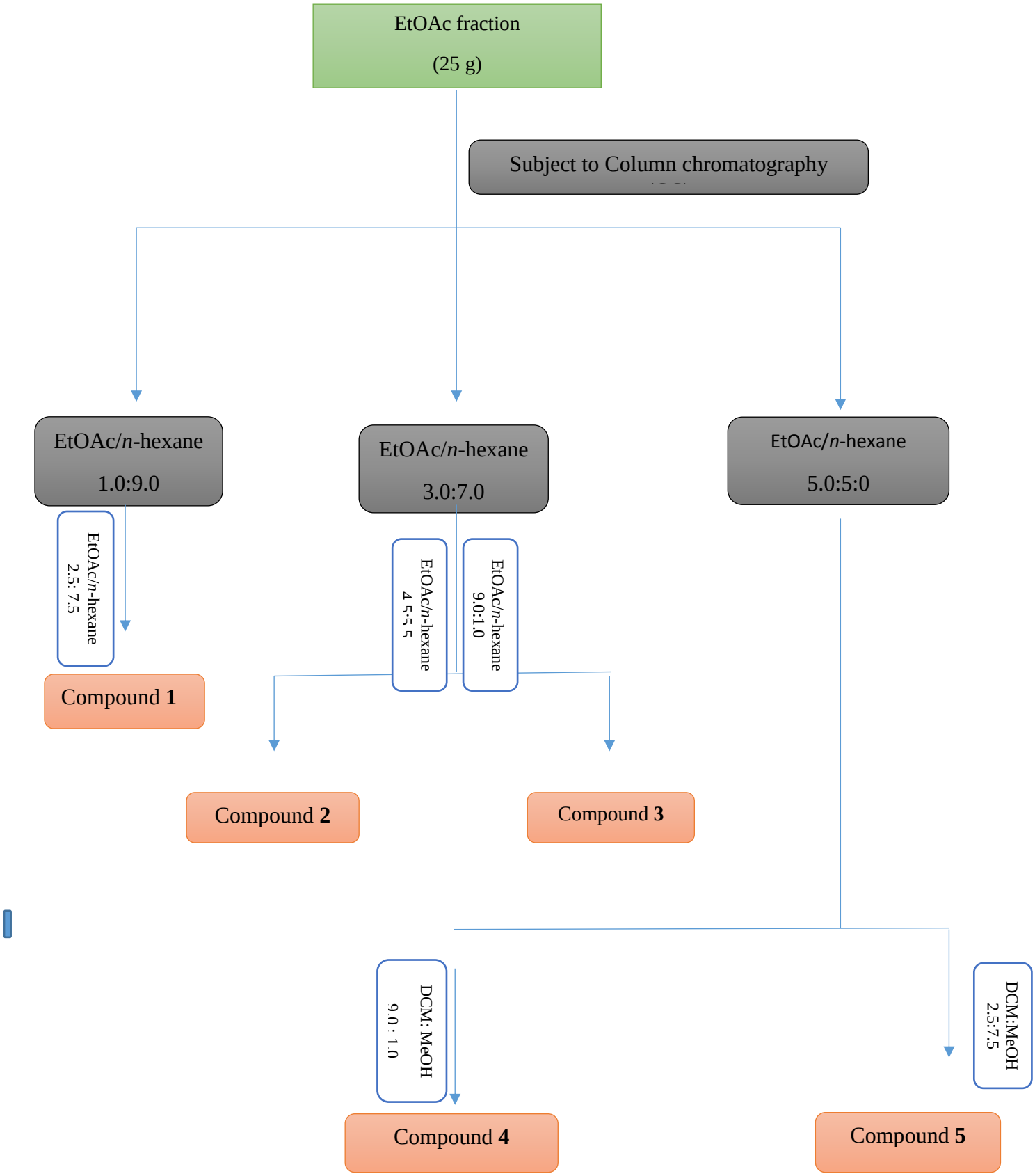
Scheme 2.4: Fractionation of EtOAc crude extracts of *S. rolfsii*



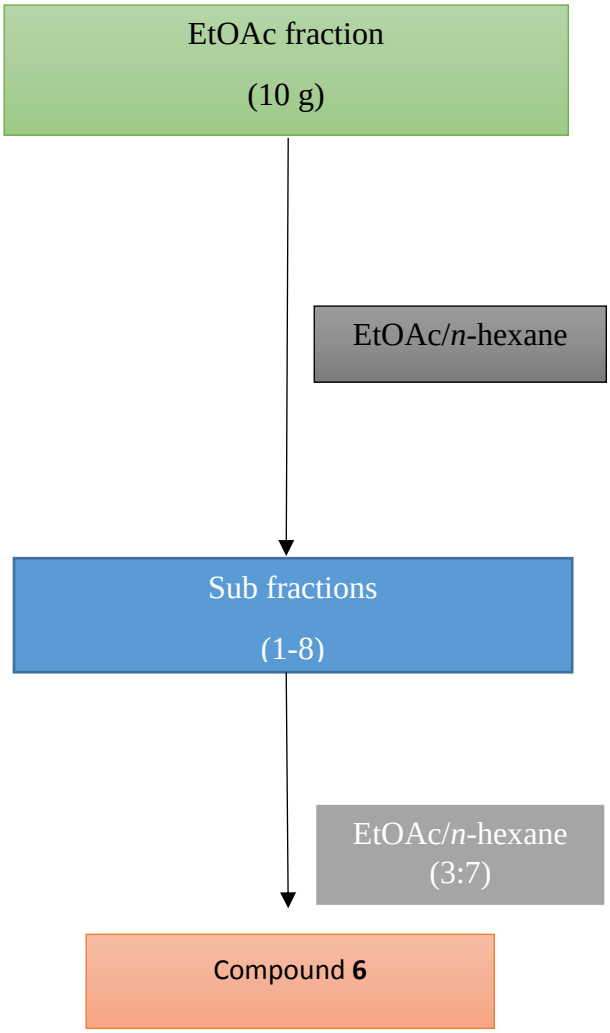
Scheme 2.5: Fractionation of EtOAc crude extracts of *A. flavus*

2.2.8. Compounds isolated from the *S. rolfsii* and *A. flavus*

The EtOAc fraction (20 g) of *S. rolfsii* was subjected to column chromatography (cc) and successively sub-fractionated in an increasing order of polarity using *n*-hexane: EtOAc (9.5:0.5 (i), 9.0:1.0 (ii), 8.5:1.5 (iii), 8.0:2.0 (iv), 7.5:2.5 (v), 7.0:3.0 (vi), 6.0:4.0 (vii), 5.0:5.0 (viii), 4.0:6.0 (ix), 3.0:7.0 (x), 8.0:2.0 (xi), 9.0:1.0 (xii) and (xiii) 100 % EtOAc). A total of six different compounds were eluted with the solvent systems, *n*-hexane: EtOAc in different ratios as follows: Compound **1-** 7.5:2.5; Compound **2-** 5.5:4.5; Compound **3-** 1.0:9.0; DCM: MeOH, Compound **4** - 9:1; Compound **5-** 2.5:7.5 (**Scheme 2.6**). Similarly, EtOAc fraction (10 g) of *A. flavus* was subjected to column chromatography (cc) and sub-fractionated in increasing order of polarity. Compound **6** was isolated from the sub-fraction, *n*-hexane: EtOAc (7.0:3.0) (**Scheme 2.7**).



Scheme 2.6: Isolation of compound from EtOAc fraction of *S. rolfsii*



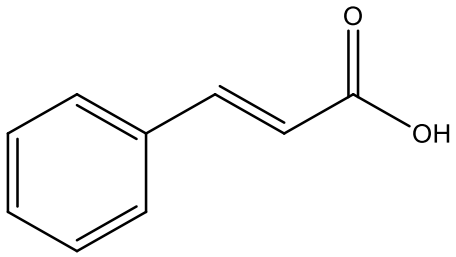
Scheme 2.7: Isolation of compound from EtOAc fraction of *A. flavus*

2.2.9. Chemical structure of new/known compounds from *S. rolfsii*

2.2.9.1. Characterization of Compound **1**

Name: cinnamic acid **(1)**

Structure:



IUPAC: 3-Phenylprop-2-enoic acid

Yield: 7 mg from EtOAc fraction

Physical State: White crystals.

MP: 122-123^{o}C

IR (KBR) Vmax cm^{-1}: 1696, 3557

UV λ_{max} (nm): 252

EIMS m/z: 148.15

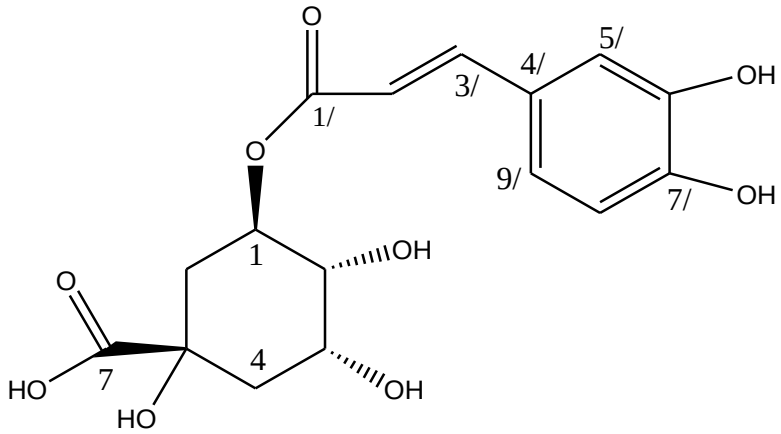
^{1}H-NMR ($CDCl_3$, 300 MHz): Table 3.7

^{13}C-NMR ($CDCl_3$, 75 MHz): Table 3.7

2.2.9.2. Characterization of Compound 2

Name: chlorogenic acid **(2)**

Structure:



IUPAC: (1S, 3R, 4R, 5R)-3-{[(2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoyl]oxy}-1,4,5 trihydroxycyclohexane-1-carboxylic acid

Yield: 10 mg from EtOAc fraction

Physical State: White powder

MP: 206-209^{o}C

IR (KBR) Vmax cm^{-1}: 3421, 1697, 1635, 1610, 1456

UV λ_{max} (nm): 362

EIMS m/z: 354.31

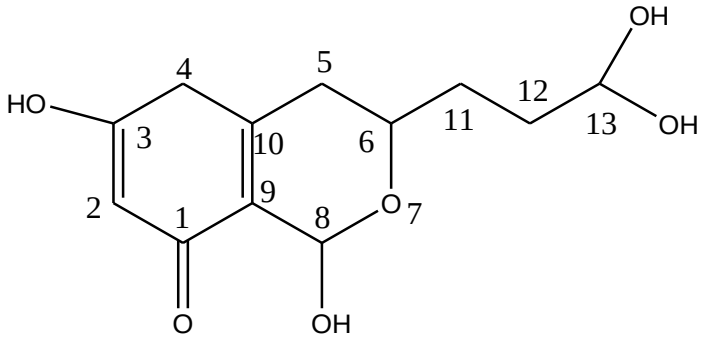
^{1}H-NMR ($CDCl_3$, 300 MHz): Table 3.8

^{13}C-NMR ($CDCl_3$, 75 MHz): Table 3.8

2.2.9.3. Characterization of Compound 3 (new)

Name: Screlotiumol **(3)**

Structure:



IUPAC: (**1**3-(3, 3-dihydroxypropyl)-1, 6-dihydroxy-3,4-dihydro-1H-isochromen-8(5H)-one (1)

Yield: 10 mg from EtOAc fraction

Physical State: yellow solid.

MP: 133-136ºC

IR (KBR) Vmax cm^{-1}: 3355-3650, 2988, 1650

UV λ_{max} (nm): 312

EIMS m/z: 156.2

^{1}H-NMR ($CDCl_3$, 300 MHz): Table 3.9

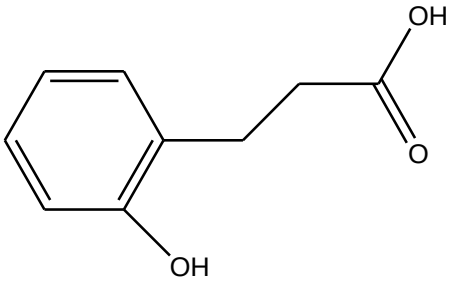
^{13}C-NMR ($CDCl_3$, 75 MHz): Table 3.9

HMBC: Figure 3.11

2.2.9.4. Characterization of Compound 4

Name: *o*-cumaric acid **(4)**

Structure:



IUPAC: (2E)-3-(2-Hydroxyphenyl) prop-2-enoic acid

Yield: 4 mg from EtOAc fraction

Physical State: White powder

MP: 210^{o}C

UV λ_{max} (nm): 350

IR (KBR) Vmax cm^{-1}: 3380, 2940, 1660, 1440

EIMS m/z: 164.58

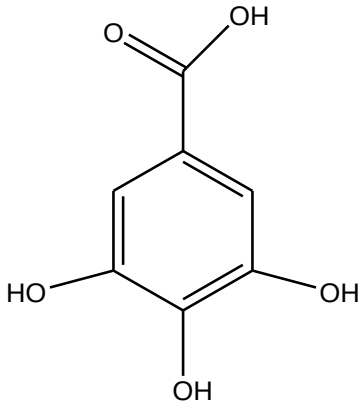
^{1}H-NMR ($CDCl_3$, 300 MHz): Table 3.10

^{13}C-NMR ($CDCl_3$, 75 MHz): Table 3.10

2.2.9.5. Characterization of Compound 5

Name: Gallic acid (**5**)

Structure:



IUPAC: 3, 4, 5-Trihydroxybenzoic acid

Yield: 5 mg from EtOAc fraction

Physical State: needle like crystal

MP: 260^{o}C

UV λ_{max} (nm): 220 and 270

IR (KBR) Vmax cm^{-1}: 39497, 1666, 1610

EIMS m/z: 170.01

^{1}H-NMR ($CDCl_3$, 300 MHz): Table 3.11

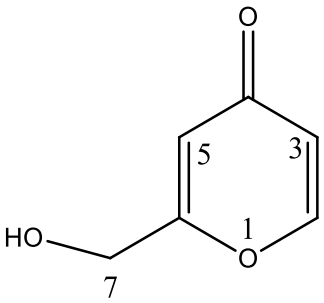
^{13}C-NMR ($CDCl_3$, 75 MHz): Table 3.11

2.2.10. Chemical structure of new/known compounds from *A. flavus*

2.2.10.1. Characterization of Compound 6 (Kojic acid)

Name: Kojic acid (**6**)

Structure:



IUPAC: 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one

Yield: 8 mg from EtOAc fraction

Physical State: white crystalline solid

MP: 155^{o}C

UV λ_{max} (nm): 252

IR (KBR) Vmax cm^{-1}: 3218 1615

EIMS m/z: 142.7

^{1}H-NMR ($CDCl_3$, 300 MHz): Table 3.27

^{13}C-NMR ($CDCl_3$, 75 MHz): Table 3.27

2.3. PHARMACOLOGICAL INVESTIGATIONS

2.3.1. *In vitro* Biological activities

The EtOAc and *n*-hexane fractions of crude extract of both fungi were screened for different properties *in vitro* such as bactericidal, fungicidal, phytotoxicity, insecticidal, brine shrimps lethality and enzyme inhibition, and *in vivo* such as acute toxicity, analgesic, and sedative biological activities.

2.3.1.1. Antibacterial assay

Materials

Pseudomonas aeruginosa, Proteus mirabilis, Serratia marcescens, Salmonella typhi, Salmonella paratyphi, Klebsiella pneumonia, and *Escherichia coli* were used for the antibacterial assays. Other materials used were Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), Petri dishes, test tubes, sterilized cotton swabs, sterile cork borers, micropipette, standard antibiotics, incubator, and test samples

Procedure

The EtOAc and *n*-hexane fractions of crude secondary metabolites produced in each medium were screened for possible antibacterial activities against the above mentioned pathogens using the agar well diffusion method [243]. The bacterial isolates were inoculated on the bacterial culture medium (nutrient agar) to obtain a fresh culture of the bacteria. After 24 hours, each bacterial isolate was inoculated in the broth medium (nutrient broth) and incubated at 37ºC for 24 hours. The bacterial culture suspensions were adjusted to McFarland turbidity standard by adding normal saline. 0.2 mL of the bacterial culture was taken from each test tube and poured on MHA Petri plates. Homogenous lawn of each bacterial species was prepared using sterilized cotton swab; and wells (6 mm) were made with the help of a sterilized borer. Stock solution of the test samples was prepared in DMSO (<1%) at concentration of 12 mg/mL, and 100 µL from stock solution was poured into respective wells. The plates were left

for 2-3 hours in laminar flow hood for better diffusion and then incubated for 24 hours at 37ºC. After incubation, the zones of inhibition were measured. Amoxicillin disk (positive control) and DMSO (<1%) (Negative control) were used as the controls.

2.3.1.2. Antifungal assay

Materials

Test fungi: *Penicillium notatum, Aspergillus fumigatus, Verticillium chlamydosporium, Acremonium* spp., *Alternaria solani*, and *Candida albicans* were obtained from the Department of Plant pathology, Agriculture University KPK, Pakistan. Other materials include potato dextrose agar (PDA), potato dextrose broth (PDB), Petri dishes, test tubes, autoclave, sterilized cotton swabs, micropipette, standard antibiotic (Miconazole), incubator, and test samples

Procedure

The EtOAc and *n*-hexane fractions of crude secondary metabolites were screened against the above mentioned pathogenic fungi. Antifungal susceptibility testing was evaluated using the agar tube diffusion method to detect the antifungal potential of the metabolites [244]. In order to obtain fresh culture, the test fungi were inoculated in potato dextrose agar and incubated for 5 days at 28ºC. For the preparation of slants, 5 mL of PDA was added to each test tube and autoclaved. Then, 66.6 µL of the test sample was added to each test tube and a small piece (5×5 mm) of 7 days old culture of test fungi was placed in the test tube. The test tubes were incubated at 28ºC for seven days. Stock solutions (24 mg/mL) were prepared in DMSO (<1%). Miconazole and DMSO ((<1%) were used as positive and negative controls, respectively. The experiments were performed in triplicate. After seven days, the percent growth inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \frac{\text{Growth in sample tube (mm)}}{\text{Growth in control tube (mm)}} \times 100$$

2.3.1.3. Phytotoxic assay

Materials

The test samples (EtOAc and *n*-hexane fractions) were screened for phytotoxic properties against *Lemna minor*. Other materials used included growth chamber, flasks, micropipette, E-medium, test samples, and standard drug (Paraquat).

Procedure

The EtOAc and *n*-hexane fractions of crude extract were tested for phytotoxic activity against *L. minor* plants [245, 246]. E medium was prepared and autoclaved at 121ºC for 15 min. Sterile dimethyl sulfoxide (DMSO) (<1%) was used to prepare a stock solution at a concentration 20 mg/mL. Using this stock solution, different concentrations viz. 10, 100, and 1000 µg/mL were prepared and used**.** 20 mL of E medium was added to each sterilized flask, and after proper examination, eighteen healthy plants of *L. minor* were transferred to each flasks containing the medium. It was ensured that each plant has a rosette of three healthy fronds. Positive control (Paraquat) and negative control (only E medium) experiments were also performed. The flasks were firmly plugged with sterile cotton and kept for seven days in growth chamber by setting the temperature at 30ºC. The light intensity was adjusted to 9000 lux, and humidity was maintained at 60% by placing a beaker of autoclaved water in the growth chamber and a photoperiod of 12 hours was maintained. The experiments were performed in replicates. Three replicas of the experiment were performed and after seven days of incubation, the fronds were visually examined, and the percent growth inhibition was calculated using the following formula:

$$\text{Growth Inhibition (\%)} = 100 - \frac{\text{No of fronds in sample}}{\text{No of fronds in control}} \times 100$$

Table 2.2: Composition of E-medium [247].

S.No.	**Constituents**	**Formula**	**Concentration(mg/mL)**
1	Manganous chloride	$MnCl_2.4\ H_2O$	3.62
2	Ferric chloride	$FeCl_3.4\ H_2O$	5.40
3	Zinc sulphate	$ZnSO_4.5\ H_2O$	0.22
4	Potassium nitrate	KNO_3	1515
5	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.12
6	Calcium nitrate	$Ca(NO_3)_2.4\ H_2O$	1180
7	Magnesium sulphate	$MgSo_4.7\ H_2O$	492
8	Potassium dihydrogenphosphate	KH_2PO_4	680
9	Copper sulphate	$CuSO_4.5\ H_2O$	0.22
10	Boric acid	H_3BO_3	2.86

12	Ethylene diaminetetraacetic acid	EDTA	11.20

2.3.1.4. Insecticidal assay

Materials: The test insects, *Callosobruchus analis*, *Tribolium castaneum*, *Forficula auricularia*, *Sitotroga cerealella and Callosobruchus maculatus* were purchased from National Institute of Food and Agriculture Peshawar (NIFA). Other materials required include methanol, growth chamber, filter paper, Petri dishes, micropipette, test samples, glass vials, and standard insecticidal drug (Permethrine). The insects were maintained at controlled conditions (at 30ºC, relative humidity of 50-70%) in a sterilized plastic container.

Sample Preparation: Stock solution was prepared by dissolving test sample at a concentration of 10 mg/mL in methanol. Different concentrations, viz., 10, 100, and 1000 µg/mL were used to test the insecticidal activity.

Procedure

Impregnated filter paper method also known as the direct contact method was applied for the determination of insecticidal activity [248]. The filter papers were cut by a sterilized cutter according to the size of Petri dishes. Different concentration of crude metabolites i.e. EtOAc and *n*-hexane fractions were added to each Petri plates containing filter paper using a

sterile micropipette. The Petri dishes were left overnight in order to evaporate the organic solvent completely. The insects of same age and size were transferred into each plate with the help of a clean brush. Permethrine and organic volatile solvent (methanol) without extract was used as the positive and negative control, respectively. The plates were transferred for incubation at 27ºC for 24 hours with a 50-70% relative humidity in a growth chamber. After the incubation period, the percentage mortality or percentage inhibition was calculated with the help of the following formula:

$$\% \text{ Mortality} = 100 - \frac{\text{No of insect alive in test}}{\text{No of insect alive in control}} \times 100$$

2.3.1.5. Brine shrimp lethality assay (BSLA)

Materials

Artemia salina, artificial sea water, de-ionized water, aluminum vials, plastic hatching chamber, magnifying glass, Pasteur pipette, and standard drugs (Etoposide) were used.

Procedure

The EtOAc and *n*-hexane fraction of *S. rolfsii* were tested against the eggs of brine shrimps (*Artemia salina*) to measure the lethality. To hatch the shrimp eggs (*Artemia salina*), artificial sea water was prepared by adding 38 g of sea salt in 1000 mL of distilled water, filtered, and the pH was adjusted to 7.4 [249]. The seawater was kept in a small plastic hatching chamber with dark partition and lit areas. Shrimp eggs (1 mg) were added to the chamber through the dark side, while the light (other side) attracted the hatched shrimp. Eggs hatched within two days into a large number of larvae. These shrimps were transferred to vials containing 5 mL of sea water at the rate of forty shrimps (40) per vial. The stock solution was prepared in DMSO (<1%) at the concentration of 20 mg/mL. The mortality of shrimps was

observed at different concentrations, viz. 10, 50, 100, 500, and 1000 µg/mL. Two vials served as the positive and negative controls. Vials only having media served as the negative control, while a cytotoxic drug, Etoposide served as a positive control. After 24 hours of incubation period, the mortality of the shrimps was checked through a magnifying glass [250].

2.3.1.6. Enzyme inhibition Assay

2.3.1.6.1. Carbonic anhydrase inhibition

Carbonic anhydrase inhibition assay was performed according to the procedure of Ashiq et al. (2015) with slight modifications [251]. The total mixture volume of 200 µL in a well contained 20 µL of enzyme (sigma Aldrich) (0.1 - 0.2 mg/mL in deionized water), 140 µL of 20 mM HEPES-Tris (Invitrogen) buffer, pH 7.4, and 20 µL of 0.5 mg/mL of EtOAc and *n*-hexane fraction in DMSO and was incubated at 25ºC for 15 min. After incubation, a pre-read was taken at a wave length of 400 nm. Then, 20 µL of substrate (4-nitrophenyl acetate, sigma Aldrich) (0.7 mM in methanol) was added and the reaction was performed at identical conditions for 30 min, and then the final read was taken at 400 nm. Acetazolamide was used as the positive control. The percentage inhibition was calculated by using the following formula. EZ-fit enzyme kinetics software was used to determine the IC_{50} values.

$$\text{Percent inhibition} = 100\text{-(absorbance of test compound} \div \text{absorbance of control)}\ 100$$

2.3.1.6.2. Urease inhibition Assay

The procedure of Akhtar et al. was used with minor modifications to perform the urease inhibition assay [252]. A solution containing 55 µL of 100 mM urea and 25 µL of Jack bean Urease was incubated with 5 µL (0.5 mM) of the test sample at 30ºC for 15 min in a 96 well microtiter plate. For the evaluation of urease inhibitory activity, indophenol was used to measure the release of ammonia. To each well, the phenol reagents (45 µL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkali reagents (70 µL, 0.5% w/v NaOH, and 0.1% NaOCl) were added. After 50 min, the increase in the absorbance was measured at 630 nm using a microplate reader (Molecular Device, USA). The change in absorbance per min was measured, and the results were processed using Soft-Max Pro software (Molecular Device, USA). The experiments were performed in triplicate at pH 8.2 (1.0 mM EDTA, 0.01 M $K_2HPO_4.3H_2O$, and 0.01 M $LiCl_2$). Thiourea (standard inhibitor) was used as the positive control. The percent inhibition was calculated using the following formula:

$$\text{Percent inhibition} = 100\text{-(absorbance of test compound} \div \text{absorbance of control) } 100$$

2.3.2. *In vivo* Biological activities

The crude EtOAc extract of *S. rolfsii* and *A. flavus* were evaluated for different *in vivo* biological activities such as acute toxicity, analgesic and sedative properties.

Experimental animals

In order to perform the pharmacological tests of the drugs, the animals were purchased form the Veterinary Research Institute (VRI), Peshawar Khyber Pakhtunkhwa, Pakistan. The guidelines prescribed by institute of laboratory animal resources, Commission on life sciences and National Research Council were strictly followed throughout the study [253].

2.3.2.1. Acute Toxicity

The crude metabolites were dissolved in propylene glycol to prepare stock solution (100 mg/mL). The mice were divided into different groups; each groups consisted of six mice (n = 6). The average weight of mice was 15-20 g. The mice were kept in an air conditioned and well ventilated room, maintained at 25ºC. They were fed with chick mash pellets. Throughout the experiment, animals were also facilitated by artificially lighting provided 24 hours. The

animals were kept for seven days before experiment for acclimatization. A dose of 5 mg/kg of body weight was injected into each mouse intraperitoneally for four consecutive days. A dose level of 10 mg/kg of body weight was administered to each group on the fifth and final day. A negative control group of mice was administered sterilized propylene glycol without the extract. During these days, the behavior of the mice was observed. The experiments were completed at the end of the seventh day. The mice were sacrificed and the blood was collected in sterilized tube, while the organs were preserved in 10% formalin solution. In the event of mice dying in any group, the mice were subjected to post-mortem examination by a senior research officer. The biochemical and hematological parameters were evaluated. The different organs of the sacrificed animals were weighed and gorsy pathology was recorded. The toxicity was graded as follows [254].

++++ (high toxicity: Three of five mice died after the first dose);

+++ (medium toxicity: Three of five mice died after the second and third doses);

++ (moderate toxicity: three of five mice died after fourth and fifth doses);

+ (mild toxicity: Lesions were observed without mortality).

2.3.2.2. Analgesic activity

Acetic acid induced writhing procedure was used to determine the antinociceptive/analgesic effect of crude EtOAc fraction. For this activity, Albino mice of both sexes and body weights of 18-22 g were selected. The mice were distributed into five groups; each group containing six mice (n = 6). Normal saline at concentration of 10 mL/kg of body weight was administered to Group I as the negative control, while diclofenac sodium at concentration of 10 mg/kg of body weight was used as a positive control (Group II). All the conditions were maintained according to the recommended guidelines. Two hours prior to the start of the experiment, the food supply was stopped [255]. The crude EtOAc extract was administered to the remaining groups, III, IV, and V at different doses, viz. 50, 100, and 150 mg/kg of body weight. After 30 min, 1% acetic acid was injected to members of all groups through the intra-peritoneal route. Abdominal writhes (constrictions) were counted after 10 min, i.e., after 5 min of acetic acid injection. The percent analgesic effect was calculated according to the following formula:

$$\% \text{ Analgesic effect} = 100 - \frac{\text{No of writhing in tested animals}}{\text{No of writhing in control animals}} \times 100$$

2.3.2.3. Sedative activity

A special apparatus was used to test the sedative activity, which consisted of an area of white wood (having a diameter of 150 cm), surrounded by stainless steel. The area was divided into four squares through black lines. Before the start of the experiment, animals were adapted under red light (40 W red bulb) with water and food provided ad libitum. The animals were divided into five groups of six animals each (n = 6). Two groups, I and II were maintained as negative (normal saline) and positive control (diazepam), respectively. Normal saline (10 mL/kg body weight) was administered to Group I, while diazepam (0.5 mg/kg b.w) was administered to group II. The extracts (50, 100, 150 mg/kg b.w) were administered to the remaining groups, III, IV, and V. After 30 min, each mouse was placed in the center of the apparatus box, and the numbers of lines crossed by each mouse was counted [256].

2.4. Assay for reversal of Multi-drug Resistance in mouse lymphoma cells

McCoy's 5A medium containing 10% heat-inactivated horse serum supplemented with L-glutamine and antibiotics was used for the growth of parent cell lines, L5178 MDR and L5178Y. The cell densities were adjusted to 2×10^6 per mL, resuspended in a serum-free McCoy's 5A medium, and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compound (4 µg/mL) was added, and incubated at room temperature for 10 min. Verapamil (10 µg/mL) was used as the positive control [257]. 10 µL (5.2 µM) of the indicator, rhodamine 123 (Sigma, St Louis, MO, USA) was added to the test samples and incubated at 37ºC for a further 20 min, washed twice, and resuspended in PBS (0.5 mL) for further analysis. The fluorescence of the cell population was measured by Partec CyFlow flow cytometer (Münster, Germany). DMSO was used as a solvent, and the tested compound was dissolved in DMSO. The treated MDR and parental cell lines were compared with the untreated cells, and the percentage of mean fluorescence intensity was determined. On the basis of the measured fluorescence values, the activity ratio R was calculated using the following equation [257].

$$\mathrm{FAR} = \frac{\mathrm{MDR}_{\text{treated}} / \mathrm{MDR}_{\text{control}}}{\mathrm{parental}_{\text{treated}} / \mathrm{parental}_{\text{control}}}$$

2.5. Docking studies

X-ray crystallographic structure of P-glycoprotein (P-gp) having a PDB accession code 4Q9L of resolution 3.80Å was retrieved from the protein data bank (PDB) [258]. This crystallographic structure was subjected to the energy refinement by the Swiss PDB viewer v 4.1.0 [259]. The structures of the isolated and characterized compounds and rhodamine123 were constructed using Chem sketch [260] and Avogadro's software [261].

The docking analyses of Compound-**2** and **3** and standard rhodamine123 were carried out using Autodock Vina [262] and i-GEMDOCK v 2.1 software [263]. The docking method was optimized by an already co-crystallized ligand of the P-gp receptor.

Furthermore, all the default parameters were used for both, Autodock Vina and i-GEMDOCKv 2.1 softwares [264-266]. The docking analysis was carried out through LIGPLOT+ version v.1.4.5 [32], PyMOL [267], and Discovery studio visualizer software [268].

3. RESULTS AND DISCUSSION

3.1. Collection of soil samples

A large number of new and known bioactive metabolites, such as antivirals, enzyme inhibitors, antibiotics, antihelmintics, anticarcinogens, insecticides, vitamins, antioxidants, immunosuppressants, and immunomodulatory compounds having industrial, pharmaceutical, and agricultural importance have been obtained from soil fungi [269, 270]. The antimicrobial properties of secondary metabolites derived from various groups of fungi are widely reported, suggesting the outstanding potentiality of this microbial community as an important source of bioactive molecules [271, 272].

Twenty-five different soil samples were collected in sterilized polyethylene bags from different localities of Malakand District, Khyber Pakhtunkhwa, Pakistan and brought to the Microbiology Laboratory, COBAM. Using the serial dilution method and by culturing the fungi in different media, a variety of fungal strains were isolated. Among them, *S. rolfsii* (15 isolates), *A. flavus* (21), *A. niger* (19), and *Nigrospora* species (4), and some other novel fungal species were also isolated. The isolated fungi were tested for bioactive properties. Among the bioactive isolates, two fungal species, *S. rolfsii* and *A. flavus* were selected for further studies (**Fig 4.1 and 4.2**).

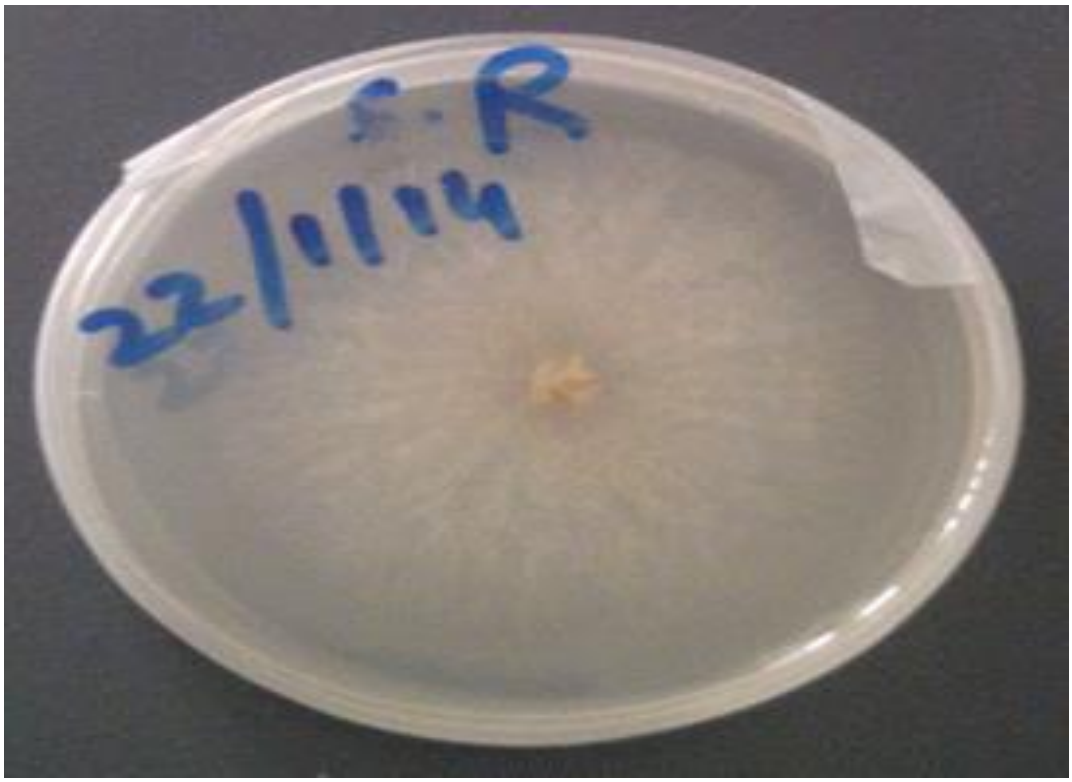


Figure 3.1: Growth pattern of *S. rolfsii* on potato dextrose agar

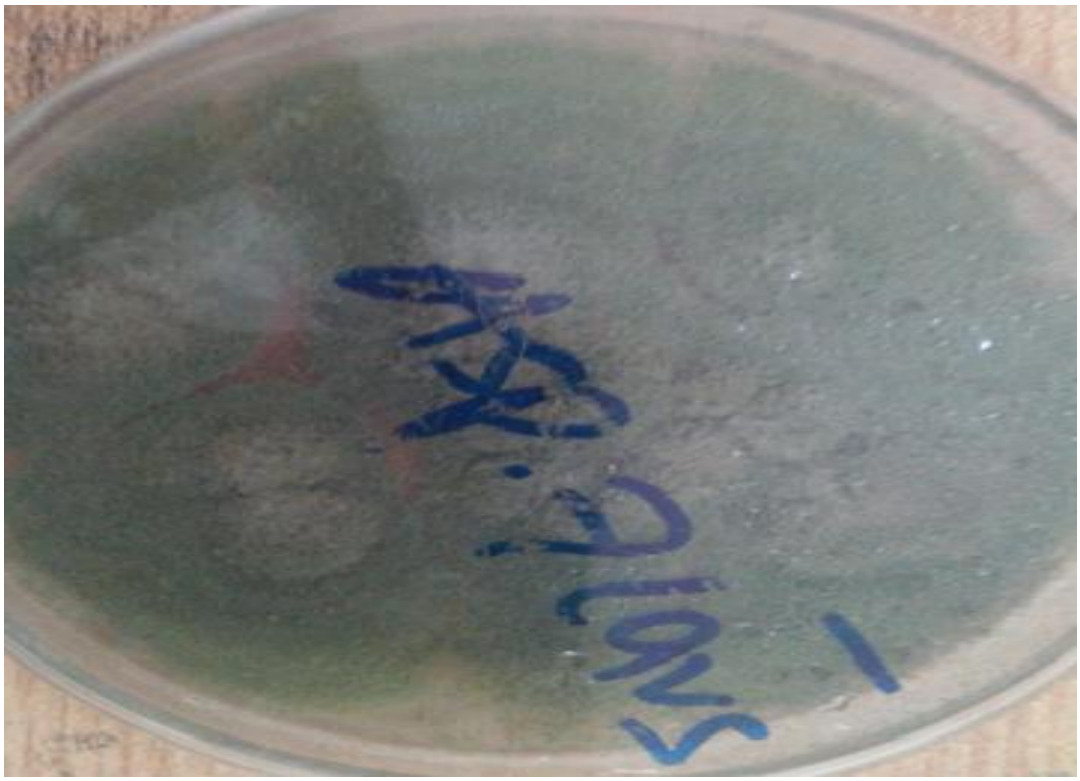


Figure 3.2: Growth pattern of *A. flavus* on potato dextrose agar

3.2.1. Optimization of growth parameters for production of bioactive secondary metabolites by *S. rolfsii*

Soil is one of the best medium for the growth of microorganisms. The most abundant microorganisms in the soil are the bacteria followed by the fungi [273-275]. Soil microorganisms serve as a rich source of novel, bioactive, and structurally unique secondary metabolites. These metabolites possess a wide range of antibacterial properties [276, 277]. This study was aimed at isolating and optimizing the growth conditions for the optimum production of bioactive crude secondary metabolites from the fungus, *S. rolfsii*. For the better exploitation of secondary metabolites from fungi, optimum culture conditions are important. The culture conditions heavily influence the growth of microbes and the production of natural products by the microbes [274].

3.2.1.1. Optimization of nutrient media

In this study, media of five different compositions were screened for optimum fungal growth and production of bioactive crude metabolites as shown in **Table 3.1** and **Fig 3.3**. Among these media, the CYB showed the maximum production of bioactive crude metabolites (413.33 ±1.53 µg/100mL), while PDB supported the production of maximum biomass of fungi (149 ±2.08 mg/100mL). On the other hand, the CYB medium did not produce the maximum growth of fungi but it supported the maximum production of bioactive crude metabolite. The NB medium supported the minimum growth as well as the production of crude secondary metabolites (82.7±3.51mg and 116±3.61 µg/100mL, respectively). The MEB and SDB media could produce almost the same quantities of biomass **(**131±3.06 and 131±1.58 mg/ 100 mL, respectively) with a varying degree of production of secondary metabolites; MEB - 388.67 ±3.21 µg/100 mL and SDB - 230.33 ±4.16 µg/ 100mL. The production of biomass and the yield of secondary metabolite are inversely related because the conditions that allow rapid cell growth could be unfavorable for the maximum production of secondary metabolites [275].

CYB is a complex medium containing carbon, potassium, sodium, zinc, copper, magnesium, and nitrogen which aid the production of secondary metabolites.

Table 3.1: Comparison of different media for biomass and crude metabolites production

S.No	Media	Biomass (mg/100mL)	Crude metabolites (µg/100mL)
1	PDB	149±2.08	342.33±2.52
2	SDB	131±1.58	230.33±4.16
3	CYB	142±3.21	413.33±1.53
4	MEB	131±3.06	388.67±3.21
5	NB	82.7±3.51	116±3.61

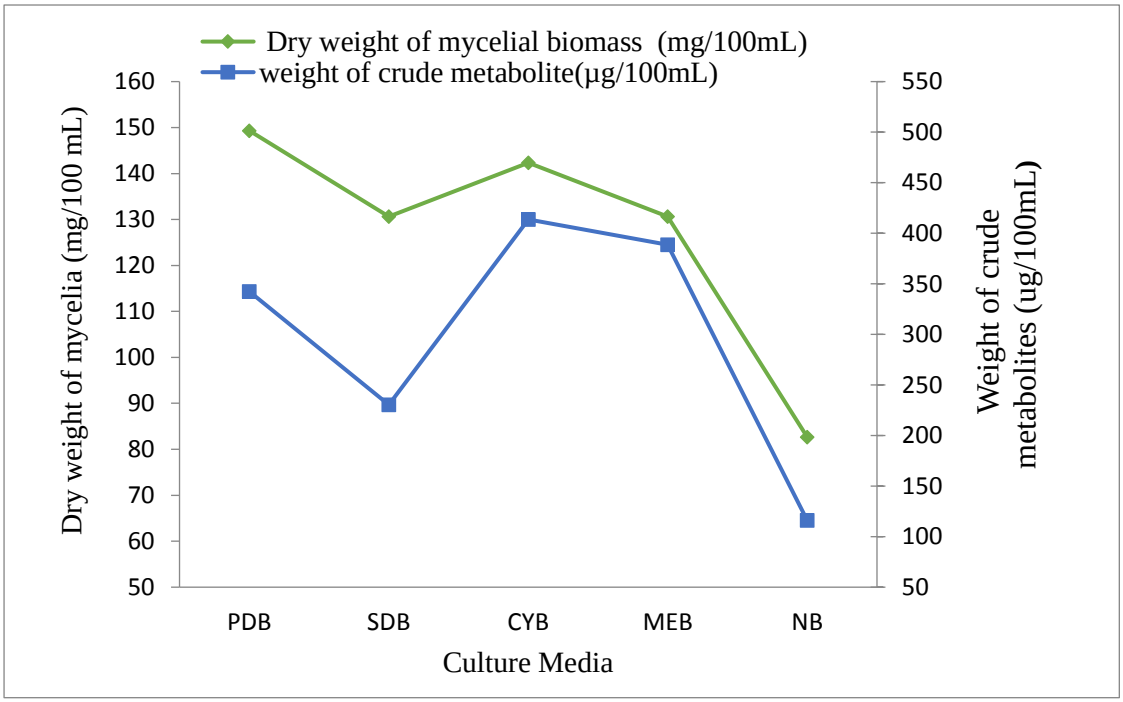


Figure 3.3: Comparison of different media for biomass and crude metabolites production

3.2.1.1.1. Determination of anti-bacterial activity of crude metabolites obtained from each medium

The crude EtOAc and *n*-hexane fractions obtained from each medium were tested against different pathogenic bacteria. The EtOAc fraction obtained from CYB was active against all pathogenic bacteria (with zone of inhibition range from 20.33 to 29.67 mm) except against *K. pneumonia* and *E. coli* (14.67±0.58 and 12.67±1.15 mm, respectively), while the *n*-hexane fraction was very effective against *P. aeruginosa, S. aureus*, and *S. typhi* (23.33 ±1.53, 18.33 ±1.15, and 24.33 ±1.15mm, respectively), moderately active against *S. marcescens* and *P. mirabilis (*16±1 and 15.67±1.15 mm, respectively), and inactive against *K. Pneumoniae* and *E. coli*. The EtOAc fraction of PDB medium was active against *P. aeruginosa, S. marcescens,* and *P. Mirabilis* (20.33±0.58, 23.33±1.53, and 23.67±1.15mm, respectively), while moderately active against *S. aureus*, *S. typhi*, *S. saprophyticus*, *S. para typhi*, and *K. pneumonia* (with zone of inhibition ranging from 11.33 to 13.67 mm). The *n*-hexane fraction was moderately active against *S. saprophyticus*, *S. typhi*, *S. marcescens*, and *P. Mirabilis (*11.0±1, *12.33*±0.58, 13.67±1.53, and 17±2 mm, respectively) and inactive against *K. Pneumoniae, E. coli, S. para typhi, P. Aeruginosa*, and *S. aureus*. Similarly, the SDB extracted EtOAc fraction was active against *S. marcescens, P. mirabilis, and E. coli* (20.7 ±0.58, 24.3 ±1.15, and 20.3 ±0.58 mm, respectively), moderately active against *S. para typhi, S. aureus, S. typhi,* and *P. Aeruginosa* (15.7 ±1.15, 12.3 ±1.15, 15.7 ±1.15, and 16.3 ±1.153mm, respectively) and inactive against *S. saprophyticus* and *K. Pneumoniae*. The *n*-hexane fraction was moderately active against *S. typhi, S. marcescens, P. aeruginosa, P. Mirabilis, and E. coli* and was found inactive against the remaining test pathogens as evidenced in **Table 3.2.** The crude secondary metabolites obtained in MEB showed good inhibitory effects against *P. aeruginosa* (23.3±2 mm), *E. coli* (20.7±0.58 mm), and *S. typhi* (20.3±1.53 mm), and low or no inhibitory effect against the remaining test organisms. The *n*-hexane fraction of NB showed no inhibitory effect against test

organisms, while the EtOAc fraction was moderately active against *P. Mirabilis* (18.7±1.53 mm), *E. coli* (17±1.73 mm), and *S. typhi* (16.3±3.1 mm) and inactive against the remaining organisms. The above results revealed that among the crude secondary metabolites obtained in various media, the EtOAc fraction obtained from CYB was most active against the test pathogens as compared to those obtained when the fungi were grown in other media. Similar result was observed in a study by Kiranmayi *et al* who reported a 30-mm inhibitory zone against *Pseudomonas* [273].

The growth parameters, incubation period, salinity, temperature, pH, and carbon and nitrogen sources also influence the production of secondary metabolites in microorganisms [276]. The yield of secondary metabolites can sometimes be increased by the optimization of physical and chemical factors required for the growth of microbes [277]. Hence, CYB medium was used to further optimize the different growth conditions, such as pH, temperature, incubation time for the optimum production of bioactive secondary metabolites.

Table 3.2: Antibacterial activity of EtOAc and *n*-hexane Fractions extracted from each medium

	Medium									
	PDB		**CYB**		**SDB**		**MEB**		**NB**	
Test Pathogens	EtOAc	*n*-hexane	EtOAc	*n*-hexane	EtOAc	*n*-hexane	EtOAc	*n*-hexane	EtOAc	*n*-hexane
P. aeruginosa	20.33 ±0.58	0±0	29.67±0.58	23.33±1.53	16.3±1.53	10.7±1.15	23±2	13.3±1.53	0±0	0±0
S. typhi	13.67 ±1.15	12.33±0.58	25.67±1.15	24.33±1.15	15.7±1.15	0±0	20.3±1.53	19.3±0.58	16.3±2.31	0±0
S. aureus	12.67±2.08	0±0	21±1	18.33±1.15	12.3±1.15	0±0	11.3±0.58	0±0	0±0	0±0
S. marcescene	23.33±1.53	13.67±1.53	25.67±0.58	16±1	20.7±0.58	10.3±2.52	19.3±0.58	17±2	0±0	0±0
S. saprophyticus	13±2	11±1	20.33±0.58	12±1	0±0	0±0	11.3±1.15	0±0	0±0	0±0
S. para typhi	12± 1	0±0	23.67±1.15	11.67±1.15	12±1	0±0	14.3±1.15	0±0	0±0	0±0
K. pneumoniae	11.33±0.58	0±0	14.67±0.58	0±0	0±0	0±0	0±0	0±0	0±0	0±0
P. mirabilis	23.67±1.15	17±2	26.33±1.15	15.67±1.15	24.3±1.15	16±2.65	19.7±0.58	11±1	18.7±1.53	0±0
E. coli	0±0	0±0	12.67±1.15	0±0	20.3±0.58	15.3±1.53	20.7±0.58	11.5±0.71	17±1.73	0±0

Positive control= Amoxicillin(10µg/Disc)

3.2.1.2 Optimization of Temperature

The temperature was optimized for the growth of fungi by growing the fungi at different temperatures, such as 20, 25, 30, 35, and 40ºC. The maximum biomass (179±2.65 mg/100 mL) and secondary metabolite (515±3.215 µg/100 mL) production by *S. rolfsii* was observed at 25ºC. A decrease in biomass (167±2.65 mg/ 100 mL) as well as crude metabolite production (499.67±3.215 µg/100 mL) was recorded when the temperature was set at 30ºC. Similarly a decrease in the biomass and crude metabolite production was observed for other temperatures such as 20, 35, and 40ºC as presented in **Table 3.3** and **Figure 3.4**. Other studies have reported that extreme temperatures affect the metabolic activities and viability of the fungal cells [278]. The highest growth as well as secondary metabolite production (20 mm against *Vibrio parahaemolyticus*) was observed at 25°C, while the lowest growth and secondary metabolite production was recorded for 15 and 20°C. There was a gradual decrease in biomass and secondary metabolite production when the temperature was increased from 25°C to 45°C. Other studies have reported that the optimum incubation temperature for the growth of fungal mycelia lies in the range of 20-25°C. However, an increase in the incubation temperature from 25 °C to 30°C enhanced both the growth of the mycelia and the production of secondary metabolites in an *Aspergillus* strain [279]. In the present study, the highest growth as well as secondary metabolite production was recorded at 25°C. These results are in complete accordance with those reported by Jain and Pundir [280].

Table 3.3: Comparison of growth and metabolites production at different temperature

S. No	Temp (°C)	Biomass (mg/100mL)	Crude metabolites (µg/100mL)
1	10	29.33±4.04	28.29±2
2	15	50±0	37.33±3.512
3	20	100.7±2.52	250.667±2.887
4	25	179±2.65	515±3.215
5	30	167±2.65	499.67±3.215
6	35	102.7±2.52	333.667±4.163
7	40	61.67±3.06	90±3.606

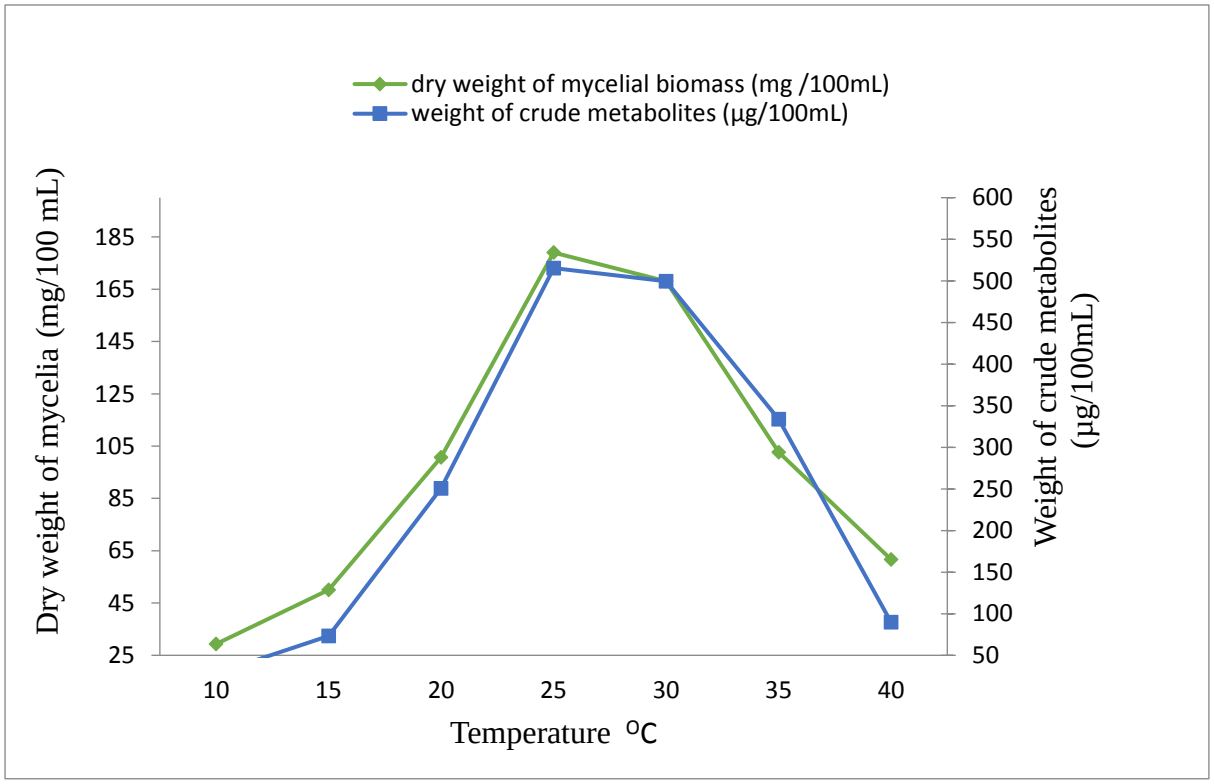


Figure 3.4 Comparison of growth and metabolites production at different temperature

3.2.1.3. Optimization of pH

The pH of the growth medium plays a key role in the growth of microorganisms in an artificial environment. Hence, the pH was optimized for the growth of the fungi in the range of pH 3-9. The production of biomass and crude secondary metabolites was recorded by measuring the dry weights of the fungal mycelia and the crude extract at the respective pH values. A maximal growth (160±5.69 mg/100 mL) and secondary metabolites production (510.3±2.52µg/100mL**)** was observed at pH 6. The biomass and secondary metabolite production gradually decreased when the pH increased to 7 or 8. Similar decrease was observed for pH 4 and 5 as well. However, no production was recorded for pH 3 and 9 (**Table 3.4** and **Fig 3.5**)

Table 3.4: Comparison of growth and metabolites production at different pH

S.No	pH value	Biomass (mg/100mL)	Crude Metabolites (µg/100mL)
1	3	0	0
2	4	96.7±4.04	139.33±4.04
3	5	109±2.31	179.67±3.51
4	6	160±5.69	510.3±2.52
5	7	150±1.53	491.67±4.73
6	8	111±3.11	209.33±3.06
7	9	0	0

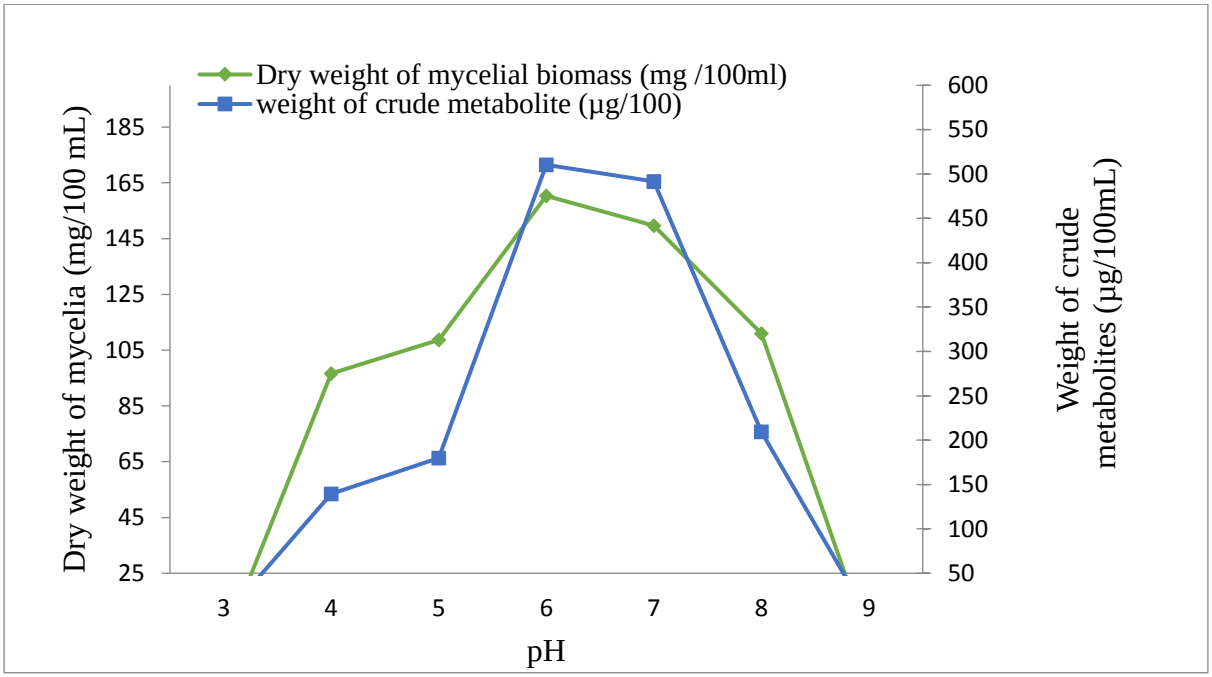


Figure 3.5: Comparison of growth and metabolites production at different pH

3.2.1.4. Optimization of incubation period

The incubation time for the production of biomass and crude metabolite was also optimized in the range of 3 – 14 days at an interval of 24 hours. An incubation period of up to 5 days was not effective for both biomasses (14±3.61 – 47.33±4.04 mg/100mL) as well as crude metabolite production (0 µg/100mL). The highest production (410.33 ±3.51 µg/100 mL) was observed for 11 days of incubation, while the maximum biomass was produced after 9 days of incubation. After the 11th day, the biomass production remained constant (212 ±2.52 to 217.7 ±3.51 mg/100 mL**),** while crude metabolite production started to decrease when the incubation time got longer (**Table 3.5** and **Fig 3.6)**. The maximum growth and metabolites production was observed for 9-11 days of incubation. The growth did not increase after 11 days, but interestingly the amount of metabolites decreased. Stinson *et al.* also observed similar results after 10 days with *Gliocladium* sp [281].

Table 3.5: Comparison of growth and metabolites production at various incubation periods

S.No	Incubation Time (Days)	Biomass (mg/100mL)	Crude metabolites (µg/100mL)
1	3	14±3.61	0±0
2	4	28±4.58	0±0
3	5	47.33±4.04	0±0
4	6	83.33±4.51	97.66±2.08
5	7	142.7±3.06	111±2
6	8	193.3±4.16	172±3.606
7	9	231±2.65	367±2
8	10	218.7±3.21	392.6±2.5
9	11	212.7±2.52	410.33±3.51
10	12	213.3±2.31	406.33±2.517
11	13	214.7±2.52	386±4.35
12	14	217.7±3.51	332±3

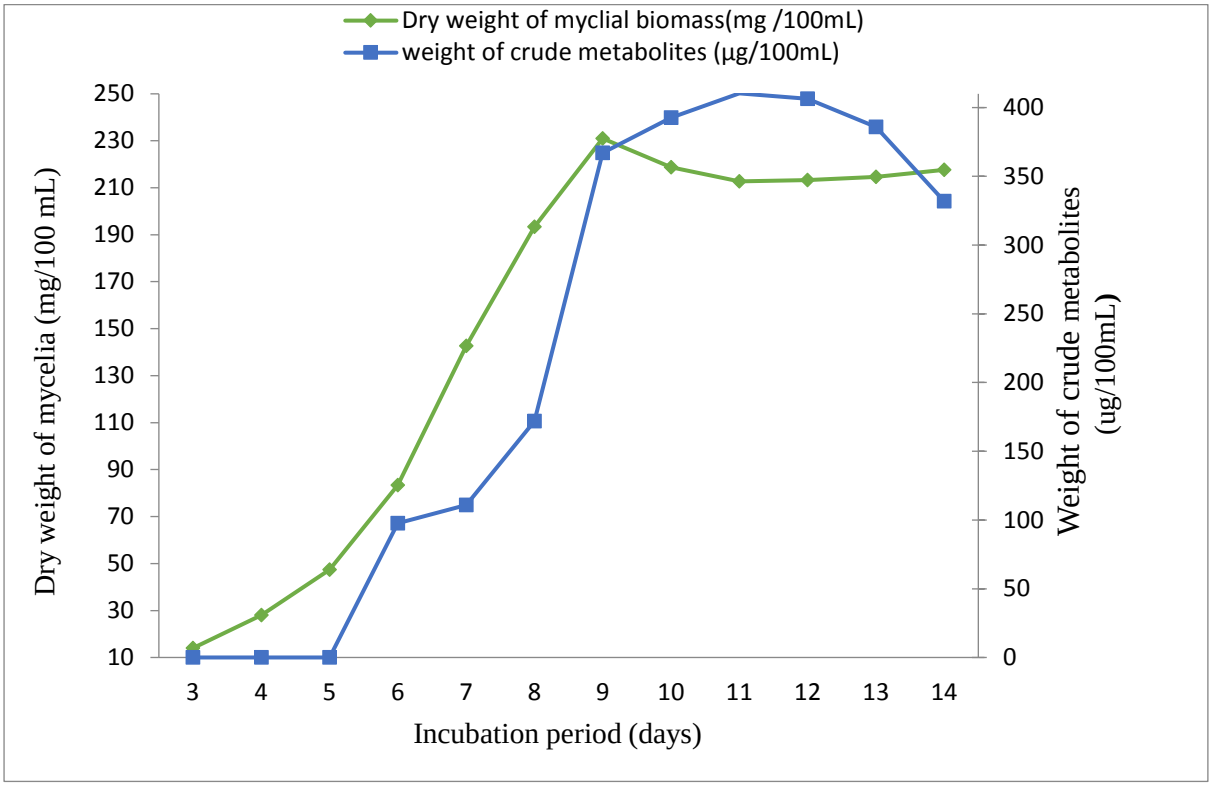


Figure 3.6: Comparison of growth and metabolites production at various incubation periods

3.2.1.5. Static/shaking incubation

The production of biomass and metabolites was also determined under static as well as shaking conditions. The dry weight of the fungi as well as crude metabolites obtained when grown in shaking condition (226 ±3.71 mg/100 mL and 408± 3.61 µg/100 mL, respectively) were almost double to that obtained under static conditions (149.7±4.16 mg/100mL and 205±4 µg/100mL, respectively) **(Table 3.6** and **Fig 3.7)**. Both the biomass and crude metabolites were produced in maximum quantities when the fungi were grown in shaking conditions as compared to that as static cultures. Madla *et al.* noted similar results that shaking induced aeration dramatically decreased the exponential phase of fungi from 20 to 5 days, and bioactive metabolites were also detected as compared to that in static cultures [282].

Table 3.6: Comparison of growth and secondary metabolites production at static and shaking condition.

S.No	Growth condition	Biomass (mg/100mL)	Crude metabolites (µg/100mL)
1	Static incubation	149.7±4.16	205±4
2	Shaking incubation	226±3.71	408±3.61

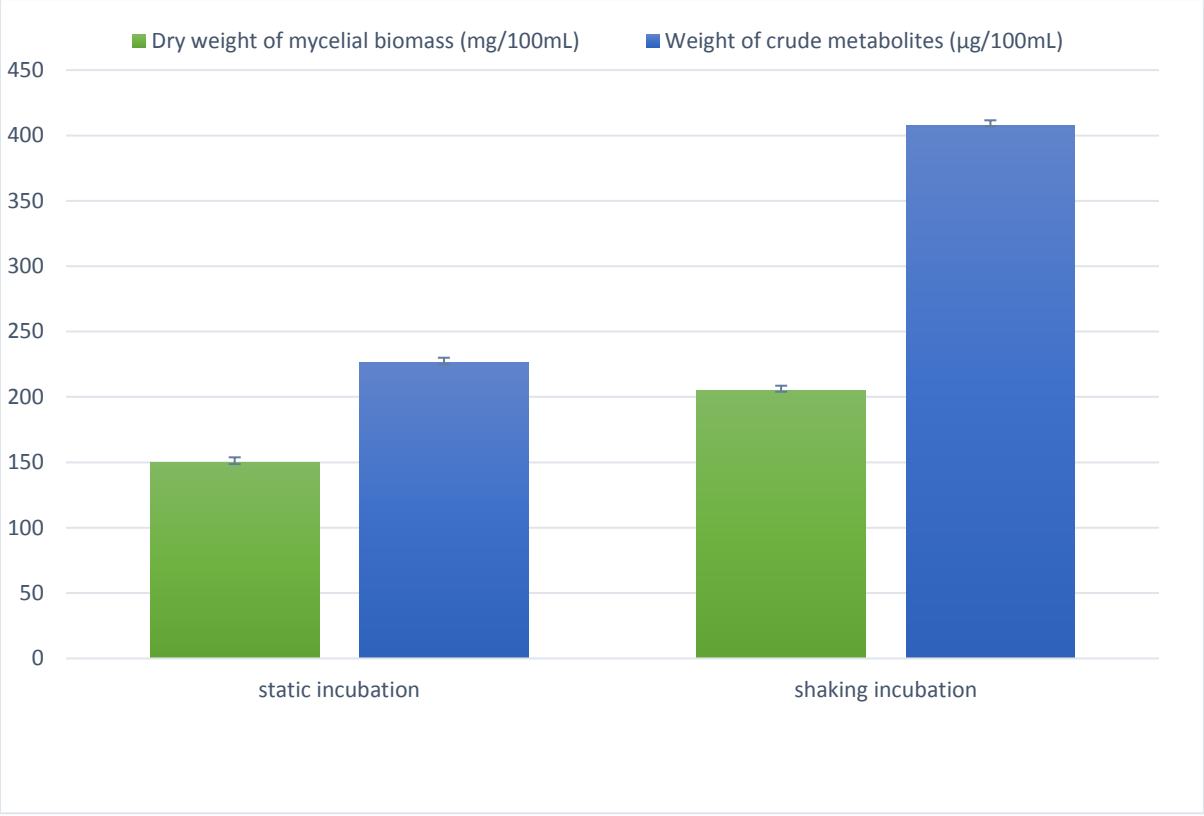


Figure 3.7: Comparison of growth and secondary metabolites production at static and shaking growth condition

3.2.2. Description of Secondary Metabolites Isolated from *S. rolfsii*

3.2.2.1. Structure elucidation of compound (1)

Cinnamic acid (**1**) was isolated as white crystals from EtOAc fraction. It's exhibited EI-MS peak at 148.15 corresponding to $C_9H_8O_2$. The IR spectra showed peaks at 3557 for OH stretching, 1696 for C=O. ^{1}H-NMR showed resonating peak at δ_H 7.05 (H-1, m), 7.69 (H-2, m), 7.67 (H-3, m), 7.51 (H-4, m), 7.31 (H-5, m), 6.47 (H-7, d, *j= 7.7*), 7.24 (H-8, d, *j*=14.8) respectively. ^{13}C NMR showed signals at δ_c 126.4 (C-1), 128.7 (C-2), 128.0 (C-3), 128.7 (C-4), 126.4 (C-5), 135.2 (C-6), 148.0 (C-7), 115.6 (C-8), 170.6 (C-9) respectively (**Table 3.7**). The structure of compound **1** was confirmed by comparing their physical and spectra data with reported one [283].

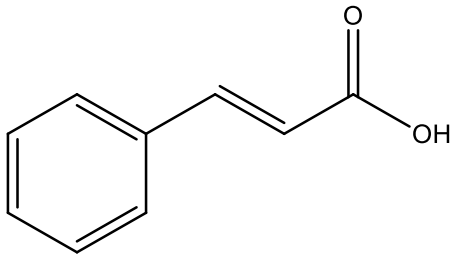


Figure 3.8: structure of Cinnamic Acid (**1**)

Table 3.7: ^{1}H and ^{13}C-NMR chemical shift values of Cinnamic acid (**1**)

Carbon No.	δ_C	δ_H (mult, *J*, HZ)	Multiplicity
1	126.4	7.50, m	CH
2	128.7	7.69, m	CH
3	128.0	7.67, m	CH
4	128.7	7.51, m	CH
5	126.4	7.31, m	CH
6	135.2	-	C
7	148.0	6.47, d, *j*= 7.7	CH
8	115.6	7.24, d, *j= 14.8*	CH
9	170.6	-	C

3.2.2.2. Structure elucidation of compound (2)

Chlorogenic aicd (**2**) was isolated from EtOAc fraction (M.p=206-209 ^{0}C). It's exhibited EI-MS peak at 354.31 conforming to $C_{16}H_{18}0_9$. The UV spectrum of this compound showed absorption peak at 362 nm. The IR spectra showed peaks at 3421 for OH stretching, 1697 for carboxylic acid stretching and 1635 for C=O stretching, while peaks observed at 1610 and 1456 showed aromatic ring stretching. ^{1}H-NMR showed resonating peak at δ_H 3.96 (H-1, m), 3.84 (H-2, m), 3.29 (H-3, m), 2.01, 1.94 (H-4, m; 2H), 1.95, 1.96 (H-6, m, 2H). 7.63 (2′-H, d, J=12.4), 7.50 (3′-H, d, J=14.2), 6.69 (5′-H, m), 6.50 (8′-H, m), 6.65 (9′-H, m). ^{13}C NMR showed signals at δ_c 67.0 (C-1), 82.5 (C-2), 63.5 (C-3), 38.5 (C-4), 77.3 (C-5), 35.8 (C-6), 177.5 (C-7) respectively. CNMR showed peak at 166.5 (1′-C), 116.3 (2′-C), 145.2 (3′-C), 129.2 (4′-C), 113.6 (5′-C), 147.2 (6′-C), 146.5 (7′-C), 117.2 (8′-C) and 120.4 (9′-C) respectively (**Table 3.8**). The structure of compound **2** was confirmed by comparing their physical and spectra data with reported one [284].

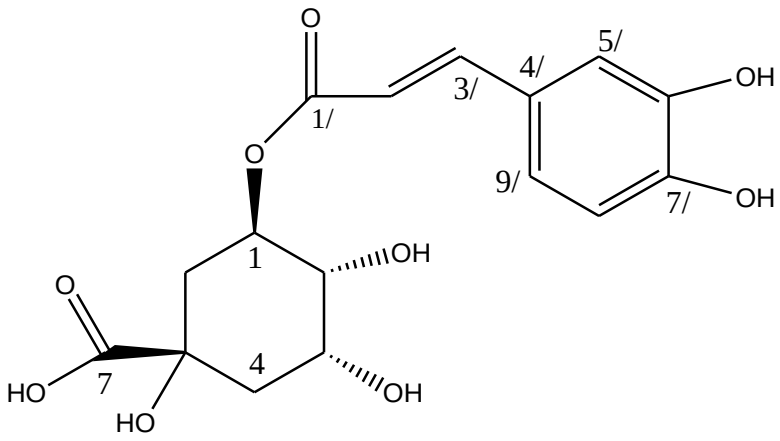


Figure 3.9: Structure of Chlorogenic acid (**2**)

Table 3.8: ^{1}H and ^{13}C-NMR chemical shift values of Chlorogenic acid (**2**)

Carbon No.	δ_C	δ_H (mult, *J*, HZ)	Multiplicity
1	67.0	3.96, m	CH
2	82.5	3.84, m	CH
3	63.5	3.29, m	CH
4	38.5	2.01, 1.94, m	CH_2
5	77.3	-	C
6	35.8	1.95,1.86, m	CH_2
7	177.5	-	C
1′	166.5	-	C
2′	116.3	7.65, d, *J*=12.4	CH
3′	145.2	7.50, d, *J*=14.2	CH
4′	129.2	-	C
5′	113.6	6.69, m	CH
6′	147.2	-	C
7′	146.5	-	C
8′	117.2	6.50, m	CH
9′	120.4	6.65, m	CH

3.2.2.3. Structure elucidation of Compound (3)

Screlotiumol (**3**) was isolated as a yellow solid from EtOAc fraction of *S. rolfsii*. Screlotiumol was identified as $C_{12}H_{16}0_5$ (M.p=133-136 ^{0}C). The UV spectrum of compound **3** showed absorption peak at 312 nm. IR (KBr, Cm^{-1}) showed absorption peaks at 3355-3650 for OH stretching, 2988 CH saturated stretching, 1650 C=O stretching. ^{1}H-NMR (400 MHz, MeOD) δ_H: 6.79 (H-2, s), 2.65, 1.99 (2H-4, m), 2.30, 1.99 (H-5, m: 2H), 3.6, (H-6, m), 4.84 (H-8, s), 1.41, 1.39 (H-11, m, 2H), 1.67, 1.66 (H-12, m, 2H), 4.20 (H-13, d, *j*=2.3) respectively. ^{13}C NMR; ($CDCl_3$, 150 MHz) δ_c: 187.2 (C-1), 108.2 (C-2), 199.9 (C-3), 38.5 (C-4), 38.6 (C-5), 66.9 (C-6), 88.5 (C-8), 136.6 (C-9), 162.2 (C-10), 25.9 (C-11), 33.0 (C-12) and 100.9 (C-13) respectively (**Table 3.9**). On the basis of the advanced spectral analysis, the chemical structure of a new compound **3** was identified as (**1**3-(3,3-dihydroxypropyl)-1,6-dihydroxy-3,4-dihydro-1H-isochromen-8(5H)-one(1). Furthermore the structure of compound **1** was confirmed by HBMC correlations.

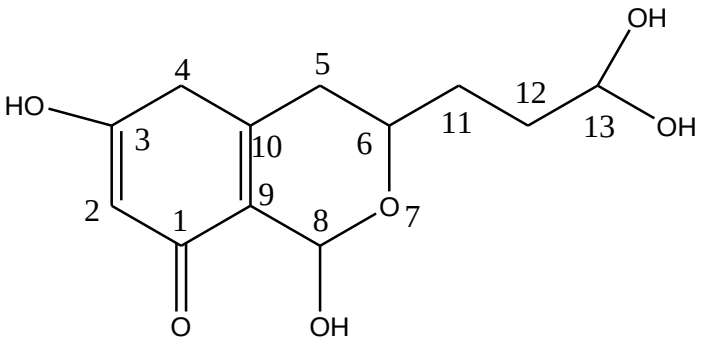


Figure 3.10: Structure of Screlotiumol (**3**)

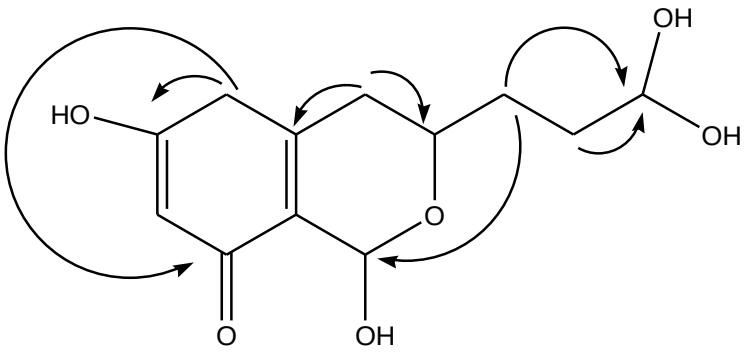


Figure 3.11: Key HBMC correlation of screlotiumol (**1**)

Table 3.9: ^{1}H and ^{13}C-NMR chemical shift values of screlotiumol (**3**)

Carbon No.	δ_C	δ_H (mult, J, HZ)	Multiplicity	HMBC
1	187.2		C	-
2	108.2	6.79, s	CH	-
3	199.9	-	C	-
4	38.5	2.65, 1.99, m	CH2	C-1, C-3
5	38.6	2.30, 1.99 m	CH2	C-6, C-10
6	66.9	3.6, m	CH	-
7	-	-		-
8	88.5	4.84, s	CH	-
9	136.6	-	C	-
10	162.2	-	C	-
11	25.9	1.41,1.39, m	CH2	C-13
12	33.0	1.67, 1.66, m	CH2	C-13
13	100.9	4.20, d (*j*=2.3)	CH	-

3.2.2.4. Structure elucidation of Compound (4)

O-Cumaric acid (**4**) was isolated as a white powder from EtOAc fraction. It's exhibited EI-MS peak at 164.58 corresponding to $C_9H_8O_3$. ^{1}HNMR showed resonating peak was observed at $\delta_{H:}$ 6.99 (H-1, d, *j*=8.7), 6.88 (H-2, d, *j*=7.5), 6.90 (H-3, d, *j*=8.7), 6.67 (H-4, d, *j*=7.7), 2.81, 2.51 (H-7, 2H, d, *j*=6.3), 2.56, 2.31 (H-8, 2H, d, *j*=6.3) respectively. ^{13}C NMR showed signals at δ_c: 129.1 (C-1), 122.3 (C-2), 127.1 (C-3), 115.9 (C-4), 157.5 (C-5), 127.5 (C-6), 26.5 (C-7), 37.2 (C-8), 117.2 (C-9) respectively (**Table 3.10**). The structure of compound **4** was confirmed by comparing their physical and spectra data with reported one [285].

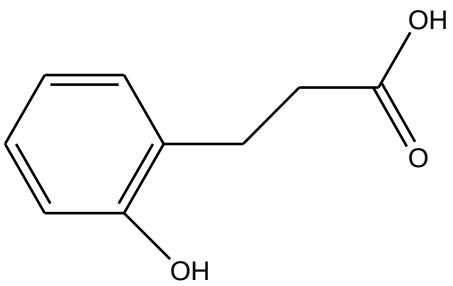


Figure 3.12: Structure of *O*-Cumaric acid (**4**)

Table 3.10: ^{1}H and ^{13}C-NMR chemical shift values of *O*-Cumaric acid

Carbon No.	δ_C	δ_H (mult, J, HZ)	Multiplicity
1	129.1	6.99, d, *j*=8.7	CH
2	122.3	6.88, d, *j*=7.5	CH
3	127.1	6.90, d, *j*=8.7	CH
4	115.9	6.67, d, *j*=7.7	CH
5	157.5	-	C
6	127.5	-	C
7	26.5	2.81, 2.51, d, *j*=6.3	CH2
8	37.2	2.56, 2.31, d, *j*=6.3	CH2
9	117.6		C

3.2.2.5. Structure elucidation of Compound (5)

Gallic aid (**5**) was isolated as a needle like crystals from EtOAc fraction. Its exhibited EI-MS peak at 170.01 corresponding to $C_7H_6O_5$. The UV spectrum of this compound showed absorption peak at 220 and 270 nm. The IR spectra reviled a broad peak at 39497 showed the presence of OH stretching, 1666 C=O stretching 1610 cm^{-1} showed presence of aromatic system. ^{1}H-NMR showed resonating peak was observed at 7.05 (H-2, s), 7.05 (H-6, s). C^{13} NMR showed signals at 121.7 (C-1), 116.1 (C-2), 146.1 (C-3), 139. 3 (C-4), 146.1 (C-5), 110.1 (C-6), 171.1 (C-7) respectively (**Table 3.11**). The structure of compound **5** was confirmed by comparing their physical and spectra data with reported one [286].

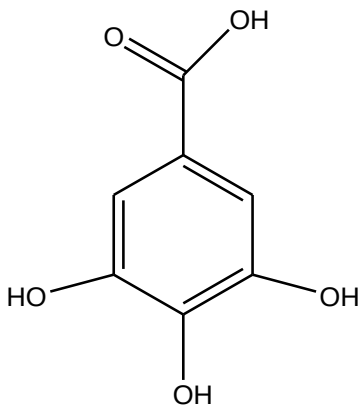


Figure 3.13: Structure of Gallic aid (**5**)

Table 3.11: ^{1}H and ^{13}C-NMR chemical shift values of Gallic acid

Carbon No.	δ_C	δ_H (mult, *J*, HZ)	Multiplicity
1	121.7	-	C
2	116.1	7.05, s	CH
3	146.1	-	C
4	139.3	-	C
5	146.1	-	C
6	110.1	-	C
7	171.1	7.05, s	CH

3.2.3. *In Vitro* Biological screening

3.2.3.1. Antifungal assay

The secondary products have a wide range of biological activities and may act as antitumorigenic, insecticidal, antibiotics, hormones, toxins, and anti-migraine agents [287]. The EtOAc and *n*-hexane fractions were screened for antifungal activities against different pathogenic fungi (**Table 3.12** and **Fig 3.14**). The EtOAc fractions were active against *A. solani* (% inhibition, 73±2.0), while moderate activity was observed against *P. notatum* and *A. alternatum* species (% inhibition, 42.68±2.08 and 50.65±0.58, respectively). No activity was observed against *T. harzianum* and *V. chlamydosporium*. All fungal species showed resistance to the *n-hexane* fraction. It indicates that the fungus *S. rolfsii* has the potential to inhibit the growth of *A. solani* to some extent, while other tested fungal species showed resistance to secondary metabolites, which may be due to the limited number of antifungal compounds produced by *S. rolfsii*. The microorganisms present in the soil compete for the available nutrients. Antagonism exhibited by microorganisms might be through the production of antifungal or antibacterial compounds or more simply due to the competition for nutrients. Competition for nutrients can lead to a great reduction of specific fungal biomass in the presence of the competing fungi and vice versa [288].

Table 3.12: Antifungal activity of EtOAc and *n*-hexane fraction of *S. rolfsii* against different fungal strains.

S.NO	Test fungi	**Percent inhibition**	
		EtOAc	*n*-hexane
1	*Penicillium notatum*	42.68±2.08	0±0
2	*Aspergillus fumigatus*	0	0±0
3	*Verticillium chlamydosporium*	0	0±0
4	*Acremonium alternatum*	50.65±0.58	0±0
5	*Alternaria solani*	73±2.0	0±0

Positive control, Miconazole at the concentration of 110 µg/mL

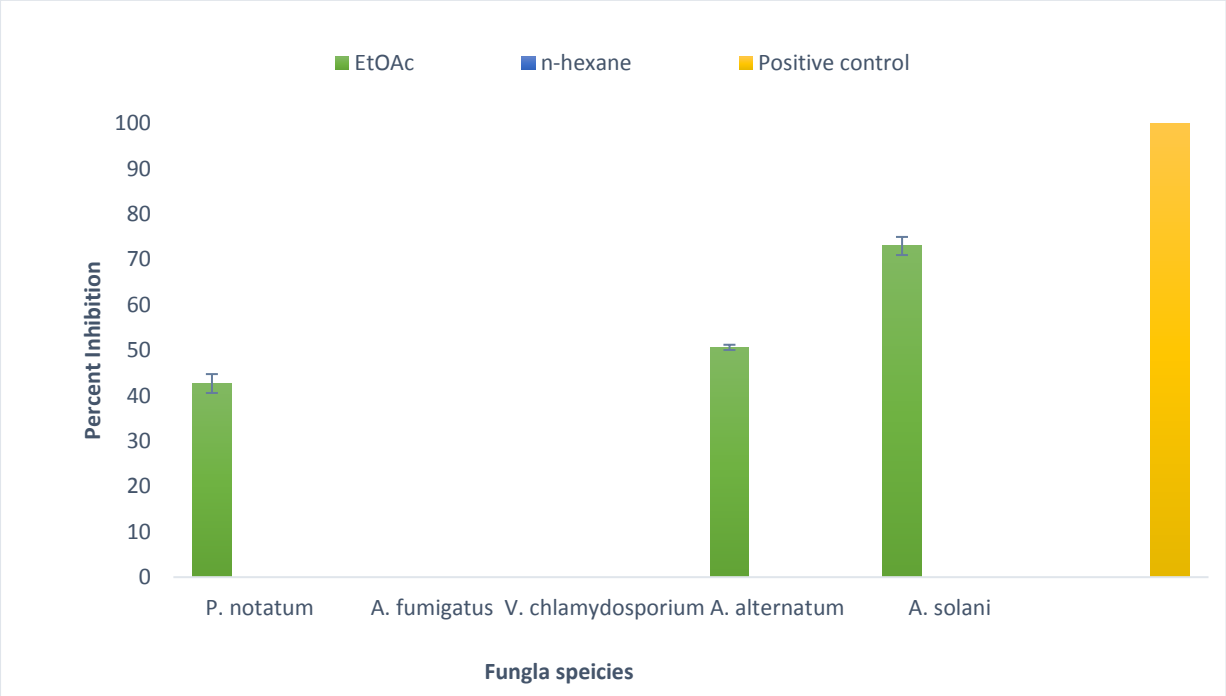


Figure 3.14: Antifungal activity of EtOAc and *n*-hexane fraction of *S. rolfsii* against different fungal strains.

Positive control = Miconazole at the concentration of 110 µg/mL

3.2.3.1. Phytotoxic assay

The phytotoxic activity exhibited by the secondary metabolites was determined against *L. minor* plants. The EtOAc and *n*- hexane fractions of the secondary metabolites were screened for phytotoxic activity. At a higher concentration (1000 µg/mL) of EtOAc fraction, 82% mortality was recorded. Low activities of 33.33% and 11.11% were observed for 500 and 100 µg/mL of the fractions, respectively. No activity was observed at a low concentration of 10 µg/mL of the EtOAc fraction. Similarly moderate activity (43.8%) was shown by the *n*-hexane fraction at a higher dose (1000 µg/mL), while at 500 µg/mL of n-hexane fraction a low activity of 11.11% was recorded. However, at still lower concentrations of 100 and 10 µg/mL no activity was observed. The results of this study are presented in **Table 3.13** and **Fig 3.15.**

Herbicides play significant role in controlling weeds because they assist the farmers in increasing the crop yields with minimal labor. But the extensive application of these herbicides can lead to environmental and health problems. Plant pathogens, especially fungi, have the ability to cause a disease in their respective hosts by the production of phytotoxic secondary metabolites known as phytotoxins [289]. *S. rolfsii* also has the ability to produce oxalate which works in a synergistic fashion to attack and destroy different plant tissues [168]. By applying Biotechnological tools, phytopathogenic fungi can be transformed into potential producers of phytotoxins. The results obtained in this study indicate that *S. rolfsii* possesses herbicidal potentials.

Table 3.13: Percent growth regulation of the *Lemna minor*

Concentration of sample (µg/mL)	**Total number of fronds**	**Percent growth regulation**		
		EtOAc	*n*-hexane	Positive control*
10	18	0	0	100
100	18	11.11	0	
500	18	33.33	11.11	
1000	18	82	43.8	

Positive control*= Paraquat at a concentration of 0.015 µg/mL

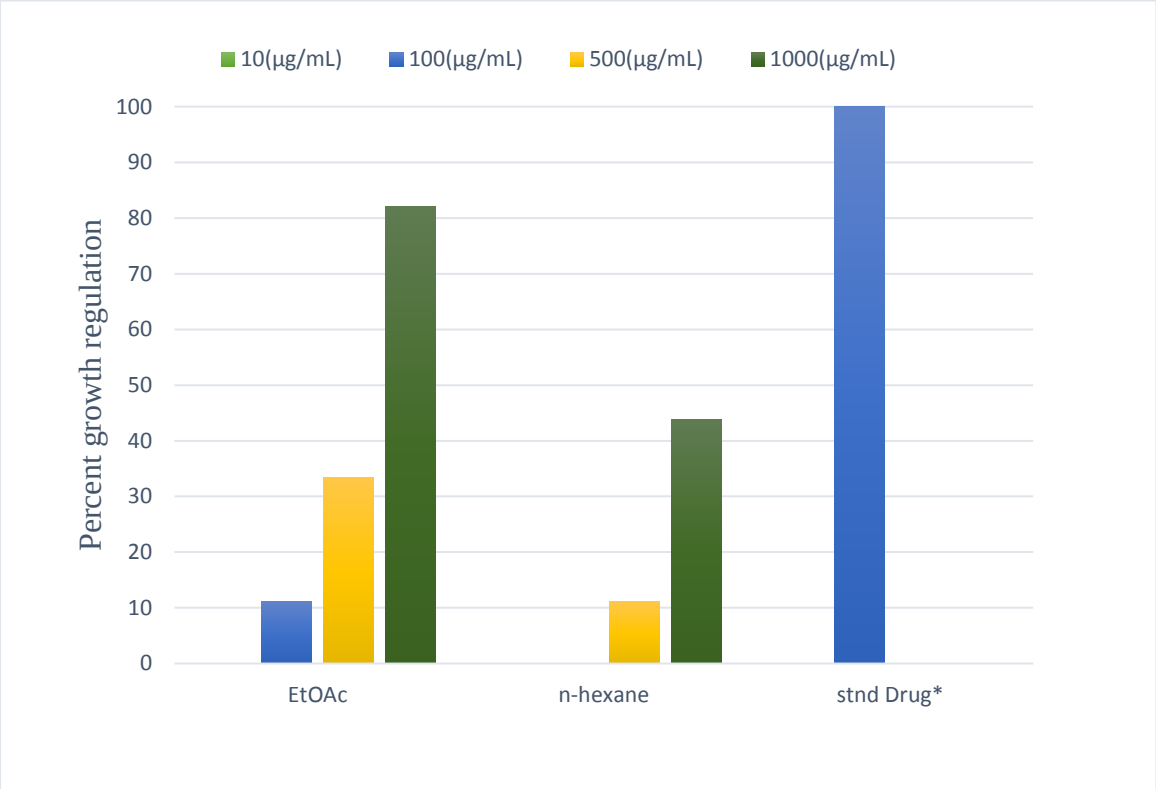


Figure 3.15: Phytotoxic activity of EtOAc and *n*- hexane fractions against *L. minor* plant

3.2.3.3. Insecticidal assay

The insecticidal tests were performed against *C. analis*, *T. castaneum*, and *F. auricularia*, and the results are presented in **Table 3.14** and **Fig 3.16 and 3.17**. The insecticidal activity was determined at different concentrations, viz., 10, 100, and 1000 µg/mL of the EtOAc fraction. The results revealed that the EtOAc fraction at higher concentration (1000 µg/mL) showed significant activity against *C. analis* (80% mortality), *T. castaneum* (100% mortality), and *F. auricularia* (100% mortality). At 100 µg/mL, the mortality rate of the EtOAc fraction decreased a little: *C. analis*, 50%; *T. castaneum*, 80%, and *F. auricularia, 100*%. Similarly at a low concentration, 10 µg/mL of EtOAc fraction showed a mortality percentage of 0% for *C. analis*, 10% for *T. castaneum*, and 40% for *F. auricularia*. The *n*-hexane fraction also showed considerable activity at a higher concentration (1000 µg/mL) with 60% against *C. analis* 70% against *T. castaneum*, and 50% against *F. auricularia*. At a moderate concentration of 100 µg/mL of the *n*-hexane fraction, low mortality rates were observed: *C. analis* 20%, *T. castaneum* 30%, and *F. auricularia* 10%. As expected, the *n*-hexane fraction was inactive against test insects at very low concentration (10 µg/mL). The fungi are well-known for their antagonistic interactions with other organisms co-occurring in the fungal habitat, especially in a decomposing system. The fungi are always engaged in competition with other organisms [290]. *S. rolfsii* is a pathogenic fungus. It is worth noting that pathogenic fungi exert a devastating effect over arthropods [291]. The secondary metabolites have immunosuppressive and toxic functionalities which offer fungi a selective advantage over the animal hosts to overcome the cellular and humoral defense systems [292].

Table 3.14: Insecticidal activity of EtOAc and *n*-hexane fraction of secondary metabolites *S. rolfsii*

% Mortality							
Name of insects	EtOAc			*n*-hexane			Permetherin (STD)
	Concentration (µg/mL)						
	10	100	1000	10	100	1000	
Callosbruchus analis	0	50	80	0	20	60	
Tribolium castaneum	10	80	100	0	30	70	100
Forficula auricularia	40	100	100	0	10	50	

Positive control: Permethrin (239.50 µg/cm^2)

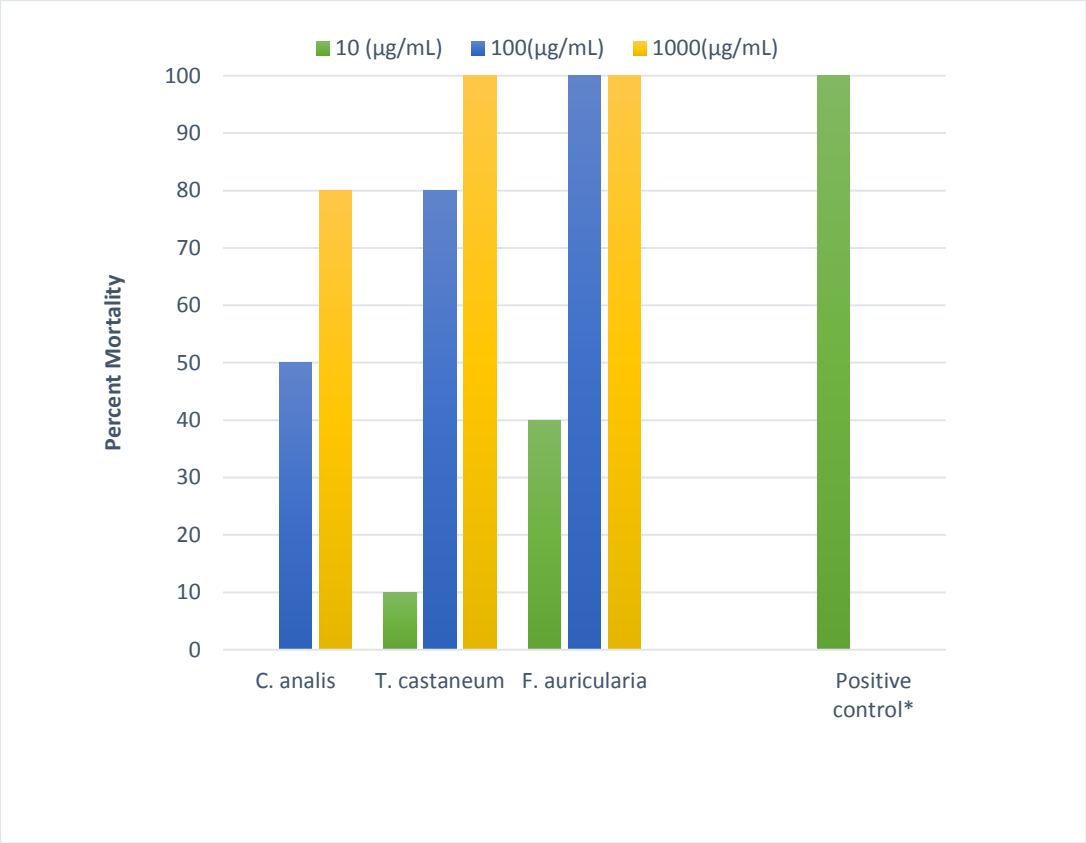


Figure 3.16: % Mortality of EtOAc fraction of secondary metabolites against different insects

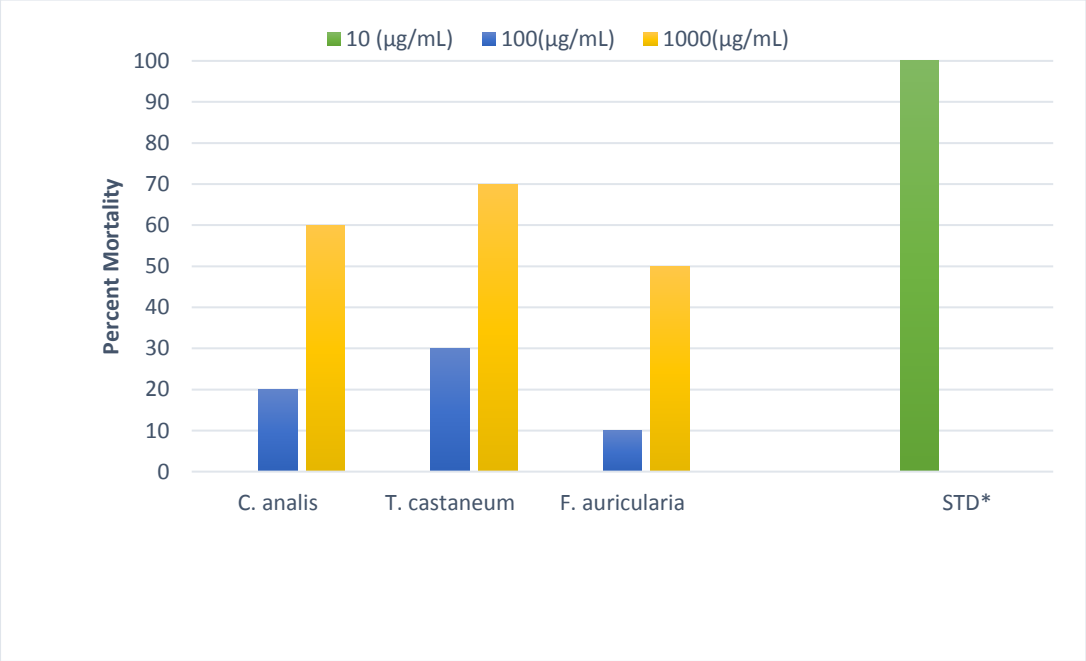


Figure 3.17: % Mortality of *n*-hexane fraction of secondary metabolites against different insects

3.2.3.4.Brine shrimp lethality assay (BSLA):

Brine shrimp lethality assay is an easy, rapid, and inexpensive method and has successfully been used to determine the cytotoxic effect of bioactive metabolites. This assay has been established as a safe, practical, and economical method for the evaluation of the bioactive properties of mycotoxins produced by fungal pathogens, synthetic compounds, marine products as well as products of higher plants [293-297]. The cytotoxic properties of the *n*-hexane and EtOAc fractions of secondary metabolites were determined, and the results are presented in **Table 3.15** and **Fig 3.18**. The mortality of shrimps was tested at different concentrations, viz., 10, 50, 100, 500, and 1000 µg/mL. It was observed that the EtOAc fraction showed significant activity at high concentrations (1000µg/mL) with a 100% mortality, while 500 µg/mL also showed a significant activity (85%). A concentration of 100 µg/mL showed moderate activity (55%), whereas 50 and 10 µg/mL showed lower activities as 48 and 25%, respectively. Similarly, the *n*-hexane fraction also showed moderate activity at higher concentrations, viz., 1000 and 500 µg/mL, with 64% and 45% mortality, respectively, while low concentrations of 100, 50, and 10 µg/mL exhibited 25, 5, and 0% cytotoxicity respectively. The results clearly show that the EtOAc fraction possesses significant bioactivity against the shrimps. The bioactive properties of natural products have been attributed to the presence of metabolites like phenolics, tannins, flavonoids, and alkaloids [298]. Fungi have been regarded as a potential source of medicinally important products such as antitumorigenic agents [299, 300]. The results observed in this study might be due to the presence of high amounts of phenolic and flavonoid compounds.

Table 3.15: Percent cytotoxicity of EtOAc and *n*-hexane fractions against brine shrimps cells

	EtOAc Fraction			***n*-hexane fraction**		**Positive control**
Concentration (µg/mL)	Total No of shrimps	No of Dead shrimps	% cytotoxicity	Number of Dead shrimps	% cytotoxicity	% cytotoxicity
10	40	10	25	0	0	
50	40	19	48	2	5	
100	40	22	55	10	25	
500	40	34	85	18	45	
1000	40	40	100	27	64.5	100

Positive control*= Etoposide at concentration of 7.4625 µg/mL

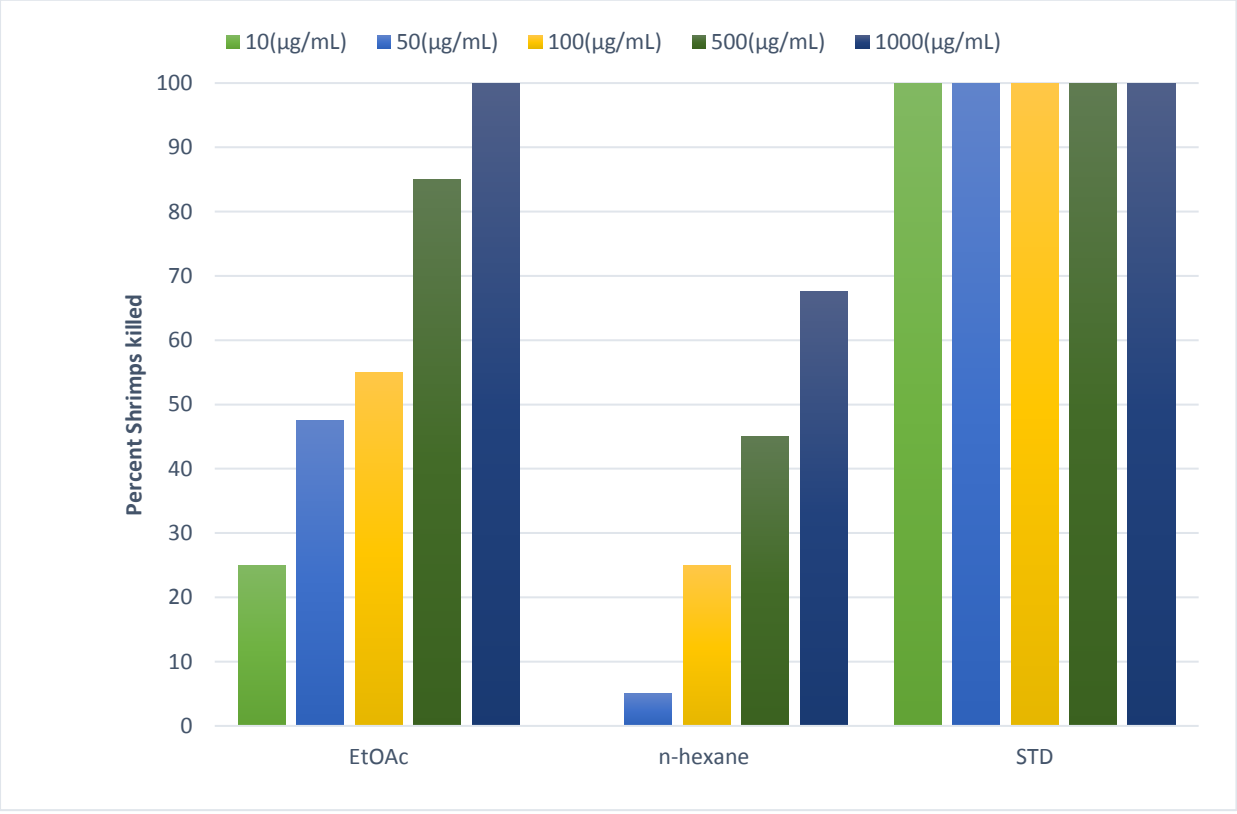


Figure 3.18: Cytotoxicity of EtOAc and *n*-hexane fraction of *S. rolfsii* against Brine shrimps

3.2.3.5.Enzyme inhibition assay

3.2.3.5.1. Carbonic anhydrase assay

The zinc metallo-enzymes, the carbonic anhydrases (CAs, EC 4.2.1.1) are present in a variety of organisms [301, 302]. Many biological and physiological processes, such as homeostasis of pH, the transportation of carbon dioxide from lungs to the metabolizing tissue, are catalyzed by these enzymes [303, 304]. The overexpression of the enzyme, such as urease, and carbonic anhydrase, lead to a number of disease. These metabolites are used in different industry, such as food, agricultural and pharmaceuticals. The secondary metabolites serve as a shield for fungi [305]. Fungi are one of the most important members of the soil microbial community, typically possessing more soil biomass than do bacteria. The inhibition of CA isozymes by aromatic/heterocyclic sulfonamides has been used clinically for the treatment of a variety of diseases, such as mountain sickness, glaucoma, congestive heart failure, gastric duodenal ulcers epilepsy, etc. [306]. *S. rolfsii* was evaluated for its ability to inhibit the enzyme, carbonic anhydrase. *S. rolfsii* exhibits a strange property against carbonic anhydrase. Both fractions, ethyl acetate and *n*-hexane, were active against CA (58 and 62.5% at 0.2 mg/mL, respectively) with $\mathbf{IC_{50}}$ values of (45.40 ± 0.75 and 52.77 ± 0.81 µg/mL respectively) (**Table 3.16 and Fig 3.19**). The secondary metabolites, i.e., oxalate, malonate, maleate, malate, pyruvate, lactate, citrate, and acetate of the pathogenic fungi, *Candida albicans* and *Cryptococcus neoformans,* showed inhibitory activity against carbonic anhydrase. [307]. *S. rolfsii* also produces oxalate which work in a synergistic fashion to attack and destroy different plant tissues [168].

3.2.3.6.2. Urease inhibition assay

The enzyme, urease (urea amidohydrolase) is also found in different organism such as fungi, algae, bacteria, and plants. Urease catalyzes the conversion of urea to ammonia and

carbamate during nitrogen metabolism in living organisms [308]. *S. rolfsii* was also evaluated for the inhibition of urease enzyme. Both the fraction showed insignificant results against the urease enzyme. EtOAc and *n*-hexane fraction showed 6.58% and 22.58% inhibition, respectively (**Table 3.16 and Fig 3.19**). The phenolic compounds were investigated against various enzymes, *in vitro*. Among these enzymes, phenolic compounds were active against urease [309]. In the present research, both the fractions of fungi did not show any significant activity against urease. This might be due to the absence of large number of phenolic compound that could induce an activity.

Table 3.16: Carbonic anhydrase and Urease inhibition by the EtOAc and *n*-hexane faction of secondary metabolites of *S. rolfsii*

S. NO	**Enzyme**	**EtOAc fraction**		***n*-hexane fraction**	
1	Carbonic anhydrase	% inhibition	IC_{50} [µM]	% inhibition	IC_{50} [µg/mL]
		58	45.40 ± 0.75	62.5	52.77 ± 0.81
2	Urease	6.58	-	22.58	-

* Acetazolamide was used as a standard inhibitor for Carbonic anhydrase inhibition
* For Urease inhibition, Thiourea was used as a standard inhibitor

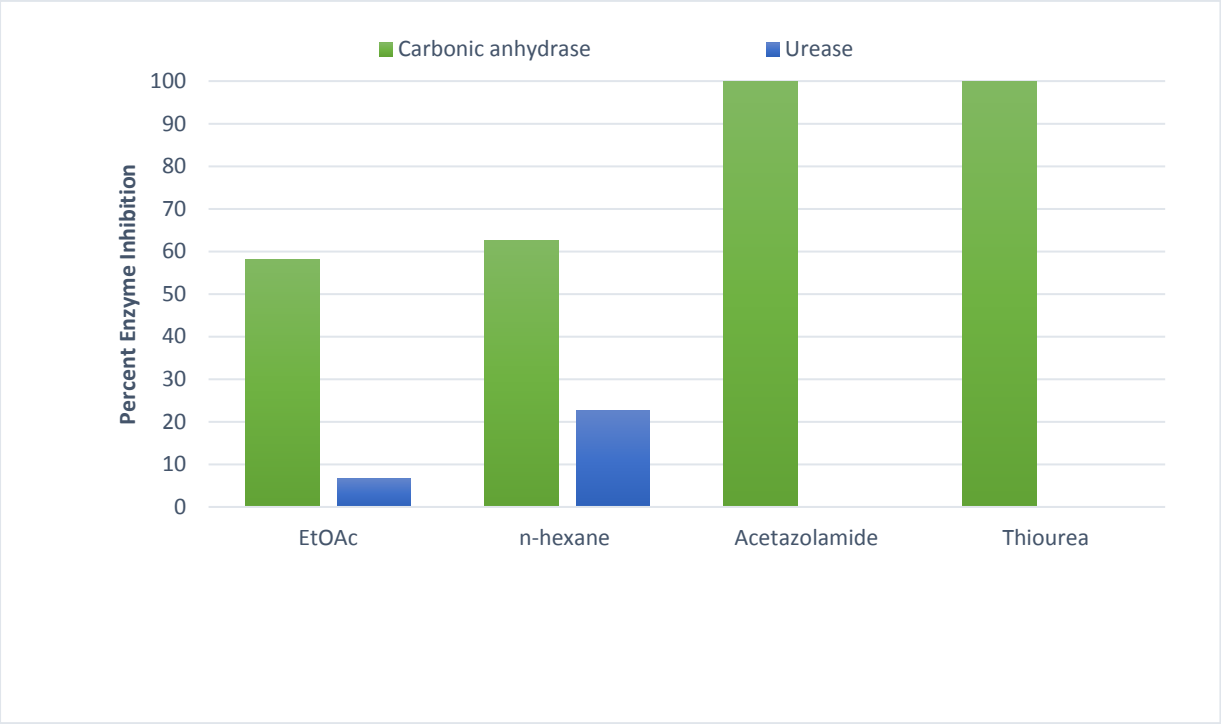


Figure 3.19: Percent enzyme inhibition of EtOAc and *n*-hexane fraction of *S. rolfsii*

3.2.4. *In-vivo* biological activities of crude EtOAc extract of *S. rolfsii*

3.2.4.1. Acute toxicity

Acute toxicity studies are usually carried out to describe the simple toxicity of secondary metabolites. The purpose of determination of acute toxicity is to evaluate the vulnerable species, detect target organs, risk valuation so that in case of acute exposure to the chemical, the required information to design and determine the dosages of suitable antidote is available [310]. The current investigation shows that, during and after the experiment, all animals were alive, so the extract has no high (++++), medium (+++) and moderate (++) toxicity. (**Table 3.17**). At the end of the experiment, blood samples were collected to determine the different parameters to evaluate any toxicity. The results show that there is a slight increase in the percent granulocytes (49.6%) from the normal range (8.6-38.9 %). The values for red blood cells (RBCs), hemoglobins (HGB), and Haematocrit (HCT) were slightly decreased from its normal range as given in the **Table 3.18**. The common clinical signs of toxicity of the extract in chicks and mice were anorexia, ataxia, dyspnoea, tachycardia, diarrhea, somnolence, and tachypnea [310, 311]. In simple words, the extract falls in the category of being mildly toxic.

Table 3.17: Acute toxicity of crude EtOAc extract of *S. rolfsii*

Treatment (crude EtOAc) (mL or mg/kg)	No. of Animal alive after 4hrs	No. of Animal alive after 24hrs	% Death after 4hrs	% Death after 24hrs
Normal saline	All	All	-	-
10			-	-
20			-	-
30			-	-
40			-	-
50			-	-

Table 3.18: Different parameters values after termination of experiment

S.No	Parameters	Before experiment	After experiment	Normal range
1	WBC	1.2×10^9/L	2.9×10^9/L	$0.8\text{-}6.8 \times 10^9$/L
2	LYM%	70%	77%	55.8-90.6 %
3	MID%	8%	5%	1.8 – 6 %
4	GRAN%	41%	49.6%	8.6-38.9 %
5	LYM#	0.5×10^9/L	0.2×10^9/L	$0.7\text{-}5.7 \times10^9$/L
6	MID#	Minimal infecting dose	1.2×10^9/L	$0.0\text{-}0.3 \times10^9$/L
7	GRAN#	1.2×10^9/L	1.5×10^9/L	$0.1\text{-}1.8 \times10^9$/L
8	RBC	7.3×10^{12}/L	2.72×10^{12}/L	$6.36\text{-}9.42 \times10^{12}$/L
9	HGB	9.0 g/dL	4.6 g/dL	11.0-14.3 g/Dl
10	HCT	30.2%	14.2%	34.6-44.6 %
12	MCH	17.5 pg	16.9 pg	15.8-19.0 pg
16	PLT	225×10^9/L	339×10^9/L	$100\text{-}600 \times10^9$/L
17	MPV	8.0 fL	13.7 fL	5.5-7.5 fL

3.2.4.2. Analgesic activity of crude EtOAc extract of *S. rolfsii*

When crude EtOAc extract at different doses was administered through the intraperitoneal at dosages of 50, 100, and 150 mg/kg body weight showed, a reduction in the mean number of writhing was observed in different test groups as given in **Table 3.19** and **Fig 3.20**. For Group I which was administered normal saline, the mean writhing was 45.22±1.166. Different test doses of crude EtOAc extract produced different percentage of writhing inhibitory effect, viz., 13.72 % (50 mg/kg), 33.26 % (100 mg/kg), and 45.06 % (150 mg/kg). The results showed that the effect produced by crude EtOAc extract was dose dependent. Diclofenac sodium (Positive control) at 10 mg dose served as the positive control and produced an inhibition of 53.18%, which is larger than the highest dose of crude EtOAc extract (150 mg/kg b.w.). The results showed that the crude EtOAc extract possess analgesic effect. Acetic acid induced pain model is a quick, sensitive, common, and simple method to determine analgesic effect of crude extract, pure compounds, and drugs [312]. The increase in the sensitization of peritoneal receptors (nociceptive) to prostaglandins during analgesic effect was investigated. During this process, the synthesis of prostanoids like PGE2 and PGF2α and lipoxygenase derivatives increases in the peritoneal fluids and serve as pain mediators [313]. It was also reported that these substances are produced by the cyclo-oxygenase (COX) pathway [314]. In the peritoneal fluid, these biochemicals are responsible for pain and appear in the form of abdominal constrictions. Apart from this, various agents are involved in the reduction of production of prostanoids, which is considered for pain inhibition through peripheral mechanism [45]. The crude EtOAc extract of the *S. rolfsii* was evaluated

for some active chemical constituents that have analgesic effects in the form of reduction in abdominal constriction (writhing).

Table 3.19: Analgesic activity of the crude EtOAc extract obtained from *S. rolfsii*

S. No.	Treatment	Dose (ml or mg/kg)	No. of writhing (10 min) (Mean+ SEM)	% inhibition of writhing
1	Saline	10	45.22 ± 1.166	-
2	Crude Ethyl acetate	150	24.83 ± 0.88***	45.06
		100	30.166 ± 1.22***	33.26
		50	39 ± 1.333***	13.72
3	STD (Diclofenac sodium)	10	21.16 ± 1.166***	53.18

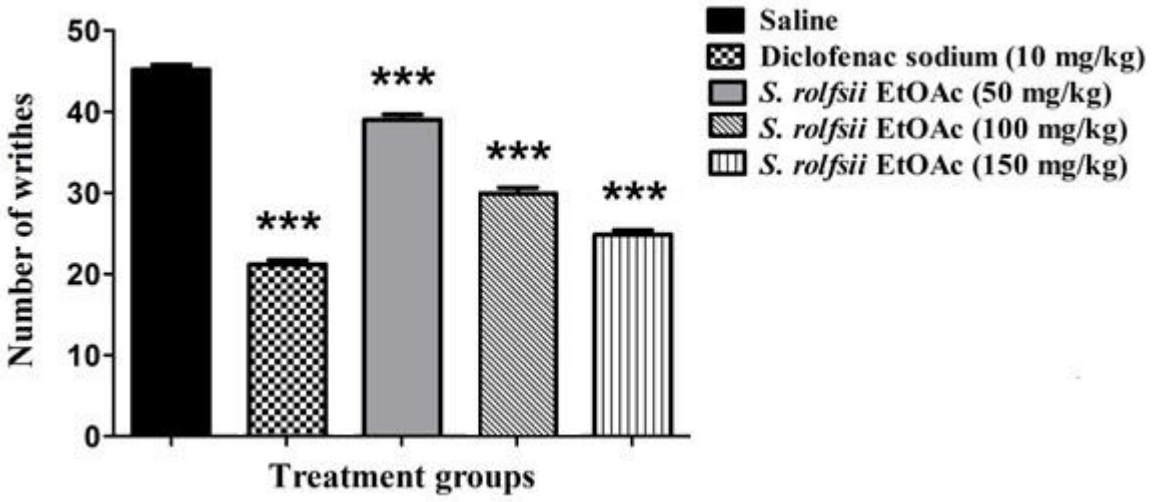


Figure 3.20: Antinociceptive activity of *S. rolfsii* crude extract in the acetic acid induced abdominal constriction assay.

Each bar represents mean number of writhes ± SEM.

One way ANOVA followed by Dunnett's *post hoc* test.

***$P < 0.001$ compared to saline treated group. $n = 6$ animals per group.

3.2.4.3. Sedative activity of crude EtOAc extract of *S. rolfsii*

Crude EtOAc extract of *S. rolfsii* were used for the determination of the sedative effect. The results indicated that *S. rolfsii* can cause significant sedative effect in an open field. The number of crossed line after 30 min is given in the **Table 3.20** and **Fig 3.21.** In these activities, any agent with sedative properties will cause a decrease in the number of movements [315]. An increase in the dose also increases the sedative effect pointing to the fact that the effects were dose dependent. The dose at 50, 100, and 150 mL or mg/kg showed 112.5±2.167, 97.5±1.33, and 89.5±1.66, respectively, of the movements. This property of the metabolites of *S. rolfsii* to suppress the locomotor activity suggests that the extract possess a central nervous system (CNS) depressant activity. The fungi serve as a main source of bioactive secondary metabolite, cyclosporin A, that has an immunosuppressive effect that is used clinically after a transplant surgery [316, 317].

Table 3.20: Sedative activity of crude EtOAc extract obtained from *S. rolfsii*

S. No.	Treatment	Dose (ml or mg/kg)	No of lines crossed
1	Saline	10	129.833 ± 2.22
2	Crude EtOAc	50	112.5 ± 2.167***
		100	97.5 ± 1.33***
		150	89.5 ± 1.66***
3	Diazepam	0.5	6 ± 0.66***

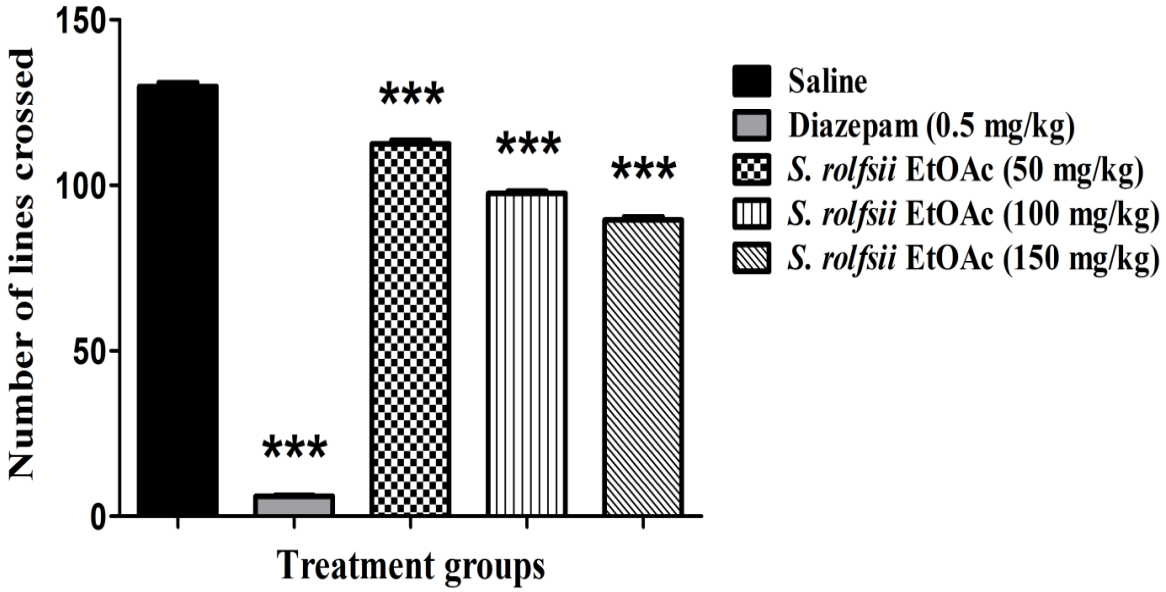


Figure 3.21: Sedative activity of *S. rolfsii* crude extract in the locomotor test (open field).

Each bar represents mean number of lines crossed ± SEM.

One way ANOVA followed by Dunnett's *post hoc* test.

*****P* < 0.001 compared to saline treated group. *n* = 6 animals per group.

3.3.1. Optimization of growth parameters for production of maximum bioactive secondary metabolites by *A. flavus*

3.3.1.1.Optimization of nutrient media

The growth of *A. flavus* was tested using five different media composition (PDB, SDB, CYB, MEB, and NB) for the optimum production of crude secondary metabolites. The results of this study are given in the **Table 3.21** and **Fig 3.22.** Among these media, PDB produced the highest biomass (552.33±4.041mg/100mL) as well as secondary metabolites (425.3 ±4.16 µg/100mL) followed by CYB (490.67±2.08 mg/100 mL and 389.7±4.73 µg/100 mL). The lowest growth and metabolite production were observed for NB (111±3.61 mg/100 mL and 132±7.211 µg/ 100 mL, respectively**).** The SDB and the MEB media supported moderate growth (328.3±3.15 and 231±7.55 mg/100mL, respectively) and metabolites production (441.67±2.887 and 385±5 µg/100mL**).** The study of Mathan et al. on the optimization of growth parameters of *Aspergillus* spp. also correlated with the data obtained in the present study. Among the media tested, maximum mycelial dry weight (74 mg/25 mL) was recorded for PDB [318]. Rabbani et al. reported that the PDB medium is the best medium for the optimum growth of *Drechslera hawaiiensis* [319].

Table 3.20: Comparison of different media for biomass and crude metabolites production

S.No	Media	Biomass (mg/100mL)	Crude metabolites (µg/100mL)
1	PDB	425.3±4.16	552.33±4.041
2	SDB	328.3±3.15	441.67±2.887
3	CYB	389.7±4.73	490.67±2.08
4	MEB	231±7.55	385±5
5	NB	111±3.61	132±7.211

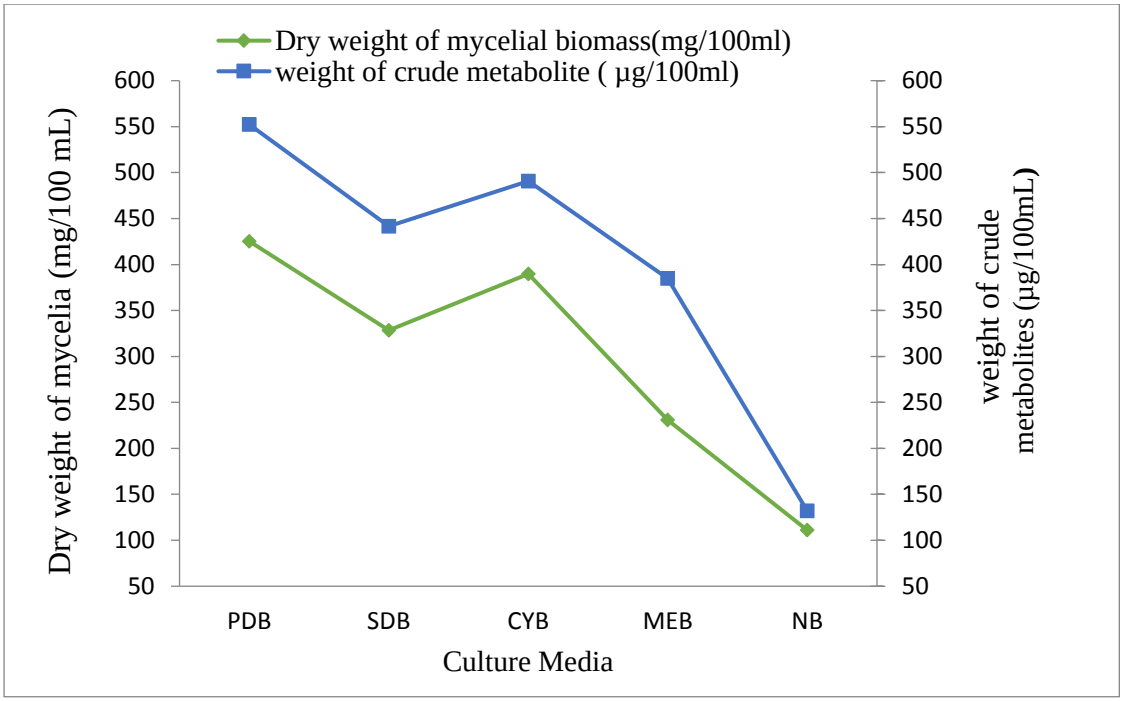


Fig 3.22: Comparison of different media for biomass and crude metabolites production

3.3.1.1.1. Determination of anti-bacterial activity of crude metabolites obtained from each medium

The crude metabolites extracted from each medium were checked against Gram-positive (*S. aureus* and *S. saprophyticus)* and Gram-negative bacteria (*S. typhi, S. marcescens, S. para typhi, Proteus mirabilis, K. pneumoniae,* and *E. coli).* Among the media studied, the crude metabolite extracted from PDB was more active against the tested bacterial pathogen as compared to those obtained in other media (**Table 3.22**). The metabolites obtained from PDB significantly inhibited the growth of *S. typhi* (20±1mm), *S. marcescens* (23±2mm), *S. para typhi* (20.3±2.8 mm), and *Proteus mirabilis* (24±1mm), while other bacterial species such as *P. aeruginosa* (17.33±0.58mm), *S. aureus* (13.7±1.5mm), *S. saprophyticus* (12±2mm), *K. pneumoniae* (16.3±2.8mm), and *E. coli* (18.7±1.3 mm) were inhibited to a moderate extent. The *n*-hexane fraction of the metabolites from PDB was moderately active against *S. marcescens* (13.7±1.3mm) and *K. pneumoniae* (17.33±1.53 mm), while it showed no activity

against other tested pathogens. The EtOAc fraction of crude metabolite obtained from CYB was active against *S. marcescens and S. para typhi* **(**20.33±1.5 and 20.33±2.1 mm, respectively), a moderate active was observed for this fraction against the remaining bacterial pathogens**.** The *n*-hexane fraction was moderately active against most the pathogens studied, but was inactive against *S. para typhi* and *P. mirabilis***.** The EtOAc fraction of SDB was moderately active against *P. aeruginosa, S. typhi, S. marcescens, S. saprophyticus,* and *K. pneumoniae* **(**17±0.58, 17±1.53, 17±2.52, 11±1, 17±2.52 mm respectively), it showed no activity against *S. aureus, S. para typhi,* and *E. coli (*no zone of inhibition*)*. Hence, it can concluded, that the *n*- hexane fraction was more active against *P. mirabilis* **(**23±1.7mm), moderately active against *S. marcescens* **(**15.3±1.5mm), and inactive against *P. aeruginosa, S. typhi, S. aureus, S. saprophyticus, S. para typhi, K. pneumoniae* and *E. coli*. The EtOAc fraction extracted from MEB was active against *P. aeruginosa and K. pneumoniae (*20±0.58 and 22±1.173mm), while it showed moderate activity against *S. typhi, S. marcescens, S. saprophyticus, S. para typhi,* and *E. coli (*zone of inhibition < 20 mm) and no activity against *S. aureus and P. mirabilis*. The *n*-hexane fraction showed moderate activity against *S. marcescens* (18.7±1.5mm) and *P. mirabilis* **(**19.7±3.1 mm) but no activity against all other pathogens. The EtOAc fraction of NB was only active against *P. mirabilis* **(**20±1mm), moderately active against *P. aeruginosa* (14±1.53mm)**,** *S. marcescens* (19±2mm)**,** and *K. pneumoniae* (11±1.53mm), and inactive against *S. typhi, S. aureus, S. saprophyticus, S. para typhi* and *E. coli.* The *n*- hexane fraction showed moderate activity against *S. aureus* (11.3±1.5mm) and *S. para typhi* **(**15.3±1.5 mm), the remaining test pathogens showed resistance to the *n*-hexane fraction. The Gram-positive and Gram-negative bacteria cause a large number of diseases ranging from simple to complex ones. Among the Gram positive bacteria, *S. aureus* is one of the most important pathogens and a leading causative organism of nosocomial infection. It infects and destroys normal healthy tissue, causing wound and skin

infections, osteomyelitis, pneumonia, lung abscess, endocarditis, and pyomyositis [320, 321]. In Pakistan, *S. aureus* is emerging as a multidrug resistance bacterium. Also *S. aureus* serves the main source of nosocomial infections. It was observed that the crude metabolite produced only in PDB was active against *S. aureus,* while that of the CYB showed moderate activity against *S. aureus.* The virulence or pathogenicity of Gram-negative bacteria is mostly dependent on the presence of a secretion system in their cells, through which they secrete nucleoproteins involved in their pathogenicity in the apoplast or inject in the host cell [322]. *E. coli* was first known to be associated with diarrhea, and now with outbreaks of foodborne diseases [318]. It was also reported that *Aspergillus* species produced the maximum mycelial dry weight (71 mg/25 mL) and also bioactive antimicrobial agents showing the maximum zone of inhibition (25 mm) against *B. subtilis*. However, PDB was found to be the best as it produced high amounts of biomass and an impressive antibacterial property against test pathogens. During the optimization process, it was observed that, the biomass and bioactive metabolite productions were directly proportional to each other [323]. PDB served as the best medium for biomass and improved production of secondary metabolites from *A. terreus* [279]. The PDB has also been reported as the best medium for biomass production and napthoquinone biosynthesis in *F. moniliforme* [318]. Some of the results also suggested that the addition of starch in PDB media enhanced the bioactivity of the compound against targeted bacterial pathogens. The maximum biomass and bioactivity were observed in PDB along with starch. In general, the EtOAc fraction of all the media tested were active than the *n*-hexane fraction. This might be attributed to the fact, that due to a difference in polarity, most of the compound dissolved in EtOAc fraction making it more active than the EtOAc fraction [324].

Table 3.22: Antibacterial activity of EtOAc and *n*-hexane fractions obtained from different media (zone of inhibition mm)

	MEDIUM									
Test Pathogens	**PDB**		**CYB**		**SDB**		**MEB**		**NB**	
	EtOAc	*n*-hexane	EtOAc	*n*-hexane	EtOAc	*n*-hexane	EtOAc	*n*-hexane	EtOAc	*n*-hexane
P. Aeruginosa	17.33±0.58	0±0	16.67±1.2	11.67±1.53	17±0.58	0±0	20±0.58	0±0	14±1.53	0±0
S. Typhi	20±1	0± 0	16.67±0.6	13±2	17±1.53	0±0	11±1.53	0±0	0±0	0±0
S. aureus	21.67.±1.53	0±0	16.33±0.2	0±0	0±0	0±0	0±0	0±0	0±0	11.3±1.5
S. marcescene	23±2	13.7±1.3	20.33±1.5	16.33±1.15	17±2.52	15.3±1.5	19±4.04	18.7±1.5	19±2	0±0
S.saprophyticus	12±2	0±0	12±2	10.33±0.53	11±1	0±0	15±1.53	0±0	0±0	0±0
S. para typhi	20.3±2.8	0±0	20.33±2.1	0±0	0±0	0±0	18±1.15	0±0	0±0	15.3±1.5
K. pneumoniae	16.3±2.8	17.33±1.53	16.33±2.1	14.33±1.15	17±2.52	0±0	22±1.173	0±0	11±1.53	0±0
P. mirabilis	24±1	0 ±0	19.67±1.5	0±0	23±2.08	23±1.7	0±0	19.7±3.1	20±1	0±0
E.coli	18.7±1.3	0±0	17.67±2.1	11±1.73	0±0	0±0	11±1.53	0±0	0±0	0±0

Positive control= Amoxicillin(10µg/Disc)

3.3.1.2. Optimization of Temperature

Along with other growth parameters, temperature also plays a remarkable role in the growth and production of bioactive metabolites. Different fungi have different optimum incubation temperature. Different studies reported that temperature is one of the important conditions that influence the growth rate of antagonists [325]. The fungi were grown in the PDB medium at different incubation temperatures in the range of 10-40ºC at intervals of 5ºC. The optimum temperature was at 30ºC at which the maximum production of metabolites occurred (507.66±4.163 µg/ 100 mL). By gradually increasing the temperature, the biomass and metabolites production increased as follows: 10ºC - 77.67±3.06 mg/100 mL and 56.09 ±5 µg/100 mL), 15ºC - 107±3.61 mg/100 mL and 101.2±3.6 µg/100 mL, 20ºC - 337.3±2.52 mg/100 mL, and 338±3.464 µg/100 mL), 25ºC (363.7±3.51 mg/100 mL and 470±3.6 µg/100 mL), and 30ºC (422±2.65 mg/100 mL and 580±4.163 µg/ 100 mL). At 35ºC, the production of biomass increased (475.7±2.52 mg/100mL) but the production of metabolites decreased (474±4 µg/100mL) (**Table 3.23** and **Fig 3.23**) The literature reports that a large number of fungi may grow quite well over the temperature range of 10-40ºC, while Sarker observed that the maximum growth and biomass of isolated fungal strains occurs at 30ºC [326, 327]. The increase of the incubation temperatures from 25 to 30ºC induces the fast growth of the cells as well as production of bioactive secondary metabolite. It was noted that in *Aspergillus*, the maximum cell growth (70 mg/25 mL) and inhibition zone (25 mm) was recorded at 30ºC. However, lowest growth and secondary metabolite production were observed at low temperature of 15 ºC and at high temperature (55ºC) [279].

Table 3.23: Comparison of growth and metabolites production at different temperature

S.No	Temp (^{O}C)	Biomass (mg/100mL)	Crude Metabolites (µg/100mL)
1	10	77.67±3.06	56.09±5
2	15	107±3.61	101.2±3.6
3	20	337.3±2.52	338±3..464
4	25	363.7±3.51	470±3.6
5	30	422±2.65	580±4.163
6	35	475.7±2.52	474±4
7	40	201.3±3.51	121±5

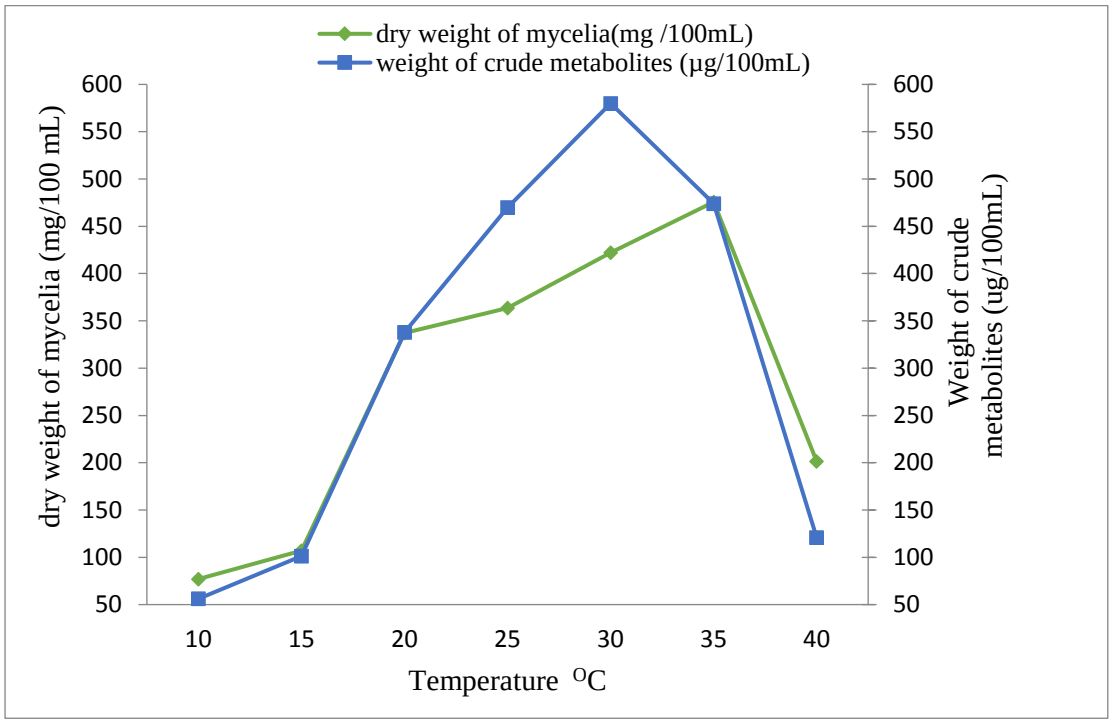


Fig 3.23: Comparison of growth and metabolites production at different temperature

3.3.1.3. Optimization of pH

The pH of the growth medium is one of the very important environmental but often neglected factors. In the current study, the fungi were grown at different pH to obtain the maximum biomass and secondary metabolites production. A wide range of pH supports the growth and metabolites to some extent except for pH 3 and 9 as shown in **Table 3.24** and **Fig 3.24**. At pH 6, the maximum quantity of biomass (453±2 mg/100 mL) and secondary metabolites (576.3±2.517 µg/100mL) were produced. At pH 7, both the growth and the production of secondary metabolites were high. Different studies also claim a variation in pH leads to a variation in the morphology. The pH of the growth media is a key factor in the production of both, biomass and metabolites [328, 329]. It was also observed that, the pH of the medium critically affects the cell membrane function, cell morphology and its structure, the uptake of various nutrient materials, and the production of compounds [330]. Fungi can tolerate a wide range of pH between pH 4 and 10 for soil activities, but the most favorable pH for the majority of fungi lies on the alkaline side of the pH scale [327]. The study conducted by Jain and Pundir support the present result that the maximum production of bioactive secondary metabolite by *A. terreus* in PDB medium occurs at pH 6.0 [280].

Table 3.24: Comparison of growth and metabolites production at different pH

S.No	pH value	Biomass (mg/100mL)	Crude Metabolites (µg/100mL)
1	3	0±0	0±0
2	4	119.7±2.542	135.67±2.8
3	5	208.7±2.31	178.67±5.567
4	6	453±2	576.3±2.517
5	7	449.7±1.53	532.67±2.08
6	8	311±3.61	301.67±3.215
7	9	0±0	0±0

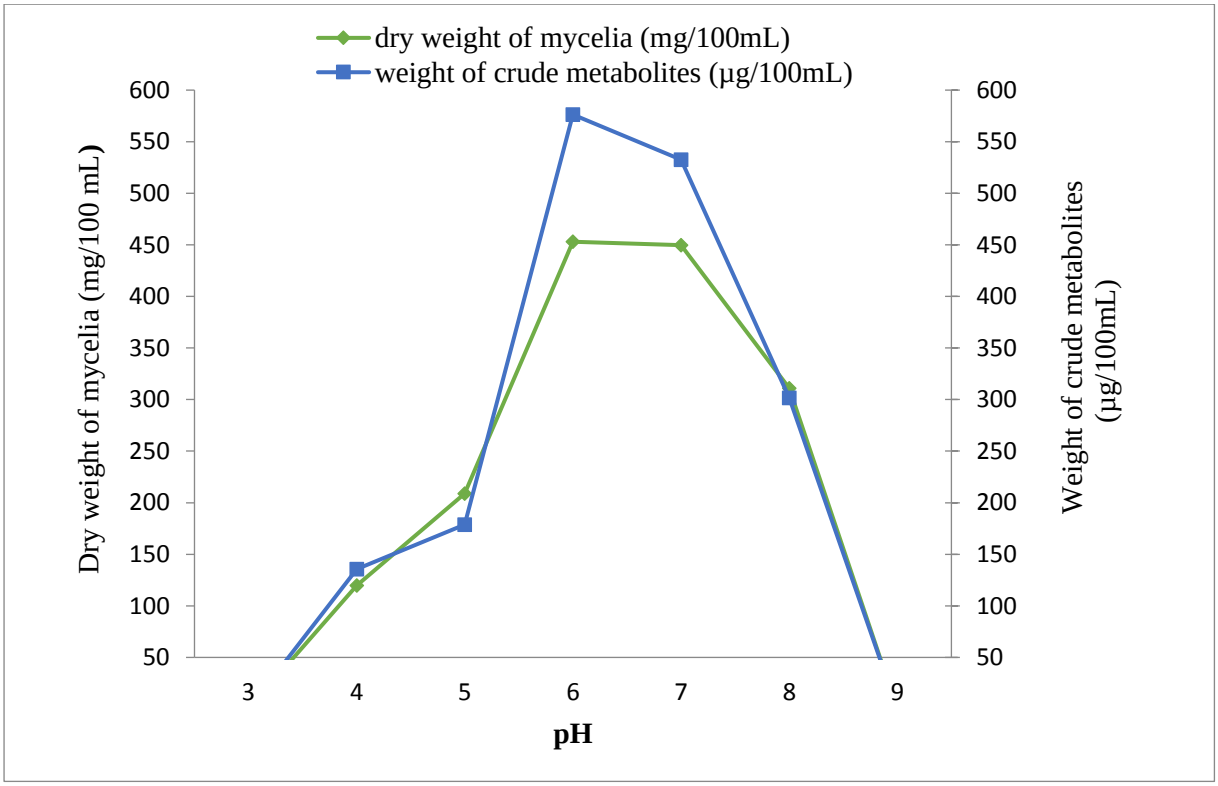


Figure 3.24: Comparison of growth and metabolites production at different pH

3.3.1.4.Optimization of incubation period

In order to get the highest growth kinetics and metabolite accumulation, incubation period must be optimized in batch fermentation [331]. In general it is a well-established fact

that secondary metabolites are secreted in the stationary phase. So, it is very important to determine the stationary phase of the specific fungus to obtained higher amounts of secondary metabolites of interest. The biomass and metabolite production were tested at different incubation time from 1-14 days as shown in **Table 3.25** and **Fig 3.25**. The biomass production increased gradually and reached the maximum at 11^{th} day (428.7±3.51 mg/100 mL). After the 11^{th} day, the biomass production remained constant. The optimum production of metabolites observed at the 9^{th} day (567±3.606 µg/100mL), which decreased gradually after the 11^{th} day. From day 1^{st}-4^{th}, no metabolite production was observed. Every fungus has its own production potential of secondary metabolites at different incubation periods [332]. A similar study showed that the *Aspergillus* species produced maximum amount of secondary metabolites on the 10^{th} day of incubation followed by the 12^{th} day. It was also observed that some fungi continued to produce secondary metabolites during the stationary phase but decreases when the incubation period increases [333].

Table 3.25: Comparison of growth and metabolites production at various incubation periods

S.No	Incubation period (Days)	Biomass (mg/100mL)	Crude Metabolites (µg/100mL)
1	3	71±1.73	0±0
2	4	116.3±4.04	0±0
3	5	146.7±2.89	97.66±3.512
4	6	2.63±3.51	210.33±3.05
5	7	331±3.21	403.666±5.132
6	8	425.7±2.52	564.66±4.72
7	9	417.7±3.21	567±3.606
8	10	219.7±1.53	535±4.5583
9	11	418.7±3.21	527±3
10	12	428.7±3.51	489.33±3.512
11	13	432.3±2.08	413.33±3.512
12	14	425.3±4.04	350±4.583

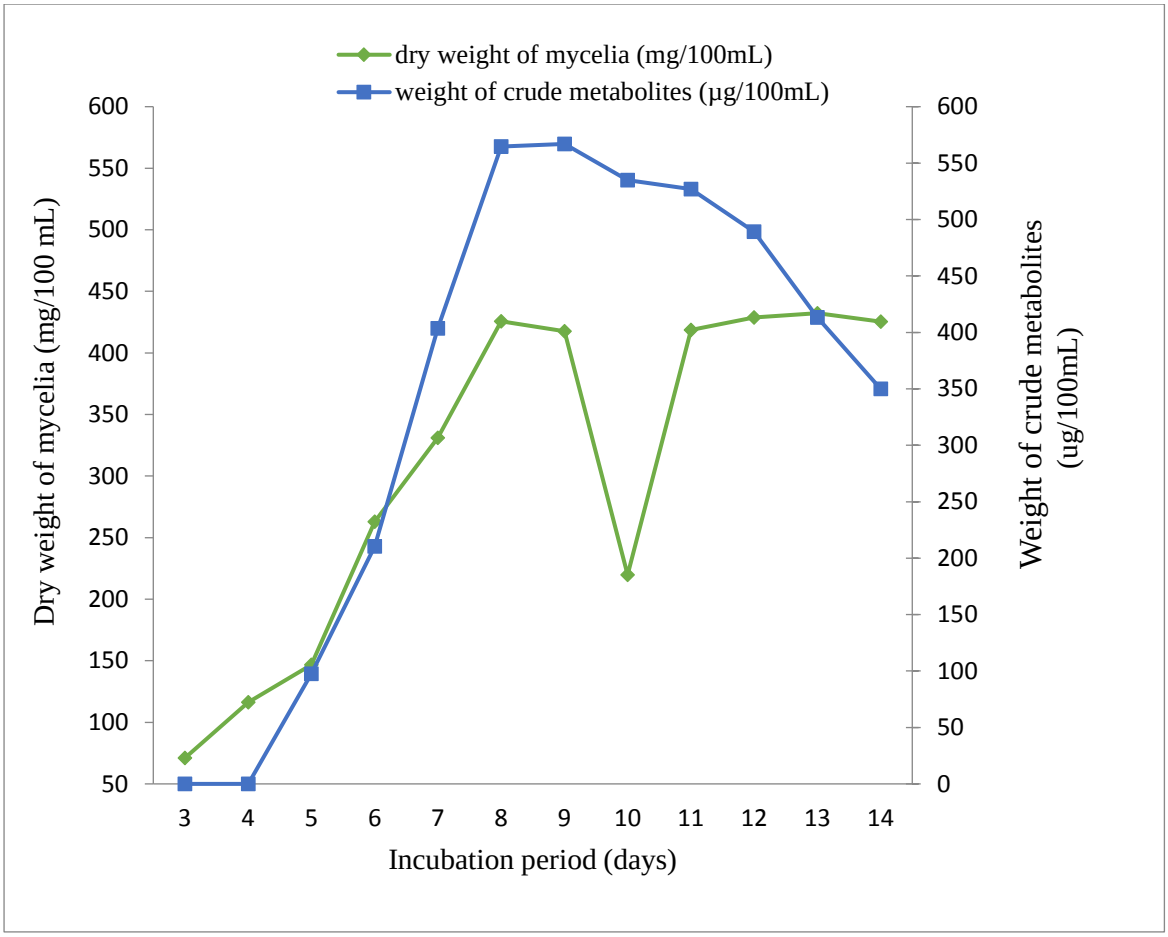


Figure 3.25: Comparison of growth and metabolites production at various incubation periods

3.3.1.5. Static/shaking incubation

The production of biomass and metabolites were also calculated on static and shaking growth condition while maintaining all the other parameters such as media, temperature, pH, and incubation period, constant. The production of biomass and crude metabolites was observed to be maximum (449±1 mg/100 mL and 571±1 µg/100 mL, respectively) under shaking conditions as compared to that at static growth conditions (247.3±4.04 mg/100 mL and 313.7±1.52µg/100 mL, respectively) (**Table 3.26 and presented in Fig 3.26**)**.** It was also investigated if the incubating condition is an important parameter for the production of bioactive secondary metabolites [334].

Table 3.26: Comparison of growth and secondary metabolites production at static and shaking growth condition.

S.No	Growth condition	Biomass (mg/100mL)	Crude Metabolites (µg/100mL)
1	Shaking incubation	449±1	571±1
2	Static incubation	247.3±4.04	313.7±1.52

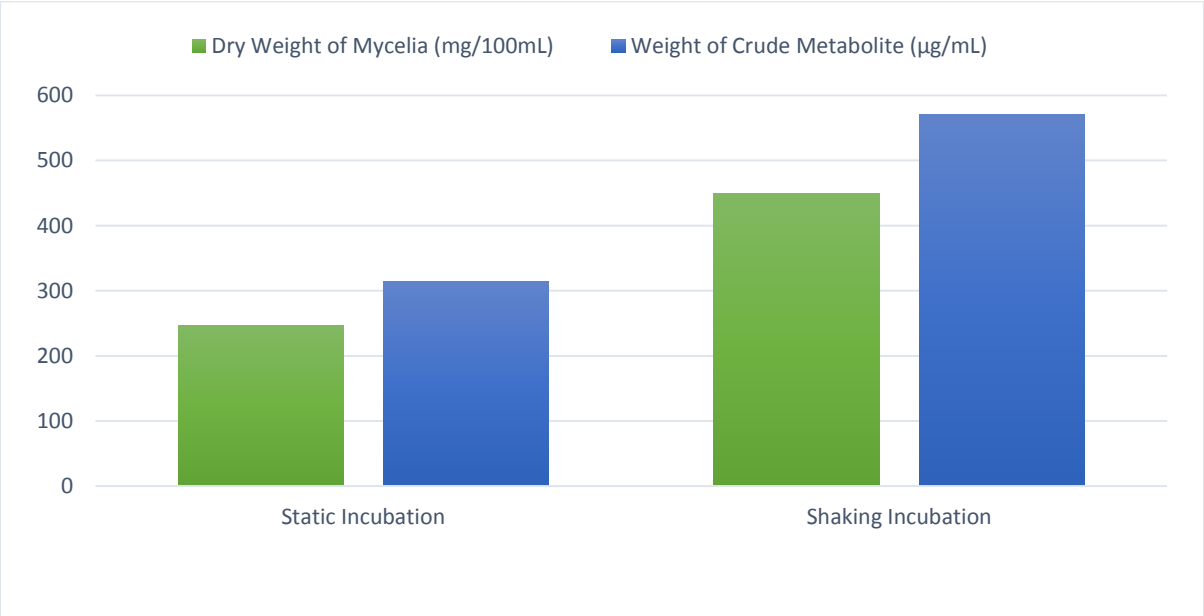


Figure 3.26: Comparison of growth and secondary metabolites production at static and shaking growth condition.

3.3.2. Description of Secondary Metabolites Isolated from *A. flavus*

3.3.2.1. Structure elucidation of compound (6)

Kojic aicd was isolated from ethyl acetate fraction as a white crystalline solid (M.p=155-159^{0}C). Its exhibited EI-MS peak at 142.7 corresponding to $C_6H_80_4$. The UV spectrum of this compound showed absorption peak at 252 nm. The IR spectra reviled peaks at 3218 for OH and 1615 cm^1 for C=O. ^{1}HNMR showed resonating peak at 6.48 (2H-2, s), 6.48 (H-5, s) and 4.39 (2H-7, s). ^{13}CNMR showed signals at 139.1 (C-2), 145.1 (C-3), 147.0 (C-4), 110.0 (C-5), 167.2 (C-6), 58.2 (C-7) respectively (**Table 3.27**). The structure of compound 6 was confirmed by comparing their physical and spectra data with reported one [335].

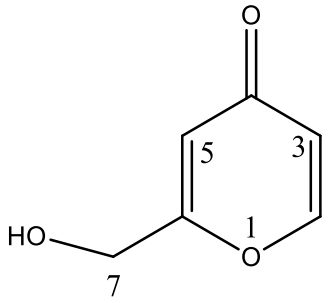


Figure 3.27: Structure of Kojic acid (**6**)

Table 3.27: ^{1}H and ^{13}C-NMR chemical shift values of Kojic acid (**6**)

Carbon No.	δ_C	δ_H (mult, J, HZ)	Multiplicity
1	-	-	-
2	139.1	6.48, s	CH2
3	145.1	-	C
4	174.0	-	C
5	110.0	6.48, s	CH2
6	167.2	-	C
7	58.2	4.39, s	CH2

3.3.3. *In Vitro* Biological screening

3.3.3.1. Antifungal assay

The antifungal potential of the EtOAc and *n*-hexane fractions was determined against different pathogenic fungi as shown in **Table 3.28** and presented in **Figure 3.28**. The EtOAc fraction showed significant activity against *Candida* spp. (80 ±1.414% inhibition), while moderate effect was exerted against *P. notatum* (44.8 ±0.77%), *A. fumigatus* (66.66 ±1.4%), and *A. alternatum* (53.33 ±0.0%). The EtOAc fraction was inactive against *V. chlamydosporium*. Fungi produce several secondary metabolites that have numerous pharmacological uses, particularly as antibacterial and antifungal agents [336]. The search for new antimicrobial drugs is critically important since the incidence of fungal diseases has grown extremely in the last few years. The production of antifungal metabolites by fungi is a natural process, because their survival in the natural ecosystem depends on their capability to inhibit the growth of other co-habitant microorganisms. Several fungi from *Aspergillus* genus are known for their capability to produce various active secondary metabolites of pharmaceutical importance, of which echinocandin B has antifungal activity [337]. *Aspergillus* spp. also produces a remarkable metabolite called kojic acid, a compound currently used in the pharmaceutical industry for various purposes due to its wide range of biological activities, including antibacterial, antifungal, anti-inflammatory, antitumor, and insecticidal effects [338]. *A. flavus* was also found to produce some bioactive secondary metabolites having antifungal properties [339].

Table 3.28: Antifungal activity of EtOAc and *n*-hexane fraction of *A. flavus* against different pathogenic fungi.

S.NO	Test fungi	**Percent inhibition**	
		EtOAc	*n*-hexane
1	*Penicillium notatum*	44.88±0.77	0±0
2	*Aspergillus fumigatus*	66.66±1.4	0±0
3	*Verticillium chlamydosporium*	0±0	0±0
4	*Acremonium alternatum*	53.33±0.0	0±0
5	*Candida albicans*	80±1.414	0±0

Positive control, Miconazole at the concentration of 110 µg/mL

Figure 3.28: Antifungal activity of EtOAc and *n*-hexane fraction of *A. flavus* against different fungal strains.

3.3.3.2. Phytotoxic assay

Fungal metabolites are recognized as a valuable source of new biologically active compounds. In our study, the EtOAc and *n*- hexane fractions of secondary metabolites produced by *A. flavus* were applied for determination of their phytotoxic effect. The results are given in **Table 3.29** and **Fig 3.29**. The EtOAc fraction was more active as compared to *n*-hexane fraction, and at a higher concentration (1000 µg/mL) showed a significant activity (90%) against *L. minor*. At 100 µg/mL, a good activity was recorded (70%), while at a low concentration (10 µg/mL), a moderate activity (40%) was observed. The *n*-hexane fraction exhibited a moderate activity (40%) at a higher concentration (1000 µg/mL) *A. flavus* is a pathogenic fungus causing different types of diseases in humans and plants. It was discovered that *Aspergillus* spp. produced cichorine, a secondary metabolite which has phytotoxic effects [340]. Phthalides, which are structurally the most diverse class, are secondary metabolites consisting of approximately 180 natural compounds. They are produced by a number of organisms, including *Aspergillus* spp. Phthalides show a broad spectrum of bioactivities, including phytotoxic activity [341-343].

Table 3.29: Percent growth regulation of the *Lemna minor*

Concentration of sample (µg/mL)	**Total number of fronds**	**Percent growth regulation**		
		EtOAc	***n*-hexane**	**Positive control**
10	10	40	0	100
100	10	70	20	100
1000	10	90	40	100

Positive control *= Paraquat at a concentration of 0.015 µg/mL

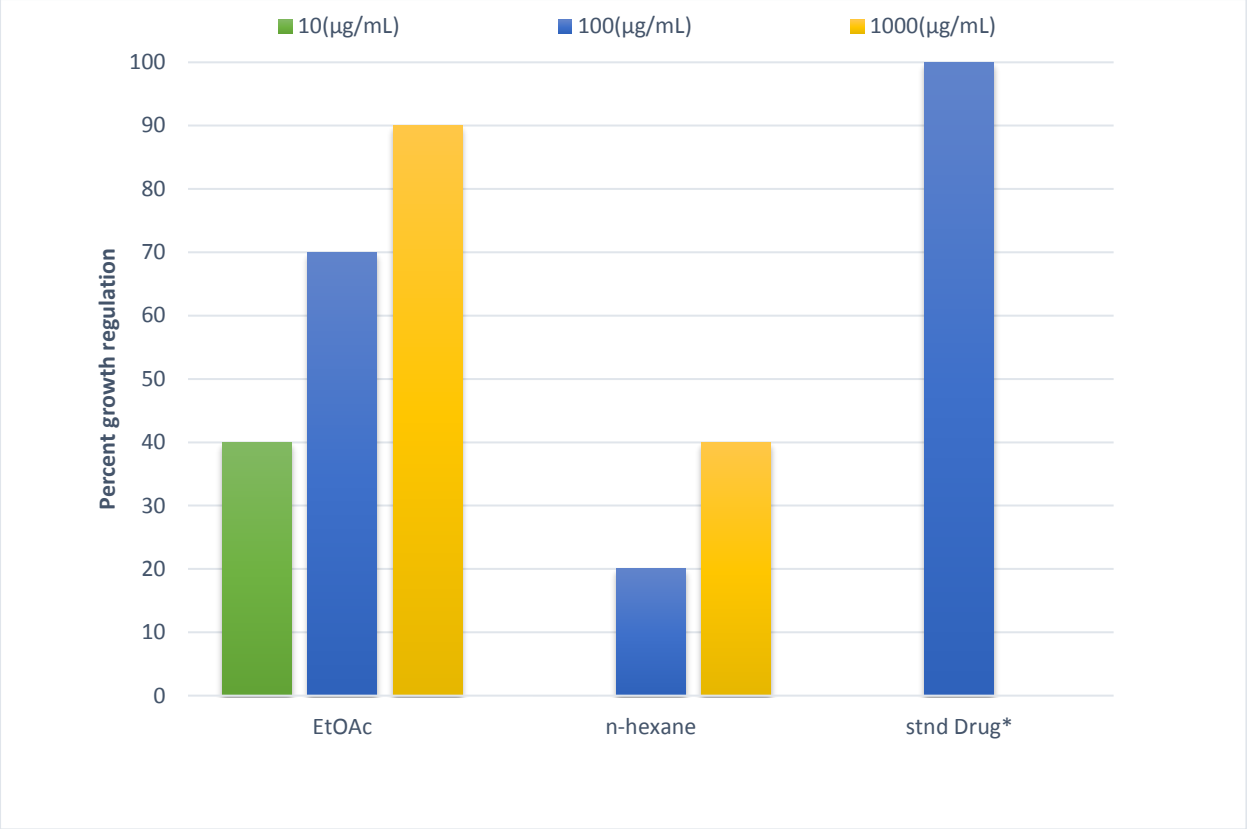


Figure 3.29: Phytotoxic activity of EtOAc and *n*- hexane fractions against *L. minor* plant

3.3.3.3. Insecticidal assay

We investigated the insecticidal potential of EtOAc and *n*-hexane fractions of crude metabolites against two test insects, *Sitotroga cerealella and Callosobruchus maculatus*. The results are presented in **Table 3.30** and **Fig 3.30-3.31**. We found that the EtOAc fraction

applied at higher concentrations (100 and 1000 µg/mL) was significantly active against *S. Cerealla*, causing mortality of 80 and 100%, respectively. At a low concentration (10 µg/mL), the EtOAc fraction was ineffective against *S. cerealla.* Similarly, the *n*-hexane fraction also exerted potent effect against *S. cerealla* at a higher concentration (1000 µg/mL), leading to 80% mortality. At 100 µg/mL, the *n*-hexane fraction induced 30% mortality. At a low concentration (10 µg/mL), the *n*-hexane fraction had no influence against *S. cerealla*. The EtOAc fraction showed a low activity against *C. maculatus* (30% and 20%) at higher concentrations (1000 and 100 µg/mL, respectively), while it was ineffective at a low concentration (10 µg/mL). The *n*-hexane fraction was not active against *C. maculatus.* We found that *A. flavus* has a strong potential to inhibit insects, which had an adverse effect on our crops. The secondary metabolite 13-desoxypaxilline, isolated from different fungal species, including *Aspergillus* spp., has an insecticidal potential [344, 345].

Table 3.30: Insecticidal activity of crude EtOAc and *n*-hexane fraction

Insects	**Total number of insects**	**% Mortality of the insects**						
		Concentration (µg/mL)						
		EtOAc fraction			*n*-hexane fraction			Permetherin (STD)
		10	100	1000	10	100	1000	
S. cerealella	10	0	80	100	0	30	80	100
C. maculatus		0	20	30	0	0	0	

Positive control: Permethrin (239.50 µg/cm^2)

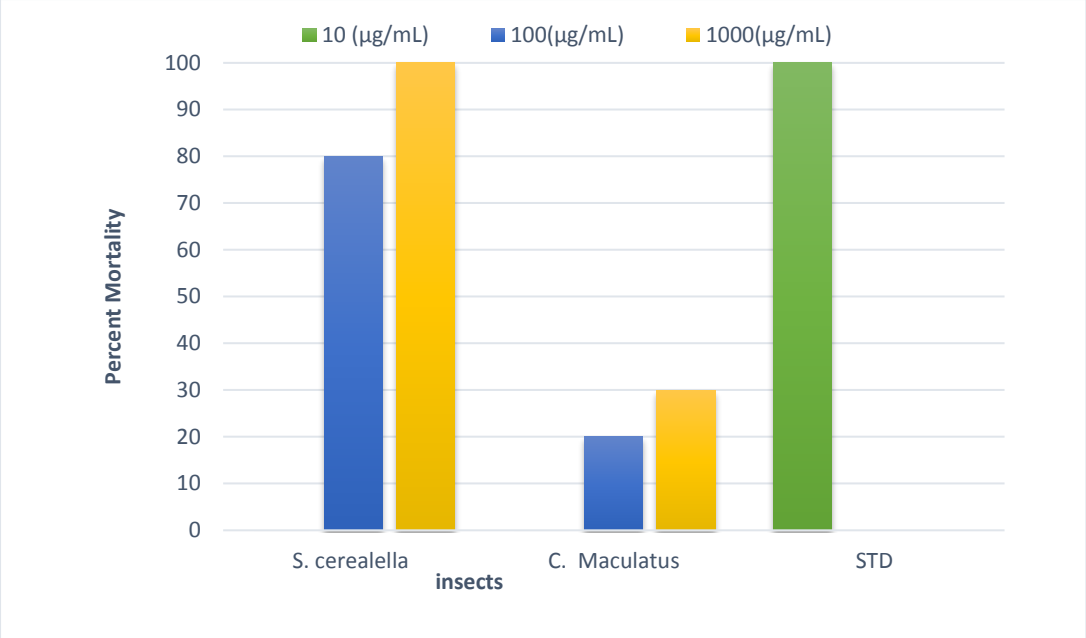


Figure 3.30: % Mortality of EtOAc fraction of secondary metabolites against different insects

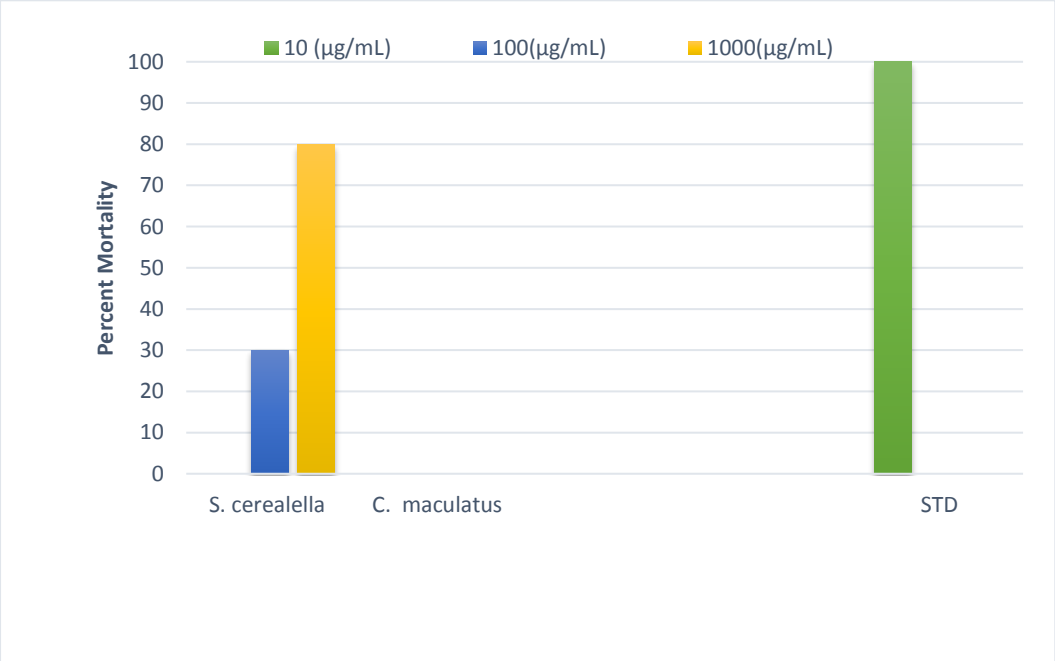


Figure 3.31: % Mortality of *n*-hexane fraction of secondary metabolites against different insects

3.3.3.4.Brine shrimp lethality assay (BSLA)

The cytotoxic properties of the *n*-hexane and EtOAc fractions of secondary metabolites were determined, and the results are presented in **Table 3.31** and **Fig 3.32**. The mortality of shrimps was tested at different concentrations, viz., 10, 50, 100, 500, and 1000 µg/mL. It was observed that the EtOAc fraction showed significant activity at high concentrations (1000µg/mL) with 85% mortality, while 500 µg/mL also showed a significant activity (65%). A concentration of 100 and 50 µg/mL showed lower activities as 45 and 20%, respectively, whereas 10 µg/mL showed no activities as 0%. Similarly, the *n*-hexane fraction also showed moderate activity at higher concentrations, viz., 1000 with 60%, while low concentrations of 500, 100, 50, and 10 µg/mL exhibited 35, 15, 0 and 0% cytotoxicity respectively. The results clearly show that the EtOAc fraction possesses significant bioactivity against the shrimps. Brine shrimps lethality bioassay is a simple cytotoxicity test based on the killing potential of a test samples on a zoological organism; *Artemia salina* [346]. It is a widely used test for determination of toxicity of fungal metabolites, plant extracts, pesticides, heavy metals etc. [347, 348]. It is a preliminary screening for further study on animal's models. It was noted that *Aspergillus* species produced a wide range of metabolites having cytotoxic effect [349].

Table 3.31: Percent cytotoxicity of EtOAc and *n*-hexane fractions against brine shrimps cells

	EtOAc Fraction			***n*-hexane fraction**		**Positive control**
Concentration (µg/mL)	Total No of shrimps	No of Dead shrimps	% cytotoxicity	Number of Dead shrimps	% cytotoxicity	% cytotoxicity
10	20	0	0	0	0	
50	20	4	20	0	0	
100	20	9	45	3	15	
500	20	13	65	7	35	
1000	20	17	85	12	60	100

Positive control*: Etoposide (7.4625 µg/mL) was used as a standard drug**.**

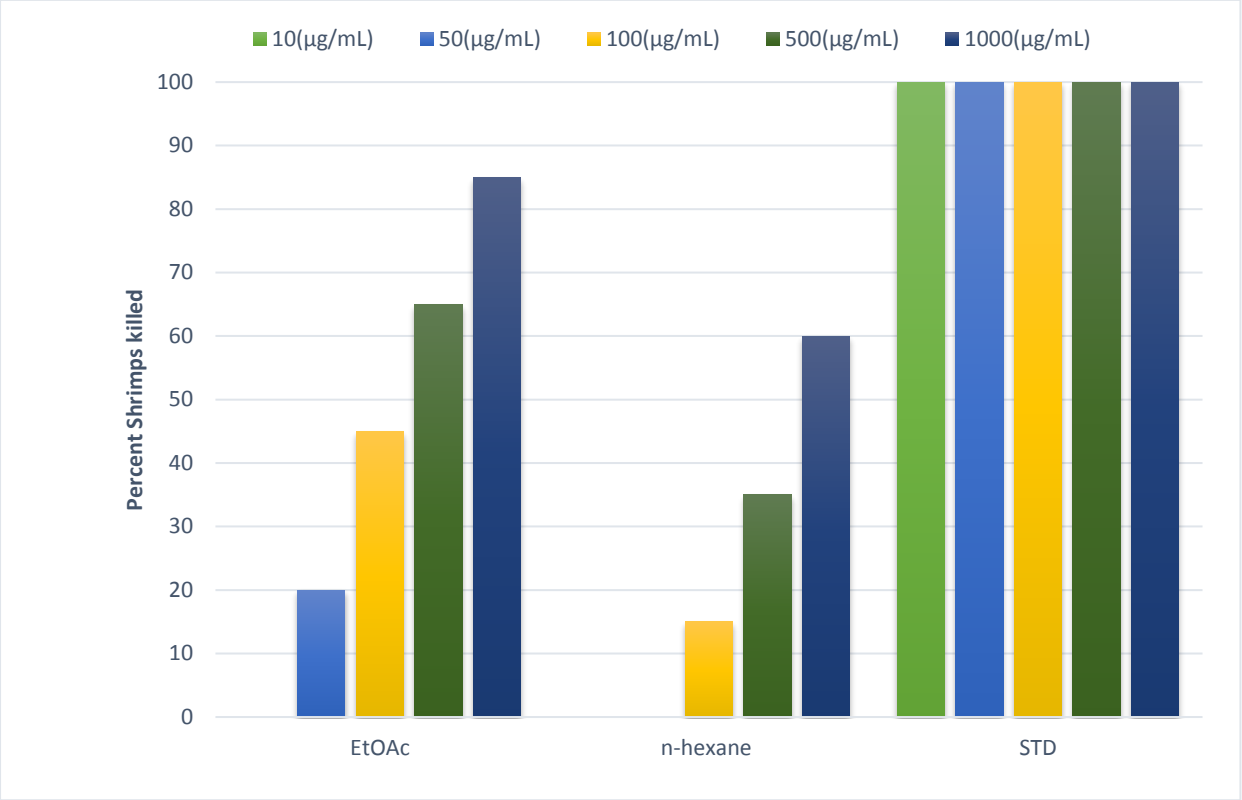


Figure 3.32: Cytotoxicity of EtOAc and *n*-hexane fraction of *A. flavus* against Brine shrimps

3.3.3.5.Enzyme inhibition Assay

3.3.3.5.1. Carbonic anhydrase Assay

Interestingly, *A. flavus* was active against carbonic anhydrase (CA). Both the EtOAc and the *n*-hexane fractions were active against CAs at 0.2 mg/mL (66 and 63%, respectively) with **IC_{50}** values of (59.89 ±1.65 and 61.3 ±1.75 µg/mL, correspondingly) (**Table 3.32** and **Fig 3.33**).

There are many good inhibitors of CAs, most of them are classical sulfonamides/sulfamates CA inhibitors and their derivatives, including ethoxzolamide (EZA), acetazolamide (AZA). Although they do not have good selectivity for CA-IX, over the sulfonamide-avid against isozyme CA-II [350-352]. Aromatic/heterocyclic sulfonamides were used clinically for the inhibition of CAs isozymes for therapy of many diseases, including gastric duodenal ulcers, glaucoma, hypobaropathy, congestive heart failure, etc. [353]. It has also been established that phenolic compounds inhibit both α-CAs and β-CAs. Lichens (a symbiotic relationship between fungi and photosynthetic organism, such as algae or Cyanobacteria) can carry out a wide range of valuable biological functions due to their ability to produce secondary metabolites, the majority of which are phenolic in nature [354, 355]. Polyamines isolated from terrestrial and marine animals, plants, and fungi have also been discovered to exert an inhibitory effect against human carbonic anhydrase [356].

3.3.3.5.2. Urease inhibition assay

In our examination, both fractions (EtOAc and *n*-hexane) of *A. flavus* showed insignificant results against the enzyme urease (313.56 and 28% inhibition, respectively) (**Table 3.32** and **Fig 3.33**). In contrast, it has been evidenced that some new secondary metabolites produced by endophytic fungi have a great potential to inhibit urease [357]. In particular, the metabolites having a flavonoid skeleton possess inhibitory capacity against this enzyme [358]. We found that both fractions used in our study were insignificantly active

against urease. This may be due to the limited number of flavonoid compounds. Similarly, some fungi also produce extracellular enzymes with a urease activity [359].

Table 3.32: Carbonic anhydrase and Urease inhibition of EtOAc and *n*-hexane fraction of *A. flavus*

NO.	**Enzyme**	**EtOAc fraction**		***n*-hexane fraction**	
		% inhibition	IC_{50} [µM]	% inhibition	IC_{50} [µg/mL]
1	Carbonic anhydrase	66	59.89 ± 1.65	63	61.3±1.75
2	Urease	13.56	-	28	-

* Acetazolamide was used as a standard inhibitor for Carbonic anhydrase inhibition
* For Urease inhibition, Thiourea was used as a standard inhibitor

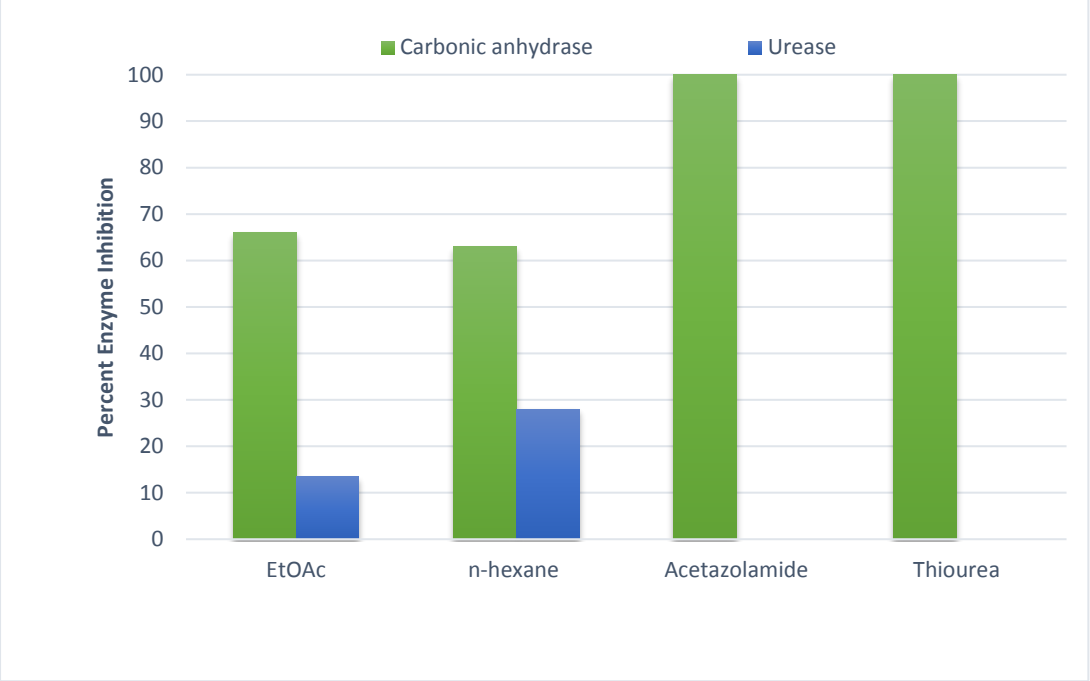


Figure 3.33: Percent enzyme inhibition of EtOAc and *n*-hexane fraction of *A. flavus*

3.3.4. *In-vivo* biological activities of crude EtOAc extract of *A. flavus*

3.3.4.1. Acute toxicity

The acute toxicity of the crude EtOAc extract of *A. flavus* was examined in our study. After the completion of the experiment, all animals remained alive. Thus, the crude metabolites of *A. flavus* also fall in the category of mild toxicity. The results are shown in **Table 3.33.** Upon completion of the experiment, blood was collected, and different hematological tests were conducted. WBC counts displayed a slight increase (10.6×10^9/L) above their normal range ($0.8–6.8 \times 10^9$/L), which may be due to the increment in the number of lymphocytes or granulocytes. However, the percentage of lymphocytes was much lower (17.7%), while the percentage of the granulocytes was higher (61.4%) than its normal range (8.6–38.9%) (**Table 3.34**) The results of the hemoglobin (HGB), hematocrit (HCT) and red blood cell counts (RBC) indicated a slight decrease from their normal values, which also suggest that the extract of *Aspergillus* species has identical effect on RBC and WBC parameters. Overall, all findings show that the extract does not exert an extremely toxic effect on mice. Based on their toxicity, mycotoxins are divided into three categories: extremely toxic, very toxic, and toxic mycotoxins [360]. Ochratoxin A and sterigmatocystin produced by *Aspergillus* species are examples of toxic mycotoxins. Literature reported that the common clinical signs and symptoms of toxicity caused by the extract in experimental animals were: anorexia, diarrhea, ataxia, dyspnea, tachypnea, tachycardia, and somnolence. The effect of ataxia on the sensory and autonomic central nervous system was evaluated on the basis of their inability to control and coordinate movement. Similarly, diarrhea, tachypnea (quick and shallow respiration), tachycardia (increased heart beat), and somnolence are signs of toxicity of the central nervous system [310, 311].

Table 3.33: Acute toxicity of crude EtOAc extract of *A. flavus*

Treatment (crude EtOAc) (mL or mg/kg)	No. of animals alive after 4hrs	No. of animals alive after 24hrs	% Death after 4hrs	% Death after 24hrs
Normal saline	All	All	-	-
10			-	-
20			-	-
30			-	-
40			-	-
50			-	-

Table 3.34: Different parameters values after termination of experiment

S.No	Parameters	Before experiment	After experiment	Normal range
1	WBC	6.0× 10^9/L	10.6× 10^9/L	0.8-6.8 × 10^9/L
2	LYM%	60.5%	17.7 %	55.8-90.6 %
4	GRAN%	40.6%	61.4 %	8.6-38.9 %
5	LYM#	6.010^9/L	19 ×10^9/L	0.7-5.7 ×10^9/L
7	GRAN#	1.9×10^9/L	6.5×10^9/L	0.1-1.8 ×10^9/L
8	RBC	5.45 ×10^{12}/L	5.80×10^{12}/L	6.36-9.42 ×10^{12}/L
9	HGB	11.0 g/dL	9.5 g/dL	11.0-14.3 g/dL
10	HCT	25.3%	30.1 %	34.6-44.6 %
12	MCH	16.1 pg	16.3 pg	15.8-19.0 pg
13	PLT	450×10^9/L	479×10^9/L	100-600 ×10^9/L
14	MPV	7.0 fL	10.5 fL	5.5-7.5 fL

3.3.4.2. Analgesic activity of crude EtOAc extract of *A. flavus*

As shown in **Table 4.30**, a reduction in the mean number of writhing in the different test groups was caused by the application of the crude EtOAc extract through the i.p. route at different doses (50, 100, and 150 mg/kg b.w). In the group in which normal saline was administered, the mean writhing was 45.4 ±1. The percentage of writhing inhibitory effect produced by different test doses of the crude EtOAc extract was 14.097% (50 mg/kg b.w), 23.28% (100 mg/kg b.w), and 33.87% (150 mg/kg b.w). The effect generated by the crude EtOAc extract was dose dependent. At a dose of 10 mg, diclofenac sodium (positive control) caused maximum inhibition (43.10), which was more profound than the one induced by the highest dose of the crude EtOAc extract (150 mg/kg).

It is suggested that the crude EtOAc extract of the *A. flavus* contains some active secondary metabolites which exert an analgesic effect in the form of a reduction in the abdominal constriction (writhing). It was also reported that some *Aspergillus* species produced some bioactive secondary metabolites which have analgesic and antiulcer activities [361]. Ibuprofen is a potent analgesic compound produced by different species of fungi [362].

Table 3.35: Analgesic activity of crude EtOAc extract of *A. flavus*

S. No.	Treatment	Dose mL or mg/kg	No. of writhing (10 min) (Mean+ SEM)	% inhibition of writhing
1	Saline	10	45.4 ± 1	-
2	Crude EtOAc	150	30.33 ± 1.44***	33.87
		100	34.83 ± 0.88***	23.28
		50	39 ± 1.83***	14.097
3	STD (Diclofenac sodium)	10	25.83 ± 1.5***	43.10

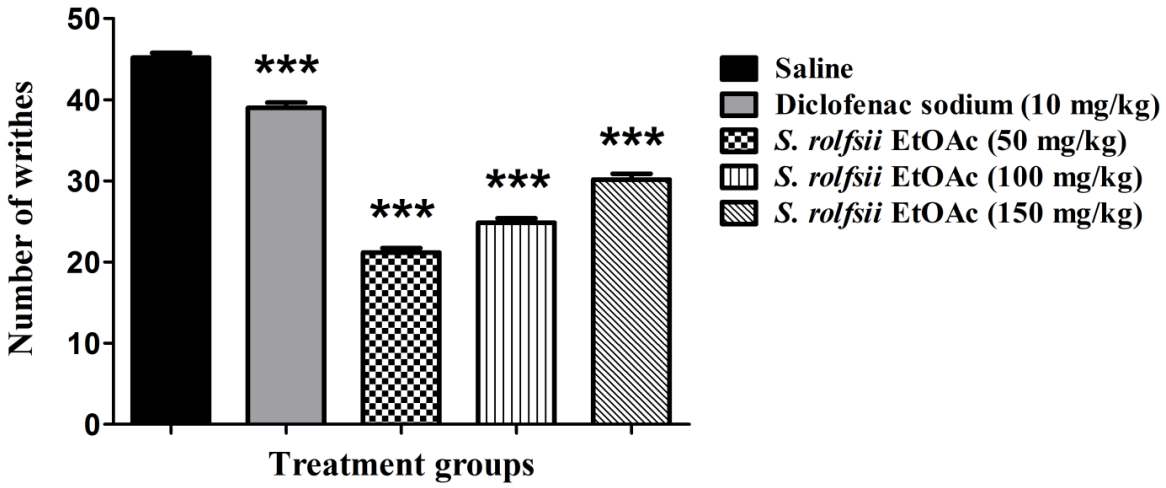


Figure 3.34: Antinociceptive activity of *A. flavus* crude extract in the acetic acid induced abdominal constriction assay.

Each bar represents mean number of writhes ± SEM.

One way ANOVA followed by Dunnett's *post hoc* test.

***$P < 0.001$ compared to saline treated group. $n = 6$ animals per group.

3.3.4.3. Sedative activity of crude EtOAc extract of *A. flavus*

The results of our investigation showed the EtOAc extract of *A. flavus* can cause a significant sedative effect in open field. The numbers of the crossed line after 30 min are given in the **Table 3.36** and presented in **Fig 3.35**. The increase in the concentration of the crude extract (50, 100, and 150 mL or mg/kg b.w) resulted in a rise of the sedative effect (108.2 ±1.22, 91.33 ±1.83, and 81.5 ±1.83, respectively). During active cell growth, fungi produce different types of secondary metabolites (toxins, ketones, alkaloids, antibiotics, fatty acids, alcohols, etc.) [363]. These metabolites have a substantial spectrum of functions, including sedative effect [364].

Table 3.36: Sedative activity of crude EtOAc extract of *A. flavus*

S. No.	Treatment	Dose (mL or mg/kg)	No of lines crossed
1	Saline	10	130 ± 2
2	Crude EtOAc	150	108.166 ± 1.22***
		100	91.33 ± 1.833***
		150	81.5 ± 1.83***
3	Diazepam	0.5	6 ± 0.66***

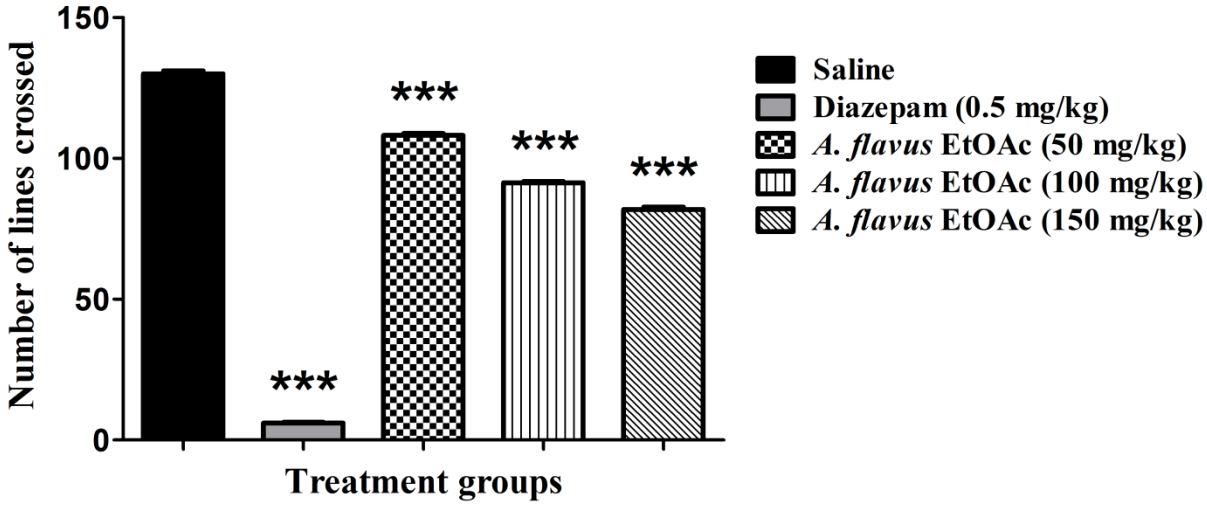


Figure 3.35: Sedative activity of *A. flavus* crude extract in the locomotor test (open field).

Each bar represents mean number of lines crossed ± SEM.

One way ANOVA followed by Dunnett's *post hoc* test.

***$P < 0.001$ compared to saline treated group. $n = 6$ animals per group.

3.4. Molecular docking studies and their Reversal of Multidrug Resistance in Mouse Lymphoma cells of Screlotiumol and chlorogenic acid from *Screlotium rolfsii*

Cancer is the second largest cause of death in developing and developed countries. Approximately six million people die due to cancer every year [365]. During the last decade extensive research has been carried out to discover effective options for the treatment of cancer. For this purpose a huge number of natural products have been isolated from natural sources, such as plants, bacteria, algae, fungi and characterized. The development of microbial biotechnology led to the development of a large number of drugs from terrestrial microorganisms, but it is still insufficient to complete the demand for therapy of new emerging diseases [366, 367]. The world scientific community has made all possible efforts to defeat cancer, and still the search of new anticancer agents is of critical significance. Natural products are a valuable source of anticancer drugs [368, 369]. Due to the development of the modern cancer biology, much of the research is concentrated on cancer-specific mechanisms and molecular targets [370]. Among natural products, secondary metabolites produced by fungi have a great pharmacological importance. These compounds possess anticancer antibacterial, antifungal, cytotoxic, and antiviral properties that can be used for the formulation of drugs [371-373]. Recent scientific techniques, such as bioinformatics, further enhanced the potential of drugs.

Two compounds, i.e., chlorogenic acid (**compound 2**) and screlotiumol (**compound 3**), were selected for anticancer and molecular docking studies.

3.4.1. Chlorogenic acid (2)

Chlorogenic acid is an extremely important natural compound used in various types of food, pharmaceuticals, beverages, cosmetics, tea products, etc. [374-376]. Beside the industrial importance, it also exhibited anticancer, antibacterial, antifungal, antiviral, and antioxidants

potential [377]. Furthermore, this substance is used as an efficient precursor compound for the development of drugs that can inhibit HIV [378].

The fluorescence activity ratio (FAR) value was used to evaluate the transporter modulating potential of *ABCB1*. In the flow cytometry, the value of SSC (side scatter count) and FSC (forward scatter count) were increased, which indicated that the compounds (chlorogenic acid) had an effect on the membrane, and the granulation of cytoplasm was intensified. The FAR values obtained in the short time experiment by using the compound chlorogenic acid suggested that it is an extremely effective MDR modulator. Verapamil, which is a calcium channel blocker and chemosensitizer, was used as a positive control. On MDR mouse lymphoma cells the compound chlorogenic acid was screened at 2 µg/mL concentration. The chlorogenic acid was strong modulators of the efflux pump activity (FAR 15.01, 2 µg/mL) (**Table 3.37**)

Docking analysis

In silico drug designing is important in the discovery of novel inhibitors against the target receptors. In the present PhD dissertation, we carried out the docking studies to explore the inhibiting potency of chlorogenic acid with the P-gp. The selected compounds (chlorogenic acid and standard rhodamine) were docked into the crystal structure of P-gp. The results revealed that chlorogenic acid gave the optimal docking result on both docking software programs. As it becomes clear from the **Fig 3.36,** these compounds are bound exactly in the region where the co-crystallized ligand of the receptor is already present. Moreover, the *in silico* study predicted that if a compound produces larger interaction (more negative) energy a good docking score is obtained, which means that the compound has a higher activity. The interaction energies of chlorogenic acid (2) were little lower than the Rhodamine123 (**Table**

3.38). Hence, we can conclude that there are certain structural features of chlorogenic acid which are responsible for the inhibitory activities of P-gp from mice.

Therefore, from the docking interactions of chlorogenic acid (**Fig 3.37**), we can deduce that it forms a total of eight hydrophobic contacts with hydrogen bonding interactions in the binding site of P-gp. The hydrophobic interactions were with the residues of Met295, Phe766, Gly770, Ser827, Val831, Gln834, Phe979, and Ala983.

There are a total of six hydrogen bonds near to the binding site including two hydrogen bonds observed from Gln986 with a distance of 3.14Å and 3.11Å. However, the other four hydrogen bonds observed from the surrounding residues are Asn292 (3.10Å), Gln721 (2.96Å), Gln769 (3.03Å), and Asn835 (3.05Å)

We may conclude that eight hydrophobic contacts and six hydrogen bonds of chlorogenic acid are responsible for such a good predicting binding capacity to the P-gp receptor.

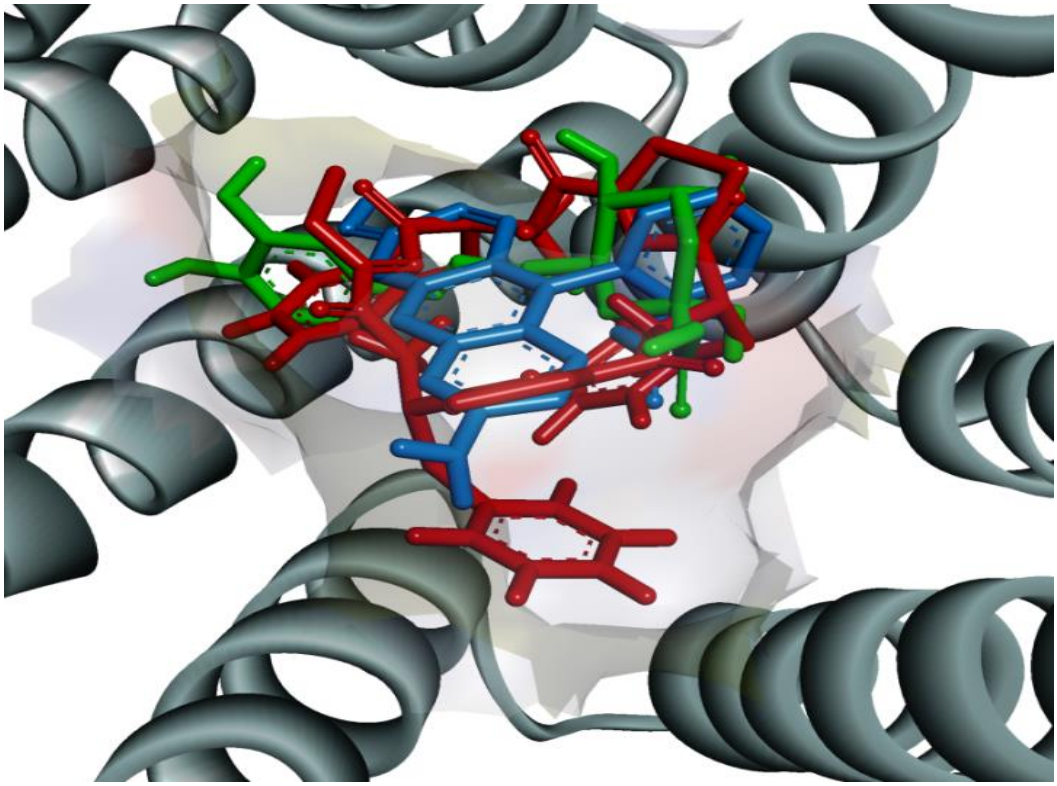


Figure 3.36: The overview of docked poses of Chlorogenic acid (**2**) in the binding region of P-gp. In the above figure 2 red color sticks represent co-crystallized ligand with superimposed green color stick Chlorogenic Acid and the blue color stick represent Rhodamine123.

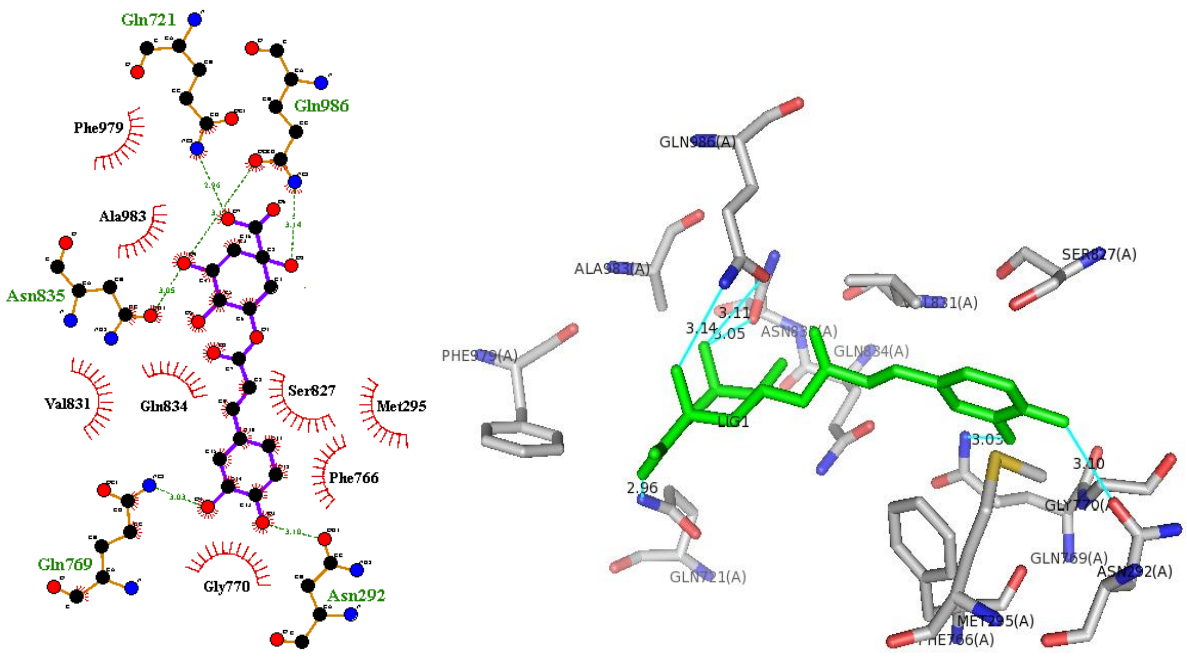


Figure3.37: The 2-D and the 3-D binding interactions of Chlorogenic acid (**2**) with the p-gp

Table 3.37: The effect of Chlorogenic acid (**2**) on the Rhodamine123 accumulation assay in L5178 MDR mouse lymphoma cell

	Sample	(final concentration) µg/ml	FSC	SSC	Mean	**FAR**	Peak Ch
1	PAR	-	2315	684	70.8	-	69.8
2	PAR	-	2134	603	65.5	-	67.3
3	MDR	-	2339	753	2.01	-	1.6
	MDR MEAN	-	2326	914	***1.64***	-	1.54
4	Verapamil	10	2329	711	*21.9*	**13.35**	27.4
8	Chlorogenic Acid	2	1848	1143	*12.55*	**15.01**	17.33
18	DMSO	0.2%	2247	759	1.02	**0.62**	0.931
19	MDR	-	2313	1076	1.27	-	1.49

Table 3.38: The docking binding energies (Kcal/mole) of Chlorogenic acid (**2**) and the standard Rhodamine123 against mice P-glycoprotein

Compound-Name	**Autodock Vina**	**i-GEM DOCK**			
	B. Affinity	Total Energy	VDW	HBond	Elec
Chlorogenic Acid	-7.6	-79	-57	-22	0
Rhodamine123	-8.2	-87	-86	-1	0

3.4.2. Screlotiumol (3)

The fluorescence activity ratio (FAR) value was used to evaluate the *ABCB1* transporter modulating potential. In the flow cytometry analysis, the value of SSC (side scatter count) and FSC (forward scatter count) were increased, which indicated that the compounds (**3**) had effect on the membrane, and the granulation of the cytoplasm was elevated. In the short time experiment, the FAR values obtained by using compound **3** indicated that compound **3** is an exceedingly effective MDR modulator. Verapamil, (chemosensitizer and calcium channel blocker) was employed as a positive control. Compound **3** was applied on MDR mouse lymphoma cells at a concentration of 2 µg/mL. It was observed that substance **3** was a strong modulator of the efflux pump activity (FAR 10.22, 2 µg/mL) (**Table 3.39**).

Docking analysis

Computational docking analysis has a vital role in the discovery of new drugs. It primarily predicts the inhibiting potency of new compounds against the target proteins of the drug. Our molecular docking studies revealed that the docking of compound–3 is in line with the *in vitro* results. The docking experiment of standard Rhodamine123 and compound–3 was carried out against the crystal structure of P-gp. The docking of compound–3 (**Table 3.40**) revealed a similar outcome as indicated in **Fig 3.38**. The interaction analysis of compound–3 (**Fig 3.39**) showed two types of contacts. One is hydrogen bonding and the other is the hydrophobic contact, if such types of interactions are present in the new compounds then it will have good mediating biological activities. There are total seven hydrogen bonds with binding site of compound–3, including two hydrogen bonds observed between Asn717 with a distance of 3.00Å and 2.95Å. The other five hydrogen bonds observed from the residues Tyr303 (2.85Å), Ser762 (3.25Å), Gln834 (2.80Å), Phe979 (2.75Å), and Gln986 (2.99Å). The hydrophobic

contacts of compound–3 in the binding site of P-gp were also identified from the surrounding residues such as Phe299, Gln721, Phe766, Met982, and Ala983.

A detailed study is further needed to explore the anticancer potential of compound–3 against the targeted receptor. The preliminary results obtained in this investigation will lead to the discovery of a new therapeutic agent for the treatment of cancer.

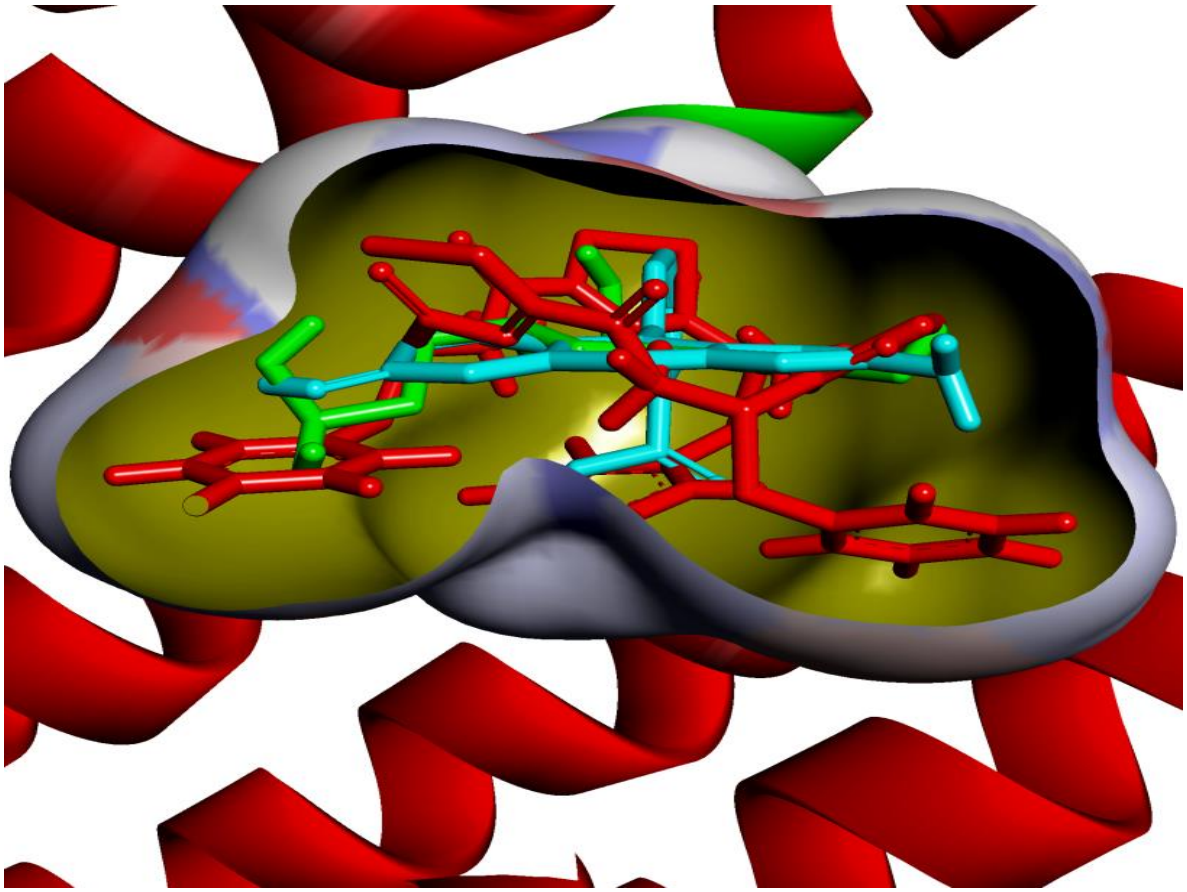


Figure 3.38: The predicted docked poses of compound-3 in the binding region of P-gp. In the above figure 2 red color sticks represent co-crystallized ligand with superimposed green color stick Screlotiumol (**3**)and the cyan color stick represent Rhodamine123.

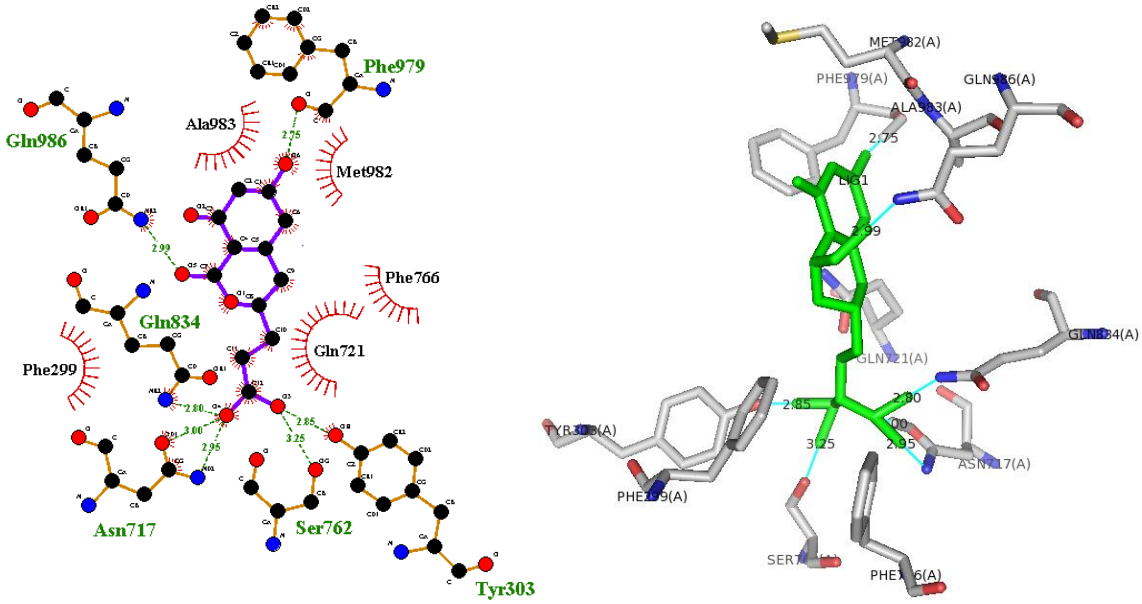


Figure 3.39: The 2-D and the 3-D binding interactions of Screlotiumol (**3**) with the p-gp

Table 3.39: The effect of Screlotiumol (**3**) on the Rhodamine123 accumulation assay in L5178 MDR mouse lymphoma cell

	Sample	(final concentration) µg/ml	FSC	SSC	Mean	**FAR**	Peak Ch
1	PAR	-	2315	684	70.8	-	69.8
2	PAR	-	2134	603	65.5	-	67.3
3	MDR	-	2339	753	2.01	-	1.6
	MDR MEAN	-	2326	914	***1.64***	-	1.54
4	Verapamil	10	2329	711	*21.9*	**13.35**	27.4
8	Screlotiumol (**3**)	2	1843	1137	*10.34*	**14.07**	16.41
18	DMSO	0.2%	2247	759	1.02	**0.62**	0.931
19	MDR	-	2313	1076	1.27	-	1.49

Table 3.40: Docking statistics (Kcal/mole) of Screlotiumol (**3**) and the standard Rhodamine123 against mice P-glycoprotein

	Autodock Vina	**i-GEM DOCK**			
Compound-Name	B. Affinity	Total Energy	VDW	HBond	Elec
Screlotiumol (**3**)	-6.8	-76	-56	-20	0
Rhodamine123	-8.2	-87	-86	-1	0

Conclusion

The search for discovery of new drugs and other industrial/pharmaceuticals important agents from the natural sources is as old as human civilization. Among the representative microbiotas, soil microorganism are the most diverse, complex, and important component in the biosphere. Due to a high level of diversity, soil microbial communities are exceedingly difficult to characterize phenotypically and genetically. They are a keystone and a basic unit of the structure and function of soil. Through microbial biotechnology, thousands of drugs are produced from terrestrial microorganisms, but they are still not enough to fulfil the demands caused by new emerging microbial diseases. Among soil microbial communities, fungi are the most important in terms of their biotechnological significance. Fungi are well known for production of biologically active secondary metabolites used in pharmaceutical, agricultural, and food industries.

The present research work comprises the optimization and characterization of two soil-borne fungi, *S. rolfsii* and *A. flavus.* Different growth parameters were optimized for the maximum production of bioactive secondary metabolites. Among the five media used, CYB was the most favorable for the production of higher quantities of the bioactive metabolites by *S. rolfsii*, whereas PDB was the most beneficial to *A. flavus*. We concluded that the medium utilized has a substantial impact on the production of bioactive secondary metabolites. Other growth parameters that contribute to the production of maximum amounts of metabolites were also optimized: pH, temperature, and incubation period and static/shaking condition.

The findings of the present study indicate the significant nature of the fungal metabolites with different biological activities generated by *S. rolfsii* and *A. flavus*. Thus this work suggests that these two soil-borne fungi could be used as sources of diverse novel bioactive metabolites. Different *in vitro* and *in vivo* activities show that these two fungi have the capability to produce

a wide range of secondary metabolites having antimicrobial, phytotoxic, insecticidal, cytotoxic, analgesic and sedative effect. Since, the EtOAc fraction was more active than the *n*-hexane fraction, it was subjected to column chromatography, and six compounds were isolated and characterized: one new and four known compounds from *S. rolfsii* and one known compound from *A. flavus*. The new compound was 13-(3, 3-dihydroxypropyl)–1, 6-dihydroxy–3, 4-dihydro–1H-isochromen-8(5H)-one. The global scientific community attempts to defeat cancer, but more efforts are still needed. Natural products are the main source of anticancer drugs. Among the isolated and characterized compounds, screlotiumol and chlorogenic acid were evaluated for their effects on the reversion of multidrug resistance (MDR) mediated by P-gp. Chemotherapeutic drugs target the multidrug resistant P-gp in cancer cells. Both tested compounds showed an excellent MDR reversing effect in a mouse T-lymphoma cell line in a dose-dependent manner. Furthermore, compounds **2** and **3** were subjected to molecular docking and showed the best results as compared to the standard. Therefore, it can also be concluded that these two compounds have anticancer effect and can be used in the treatment of cancer. With the help of bioinformatics tools this activity can be substantially enhanced.

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