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Inhibitory Activity of *Saussurea costus* (*Falc*.) Root Extract against *Aspergillus niger* Isolated from Stored Rice Grains and Detection of Toxins Produced by Fungus

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By

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

(يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ

وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ)

صَدَقَ اللَّهُ العَلِي العَظِيمِ

سورة المجادلة

الآية (11)

Dedication

To the one who gave me life, my creature, Allah

To the prophet of mercy and source of knowledge... Our prophet Mohammed and his family

To the imam of our time... Imam Mahdi

To the persons who raised me as a child and encouraged me in my life, my father and mother

To my support in life... my brothers

To all my family and friends

Fatima

Thanks and appreciation

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Summary

The current study involved the identifying and diagnosing of seed borne fungied fungi found in rice grain collected from various local markets in 2022 October to 2023 March in Hilla city, located in the Babylon Governorate of Iraq. A total of 121 fungal isolates were obtained from 90 rice grain samples and identified based on their cultural and morphological characteristics.

Among the isolates, 31 were identified as *Aspergillus niger*, accounting for 25.61% of the total isolates and appearing in 34.44% of the samples. *Aspergillus flavus* comprised 12 isolates, representing 9.91% of the total isolates and appearing in 13.33% of the samples. A total of 7 isolates of *Aspergillus terrus* were identified, making up 5.78% of the isolates, while 11 isolates of *Alternaria*. accounted for 9.09% of the isolates.

From the 49 different isolates isolated, 31 pure cultures of *Aspergillus niger* were obtained. Initial identification was performed using traditional taxonomy based on morphological features. Subsequently, 24 isolates of the *Aspergillus* niger group were successfully identified using the amplification amplicone ITS1-5.8S-ITS2 with flanking primer regions ITS5 and ITS4. All isolates exhibited monomorphic characteristics, with 20 isolates identified as *Aspergillus niger*, 2 isolates as *Aspergillus brasillensis*, and 2 isolates as *Aspergillus tubigensis.*

Fifteen random isolates of *Aspergillus niger* were subjected to genetic analysis to detect the presence of the fumonisin (FB) toxin gene using polymerase chain reaction (PCR). Out of these 15 isolates, 8 showed distinct bands measuring 651bp on agarose gel electrophoresis. Additionally, out of the total 15 isolates, only 7 exhibited distinct bands (420bp) on agarose gel electrophoresis for the ochratoxin A (OTA) toxin.

The study also investigated the antifungal activity of crude alkaloid compounds extracted from the roots of *Saussurea costus* against *Aspergillus niger* isolated from rice grains obtained from local markets in Hillah, Iraq. The antifungal activity was assessed using three concentrations (5, 10, and 15 %) through food poisoning methods. The results demonstrated that the crude alkaloid compounds from *Saussurea costus* (*Falc*.) Lipsch. roots significantly inhibited the growth of *Aspergillus niger* compared to the negative control (10% DMSO)and positive control(fungicide Carbendazim 500g/l).The mycelial inhibition percentages ranged from 66.0% at 5 %, 71.6% at 10 %, to 100% at 15 %.

Similarly, the crude flavonoid compounds exhibited significant antifungal activity, with 62.3% mycelial inhibition at 5 % and 100% inhibition at both 10 % and 15 % concentrations.

Also,the crude terpenoid compounds exhibited significant antifungal activity, with 51.0% mycelial inhibition at 5 % and 100% inhibition at both 10 % and 15 % concentrations. These results differed significantly from the control treatment and showed a similar effect to the positive control.

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Table of Abbreviations

Term	Meaning
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	AflatoxinG1
AFG2	AflatoxinG2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
BEN	Balkan Endemic Nephropathy
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribo Nucleic Acid
Fum	Fumonisin
IARC	international a gency research on cancer
NaOCI	Hypochlorite
NRPS	Neural Information Processing System
OmtA	Omethyltransferasel
OTA	Ochratoxin A
OTB	Ochratoxin B
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar

Term	Meaning
PKSs	polyketide synthases
RNA	Ribonucleic acid
UPGMA	Unweighted Pair Group Method With Arithmetic
UV	Ultraviolet
Verl	Versicolorin A dehydrogenase
nor1	Norsolorinic Acid Reductase
WHO	World Health Organization

Chapter One

Introduction

Chapter One

1.1 Introduction

Rice, *Oryza sativa* L., is an important summer grain crop in the world It is the third after wheat and barley and is grown in the countries of Asia, America, Australia, Africa and Europe, as it belongs to the Poaceae family and is the main food for more than half of the world's population. Its grains contain starch, protein, fat, iron, calcium, phosphorus and vitamins (Motlagh, 2011). It is impossible to prevent the quality and quantity of rice from being lost due to microorganism seed borne fungiion in storage conditions that are not suitable (Oh *et al.*, 2007). The rice crop faces many problems during harvesting, after harvest and storage, and the most prominent of these problems are fungal infections, as the *Aspergillus* fungus is at the forefront of fungi that attack rice grain due to its ability to grow and produce spores when the crop is exposed to moisture and high temperatures (Makun *et al.*, 2007).

Rice is typically cultivated in favorable climatic conditions that promote fungal infection and growth, resulting in potential mycotoxin seed borne fungiion both before and after harvest (Sales and Yoshizawa, 2005), However, compared to numerous other grains (Tanaka *et al.*, 2007).

Microorganisms play an important role in affecting the quality of seed of which fungi are the largest group. These pathogens are disastrous as they reduce seed vigor and weaken the plant at its initial growth stages, Controlling seed-borne diseases caused by fungi is comparatively challenging due to the establishment and dormancy of fungal hyphae. In addition to being seed-borne pathogens, fungi can also grow on stored products (wheat, corn, rice etc) (Uma and Wesely, 2013).

Fungi have the potential to reduce seed viability, leading to seed discoloration and the production of harmful toxins. Stored rice grain are particularly susceptible to fungal attacks, especially under moderate temperatures and high humidity. The spoilage of stored rice can be attributed to the presence of storage fungi introduced during post-harvest handling processes (Javaid *et al.*, 2002). These microorganisms can cause significant economic damage by spoiling food, resulting in undesirable tastes, discoloration, and the breakdown of food structures. Moreover, fungi show a serious function in food spoilage through the production of toxic secondary metabolites known as mycotoxins (Blagojev *et al.*, 2012).

Mycotoxins exist hazardous secondary metabolites supplied by filamentous yeasts that establish a threat to individual and animal condition (De Ruyck *et al.*, 2015). They naturally seed borne fungie various important plant products such as oil grains, dried fruits, nuts, spices, and grains like rice, corn, wheat, and barley (Kamkar *et al*., 2014; Mozaffari *et al*., 2014) . The most well-known mycotoxins established on their incident in diet garners and their toxicological significance involve deoxynivalenol (DON), fumonisins (FUM), ochratoxin A (OTA), zearalenone (ZEN), aflatoxins (AFS), and citrinin (CIT)(De Ruyck *et al.*, 2015). To date, over 400 mycotoxins have been identified (Zain, 2011).

Depended on the level and kind of mycotoxin consumed, as well as the rate of contact, mycotoxicosis may arise. The toxicologic effects can scale starting severe stomach discomfort and vomiting to constant conditions like immune system repression, enlarged liver, growth impairment, cancer, and, in extreme cases, death (Ayeni *et al.*, 2021). Grains for example rice, corn, millet, and sorghum molasses are frequently seed borne fungied by mycotoxins(Abdus-Salaam *et al.*, 2016).

In most nations, the use of plants in medicine is widespread and common, predating the introduction of modern drugs like antibiotics. Throughout the world, the crude components of plants and their extracts are utilized in medicinal products. It is also estimated that a quarter of current medications are derived from medicinal plants, with flowering plants comprising a significant portion. More than 250,000 varieties of flowering grains serve as resources for developing new drugs. Traditional medicines continue to be relied upon by over 80% of the global population for various ailments, and ordinary produce have been employed for medical purposes worldwide on behalf of thousands of years (Mohammed *et al.*, 2020).

Medicinal plants and microorganisms contain various classes of biologically active compounds such as Alkaloids, Terpenoids, Glycosides, Phenols and flavonoids (Chitemerere and Mukanganyama, 2011). Plant extracts have emerged as a potential alternative for preventing and treating fungal diseases as they have shown inhibitory effects on the growth of a wide range of fungi. This is particularly important given that the resistance of pathogenic fungi to antifungal drugs is a major public health problem (Shirwaikar *et al.*, 2010).

1.2 Problem of the Study

Fungi can lead to decreased the seed viability,its quality, and efficacy of bioactive compounds when stored.

1.3 Aims of the Study

Evaluation the antifungal activity of *Saussurea costus* against fungi isolated from rice grains, Molecular identification, and mycotoxin genes detection.

1.4 Objectives of the Study

1. Collection of rice grain from local markets in Hilla city
2. Isolation and Morphological identification to Fungi responsible for grains deterioration of grain during storage**.**
3. Molecular identification to Fungi responsible for grains deterioration of medicinal plants during storage and their myco toxins.
4. Sequence analyses DNA to some pathogenic fungi responsible for grains spoilage during storag.
5. Molecular detection mycotoxin genes of *Aspergillus* isolates producing mycotoxins (Aflatoxin, Ochratoxin, and Fumonosine).
6. Extraction of secondary metabolites compounds such as Flavonoid, Alkaloid, and terpenoid from medicinal plant *(Saussurea costus)* in order to biological control to Fungi Responsible for grain deterioration during storage.
7. Antifungal Efficacy of the crude Alkaloid, Flavonoid, and Terpenoid Extracted from *Saussurea costus* (*Falc*.) Lipschitz Roots against *Aspergillus* isolated from Rice Grains

Chapter Two

Literature Review

Chapter Two Literature Review

2. Literature Review

2.1 Oryza sativa

Scientific classification

Kingdom: Plantae

Phylum: Tracheophyta

Class: Liliopsida

Order: Poales

Family: Poaceae

Genus: *Oryza*

Species:*Oryza sativa* L.(Pullaiah and Galbraith, 2023) .

Rice belongs to Gramineae, there are only two species of cultivated rice in the world, Asian rice (Oryza sativa) and African rice (Oryza glaberrima). Oryza sativa produces higher yield than African rice, and softer grain easier to mill(Leewijit *et al.*, 2016).

As a grain grain, domesticated rice is the most widely consumed staple food for over half of the world's human population(Geng *et al.*, 2021), particularly in Asia and Africa. Rice,

a monocot, is normally grown as an annual plant, although in tropical areas it can survive as a perennial and can produce a ratoon crop for up to 30 years. The traditional method for cultivating rice is flooding the fields while, or after, setting the young seedlings. This simple method requires sound irrigation planning, but it reduces the growth of less robust weed and pest plants that have no submerged growth state, and deters vermin. While flooding is not mandatory for the cultivation of rice, all other methods of irrigation require higher effort in weed and pest control during growth periods and a different approach for fertilizing the soil (Iqbal, 2022) .

The rice plant can grow to 1–1.8 m (3–6 ft) tall, occasionally more depending on the variety and soil fertility. It has long, slender leaves 50–100 cm (20–40 in) long and 2–2.5 cm (3⁄4–1 in) broad. The small wind-pollinated flowers are produced in a branched arching to pendulous inflorescence 30–50 cm (12–20 in) long. The edible seed is a grain (caryopsis) 5–12 mm (3⁄16–15⁄32 in) long and 2–3 mm (3⁄32–1⁄8 in) thick. Rice is a grain crop belonging to the family Poecae. Rice being a tropical crop can be grown during the two distinct seasons (dry and wet) of the year provided that moisture is made available to the crop(Kawure *et al.*, 2022).

A study indicated (Leewijit *et al.*, 2016) Isolation of Soil and Endophytic Fungi from Rice (Oryza sativa L.)to identification fungi from rhizosphere soil and endophytic fungi from rice (Oryza sativa L.). thase results showed that rhizosphere soil fungi isolates found were 11 species as follows:- *Aspergillus* spp., *Penicillium* spp., *Trichoderma* spp., *Chaetomium* spp., *Curvularia* spp., *Fusarium* spp., *Gliocladium* sp., *Phytopthora* spp., *Rhizoctonia* spp., *Rhizopus* spp. *Xylaria* sp.

The assessment (Monajjem *et al.*, 2014) results showed , evaluation seed-born sungi of sice (*Oryza sativa* L.) and that effect seed quality, that among the seed-borne fungi, two species *A. niger* and *A. flavus* exhibited the highest severity.

2.2 *Saussurea costus*

Scientific classification

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: *Saussurea*

Species: *costus* (*Saussurea costus (Falc.)* Lipsch.)(Rathore *et al*., 2021).

The genus Saussurea contains around 400 species that are distributed worldwide. Atleast 300 species are found only in India, China and the Tibet region(Zhao *et al*., 2017) . Many species ofthe genus *Saussurea* are used in traditional medicines due to their biological properties ,*S. costus*, commonly known as 'AL_kust al_hinda', is an important medicinal species of this genus. Onthe Indian subcontinent, A. costus is distributed in parts of the western Himalayas andgrows at an altitude ranging from 2600 to 4000 m(Butola and Samant, 2010; Rathore et al., 2021) .

It is found abundantly in the Kashmirregion and is commercially cultivated in Uttarakhand, Himachal Pradesh and Sikkim. *A.costus* was observed extinction threats due to overexploitation for trade , The plantis a tall perennial herb reaching heights of up to 1 to 2.2 m and has dark-purple-coloredflowers arranged in terminal and axillary heads. The dried roots of A. costus are knownas Mu Xiang in the Traditional Chinese system of medicine(Kuniyal *et al*., 2015; Su *et al*., 2016) . The plant *A. costus* hasbeen extensively used in different traditional systems of medicine since ancient times, sonumerous pharmacological activities of this plant are found in the literature data ,*A. costus* rhizomes have been widely used in the treatment of various ailments, such asleprosy, cough and cold, malaria, persistent hiccups, stomachache, toothache, typhoidfever, chest congestion, etc , In addition, it is used as an antispasmodic in asthmaticpatients and also in the treatment of cholera, gout, erysipelas, etc , The compoundsisolated from A. costus have been found to be effective against a wide range of cancers,such as ovarian, pancreatic, prostatic, colon and bladder cancer, leukemia, etc, Otherimportant reported biological activities of *A. costus* include antimicrobial, antiulcer, hepato-protective and cardioprotective effects, among others (Bhushan *et al*., 2023) .

The water extracts are shown highly effective against *Aspergillus niger*, *A.favus* and *Candida albicans* tested, it exhibit this effect in all the extracts treatment, cold or hot when used for private high concentrations of them. As aresult, the extract of *S.costus* on a hot treatment is demonstrated an effective on the fungus *A.niger*, and the S.*costus* cold for both types of *S.costus* was a highly effective from a hot treatment for *A.flavus*(ALkattan and AL-Sheikh, 2011).

Also, the growth rate of Candida albicans have been affected by ail treatments of Costus extract, the methanol extract of roots of S. costus are rich in some bioactive phytochemical compounds such asalkaloids, phenols/polyphenols, flavonoids, terpenoids, tannins, coumarins, quinines, steroids, cardiac glycosides and resins. The antimicrobial screening revealed that, among 12 referenced microbial isolates 10 bacteria and 2 fungi , (4 Gram-positive bacteria exhibited high susceptibility with the methanol and ethanol extracts of *S. costus*, namely(Bacillus cereus ATCC 10876 , Staphylococcus), (saprophyticus ATCC 43867 ,Staphylococcus) , (epidermidis ATCC 12228 and Staphylococcus aureus ATCC 29213) Also, 1 fungal strain (*Aspergillus* niger ATCC 6275)(Abdallah *et al.*, 2017).

2.3 Secondary plant metabolites

Various biological effects have been demonstrated by secondary metabolites, forming the scientific foundation for the use of herbs in traditional medicine by ancient communities. These metabolites possess antibiotic, antifungal, and antiviral properties, allowing them to safeguard plants against pathogens. Moreover, they serve as crucial compounds that absorb UV radiation, thereby preventing significant leaf damage caused by light exposure(Adiloğlu, 2018).

Natural products, including organisms such as plants, animals, or microorganisms, exhibit health benefits for both humans and animals. In

developing countries, it is estimated that 80% of the population still relies on traditional or folk medicines derived mainly from plants for disease prevention and treatment. Traditional medicine using plant extracts has proven to be more economically viable, clinically effective, and associated with fewer side effects compared to modern drugs. The pharmaceutical industry has increasingly focused on exploring the phytochemical constituents of medicinal plants as evidenced in literature.

Plant-derived secondary metabolites encompass a wide range of small molecules or macromolecules synthesized in plants, including steroids, alkaloids, phenolic compounds, lignans, carbohydrates, and glycosides. These substances possess diverse beneficial biological properties for humans such as anti-allergic, anticancer, antimicrobial, anti-inflammatory, antidiabetic and antioxidant activities related to diabetes mellitus (Tran *et al*., 2020).

One of the main public health concerns is the resistance of pathogenic fungi to antifungal drugs. However, there is evidence to suggest that plant extracts can be effective in inhibiting the growth of a broad range of fungi . In terms of preventing and treating fungal diseases, natural sources can provide a beneficial alternative. Hence, it is important for humans to seek out environmentally-friendly and less harmful means of controlling fungi, with the ultimate aim of minimizing the utilization of fungicides and pesticides (Al-Snai, 2019).

Medicinal plants have a wide range of pharmacological effects, and these effects primarily depend on the chemical components found in these plants. Generally, there are two categories of phytochemical constituents in plants: primary and secondary metabolites. Primary metabolites play a role in fundamental metabolic processes and are present in all living cells to some extent. On the other hand, secondary metabolites are produced through subsidiary pathways like the shikimic acid pathway.

When studying the medicinal properties of herbal remedies, the focus is mainly on these secondary metabolites. Traditional medicine and folk remedies have

long recognized the important role of secondary plant metabolites in treating various ailments. In modern medicine, these compounds serve as starting points for developing medications to treat a wide range of diseases, including migraines and cancer (Hussein and El-Anssary, 2019). Czapek later referred to them as end-products (Bourgaud *et al.*, 2001). Suggesting that they are derived from nitrogen metabolism through what he called 'secondary modifications,' such as deamination.

Different classes exist for classifying secondary plant metabolites based on their chemical structures:

2.3.1 Flavonoids

In nature, flavonoid compounds are products extracted from plants and they are found in several parts of the plant. Vegetables for their growth and defence against plaques (Havsteen, 2002) . Use flavonoids groups of small phenolic compounds that are widely found in plants, called low-molecular-weight phenolic compounds which are part of the plant kingdom. They form one of the most distinct classes of compounds in higher plants. Numerous angiosperm families easily recognize many flavonoids as pigments for flowers. Nevertheless, these compounds are not limited to flowers but exist in all parts of plants(Dewick, 2002). Flavonoids can also be abundantly found in plant-derived foods and beverages like fruits, vegetables, tea, cocoa, and wine. Therefore, they are known as dietary flavonoids. There are various subgroups within flavonoids including chalcones, flavones, flavonols, and isoflavones. Each subgroup has its own primary sources. For instance, onions and tea are significant dietary sources of flavonols and flavones.

Flavonoids in bacteria, animals, and especially plants hold a multitude of biological functions. They are generated at distinct locations within plants specifically. Their contribution extends to the pigmentation and scent of blossoms while also aiding in pollinator attraction and facilitating the spread of

fruits for seedlings' growth (Griesbach, 2005). Beyond that, flavonoids participate in seed and spore sprouting.

They also serve as defensive agents against various plant stressors both living (biotic) and non-living (abiotic) while simultaneously acting as potent filters for ultraviolet radiation (Takahashi and Ohnishi, 2004), Roles of flavonoids extend to functioning as signaling molecules, compounds causing harm to other organisms (allelopathic compounds),disease-resistant substances (phytoalexins), detoxifiers, and compounds defending against microbes , Moreover, they've been discovered to enhance resistance to frost and drought conditions. These compounds may even have a functional part in adapting plants to heat and increasing their tolerance to freezing temperatures (Samanta *et al.*, 2011).

2.3.2 Alkaloids

Alkaloids are naturally occurring compounds that contain heterocyclic nitrogen. They are derived from primary metabolites such as tryptophan, tyrosine, and lysine, and their biosynthesis pathways can be quite complex, resulting in intricate chemical structures. Alkaloids have been used for medicinal purposes for over 3000 years, with applications including purgatives, antitussives, and sedatives for treating snakebites, fever, and mental illness.

The plant kingdom is abundant with over 5500 distinct alkaloids, making it the most substantial category of secondary metabolites. These substances display a variety of impacts and are utilized in healthcare, recreational drugs, and spiritual ceremonies. Certain alkaloids such as papaverine and indoquinoline exhibit inhibitory effects on viruses, bacteria, and yeast. Quinine, another representative of alkaloids, is recognized for its anti-malaria abilities.

The pharmacological potentials of these alkaloids encompass local anesthesia effects, analgesia provision, stimulation of cardiac and respiratory

systems, muscle relaxation potentialities, vasoconstriction phenomena as well as toxicity. Additionally, they demonstrate hypertensive, antineoplastic and hypotensive attributes. Documented evidence exists about their effectiveness against herbivores, cytotoxic properties in vertebrates alongside mutagenic or carcinogenic tendencies. Alkaloids also offer antifungal benefits along with molecular targeting capacities, antibacterial actions, allelopathic traits and antiviral competencies. Ingestion of numerous alkaloids can lead to fatal outcomes in animals. Insecticides often deploy alkaloids like nicotine and anabasine(Hoffmann, 2003; Seigler, 1998).

Acting as protective shields within plants against herbivores and disease-causing organisms constitutes another role played by alkaloids. Considering their powerful biological activity approximately 12,000 known types have found applications in pharmaceuticals as stimulants or narcotics while some are employed as toxins. The practice of using plants rich in these compounds for creating dyes or spices or for medicinal purposes or producing poisons can be traced back to ancient times (Jain *et al.*, 2019; Okunade *et al.*, 2004).

2.3.3 Terpenoids

Terpenes and terpenoids, which are a diverse group of naturally occurring compounds in plants, are classified based on the number of isoprene units they contain. They can be mono, di, tri, tetra, or sesquiterpenes. Abundant in plants, terpenes are the major components of essential oils derived from plants like tea, cannabis, thyme, citrus fruits, and Spanish sage(Lane and D'Mello, 2019) . These compounds serve various functions in plants including signaling, pigmentation, solvent properties, flavoring, and medicinal applications (Yang *et al.*, 2012). Furthermore, terpenes have numerous therapeutic effects such as anticancer, antimicrobial, antiviral, antifungal, analgesic, antihyperglycemic properties. They also exhibit antiparasitic and anti-inflammatory effects while enhancing skin penetration and preventing inflammatory diseases. In medicine,

terpenes are utilized for the production of various medicinal drugs (Franklin *et al.*, 2001). They also act against pathogens, herbivores as well as mycorrhiza and pollinators (Falara *et al.*, 2014). They also act against pathogens, herbivores as well as mycorrhiza and pollinators (Lane and D'Mello, 2019). Plants produce hundreds of different terpenoid compounds with a wide range of characteristics such as functioning as phytohormones or antioxidants while also modifying proteins (Pichersky and Raguso, 2018) .

Phylogenetically, terpenoids are conserved and play a role in attracting organisms.

Humans have utilized several terpenoid compounds, including b-carotene, to produce important substances like vitamin A. Terpenes found in food greatly influence our eating habits. In food manufacturing, terpenoids like bixin, astaxanthin, and lycopene are used as pigments. Volatile terpenoid compounds contribute specific flavors to food; for example, zingiberene is responsible for the ginger flavor. Many herbs such as lemon grass and spices like saffron contain volatile terpenoids as their primary flavor components. Alcoholic drinks also contain these compounds(Stewart, 2013).

In 2002, the global revenue from pharmaceutical drugs containing terpenes reached approximately 12$ billion. Among these medications are Artemisinin, an antimalarial drug, and Taxol®, an anticancer drug. Terpenoids have a wide range of uses in combating cancer, inflammation, malaria, and various infectious diseases caused by bacteria and viruses. The marine environment has yielded numerous terpenoid compounds with diverse structures and bioactivities, with more discoveries anticipated in the future. While total chemical synthesis plays a significant role in producing certain terpenoid drugs, it has also contributed greatly to the advancement of terpene-based medications (Wang *et al.*, 2005).

Terpenes, or isoprenoids, consist of a regular arrangement of isoprene units connected head-to-tail. The terpene family includes important compounds

such as squalene (an unsaturated hydrocarbon found in humans and sharks), as well as the side chains of vitamins A, E, and K. Terpenes find applications in the production of fragrances, cosmetics, insect repellants, perfumes, and also possess various therapeutic uses (Kandi *et al.*, 2015). Many of these compounds exist naturally in small quantities. However, advancements in biology and metabolic engineering have provided strategies to generate sufficient quantities of terpenoids for pharmaceutical purposes (Proshkina *et al.*, 2020).

2.4 Fungi associational grains rice:

During the storage of rice grain , the most commonly isolated fungal species belong to the genera *Aspergillus* and *Penicillium*. Some of these species have the ability to produce mycotoxins, which can have detrimental effects on the health of humans and animals. One of the most notorious mycotoxin-producing by species *Aspergillus flavus*, which is responsible for producing highly carcinogenic aflatoxins. (Mannaa and Kim, 2016).

The major fungi associated with rice grains were: *Curvularia lunata,Bipolaris oryzae* , *Aspergillus flavus*, *Rhizopus* , *F. oxysporum* and *Fusarium moniliforme* (Aidoo *et al.*, 2015).

There are also fungal species that can tolerate low temperatures known as psychrophiles, and others that can bare high temperatures referred to as thermophiles While after harvest, hygrophilic fungi disappear as mesophytic and xerophytic fungal species such as *Aspergillus* spp. and *Penicillium* spp., germinate, grow and produce mycotoxins at relative humilities of 80 to 90%, and 80% and less, respectively (Mannaa and Kim, 2017). A previous study conducted by, (Oh *et al.*, 2007) examined the occurrence of fungi and bacteria on rice grain stored in rice-processing facilities. They found that *Penicillium* and *Aspergillus* were the dominant fungal genera, despite significant variations in fungal diversity across different regions.

In another study focusing on microbial communities and aflatoxin seed borne fungiion, three main species of *Aspergillus* were identified: *A. flavus, A. c*and*idus,* and *A. fumigatus* (Sang *et al* ., 2010).

These findings were consistent with other studies, which identified *Aspergillus*, *Fusarium*, and *Penicillium* as the predominant genera (Taligoola *et al.*, 2004), and *A. c*and*idus* and *A. flavus* as the predominant species (Park *et al.*, 2005).

Furthermore, it has been widely noted that various *Aspergillus* species, with special emphasis on *A. flavus*, are typically found residing in stored food commodities; grains are predominantly affected. The ubiquity of *A. flavus* is demonstrated on a global scale; the fungus is known to infiltrate rice crops universally.

A more comprehensive understanding of *A. flavus* reveals its tendency to flourish as a saprophyte - an organism that feeds on dead or decaying organic material - within soil environments. (Kim *et al* ., 2008). This ecological niche allows it to form associations with diverse types of decomposing organic substrates. This particular species exhibits impressive adaptability due to its capacity to endure extreme variations in temperature and remarkably low humidity levels. These traits permit it to proficiently colonize the exterior surface of stored rice grain . In addition to targeting rice crops, there is also evidence indicating that other agricultural products such as peanuts, maize and tree nuts are susceptible to infection by *A.flavus*.

Another significant factor contributing to the severity of seed borne fungi on caused by this fungus is the production of aflatoxin - a toxic secondary metabolite derived from *A.flavus* (Taligoola *et al.*, 2004).

2.5 General Characteries of *Aspergillus*

Micheli of Florence was the first to recognize the genus *Aspergillus* in 1729, when he saw a likeness between an *Aspergillus* . sporulating head and an aspergillum that used to sprinkle holy water. In 1856, Virchow published the first precise microscopic descriptions of the creature (Bennett *et al.*, 2019).

Kingdom: Fungi

Division: Ascomycota

Class: Eurotiomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: *Aspergillus*

Aspergillus niger, a filamentous fungus, exhibits plant-like characteristics with its hyphae. The initial color of their growth is white but transforms into black within a few days, indicating the production of conidial spores. Pale yellow borders and radial fissures can be observed in the colonies. Microscopic identification of *Aspergillus niger* involves examining its smooth, colored conidiophores and conidia. Conidiophores are projections stemming from a septate and hyaline hypha, while the conidial heads display a radial pattern and split into biseriate columns. The conidiophore vesicle generates sterile medullae cells that support phialides on the conidiophores. These conidiophores range in length from 400-3000um, appearing smooth and hyaline. They darken at the apex and terminate in a globose vesicle measuring 30-75um in diameter. Covered by medullae and phialides, the vesicle produces rough-textured, dark brown conidia with a diameter of 4-5um (Faith,. 2021)

Aspergillus species like *A. niger* and *A. flavus* commonly seed borne fungie agricultural products throughout different stages: pre-harvest, harvest, processing, and handling. These fungi can induce alterations in sensory perception, nutrition content, and quality aspects of products including

pigmentation disparities, discoloration, decay, off-odors, and off-flavors. Nonetheless, the most worrisome consequence associated with their presence is the seed borne fungiion of foods and feeds with mycotoxins. Other *Aspergillus* species such as *A. parasiticus*, *A. ochraceus*, *A. carbonarius*, and *A. alliaceus* also have potential to seed borne fungie agricultural goods. Typically encountered as storage molds on plant-based items, these opportunistic pathogens pose a threat (Kozakiewicz, 1989).

Produce by the genus *Aspergillus* and its members several mycotoxins, which have a significant agricultural, epidemiological, and economic impact (Plascencia-Jatomea *et al.*, 2014). This genus of fungi produces important secondary metabolites with industrial value (Singh *et al.*, 2017) , and therapeutic significance like lovastatins and antibiotics (Vadlapudi *et al.*, 2017).

Several clinical presentations were seen, such as an asthma exacerbation, a fungus ball in the sinus chronic invasive, granulomatous sinusitis and otomycosis, , keratitis and endophthalmitis , cutaneous and wound infections, infections invasive pulmonary aspergillosis, and osteoarticular. *Aspergillus* was the most common cause of the disease(Gautier *et al.*, 2016). The filamentous fungus *Aspergillus* comprises approximately 340 officially identified species, with only a small number of them being utilized for large-scale enzyme and food fermentation, organic acid, and bioactive chemical synthesis (Park *et al.*, 2017).

High moisture content during storage and the presence of insects and mites can cause seed borne fungiion of corn grain by *Aspergillus* , leading to reduced quality and nutritional value of the grain and mycotoxin seed borne fungiion. Toxigenic *Aspergillus* are the primary cause of mycotoxin seed borne fungiion, which can have detrimental effects on human and animal health due to its ability to persist in the food chain (Pattron, 2006).

2.6 Growth and Distribution of *Aspergillus* spp.

The group *Aspergillus* comprises fungi in their asexual state, commonly known as conidial fungi, although some have a teleomorph or sexual state in the Ascomycota. DNA evidence confirms that all *Aspergillus* species belong to the Ascomycota(Klich, 2002) . *Aspergillus* species are versatile microorganisms that can thrive in a wide range of environments, including those with high osmotic pressure caused by high concentrations of sugar, salt, or other solutes. These fungi are highly aerobic and can be found in nearly all oxygen-rich habitats, where they typically grow as molds on the surface of a substrate due to the high oxygen tension. *Aspergillus* are known to utilize a variety of carbon-rich substrates, including monosaccharides like glucose and polysaccharides like amylose. As common contaminants of starchy foods, such as bread and potatoes, *Aspergillus* can be found growing on or within many plants and trees (Klich and Pitt, 1988).

Many species of *Aspergillus* exhibit oligotrophy, showcasing their ability to thrive in environments lacking essential nutrients or nutrient-depleted settings. A prime instance of this is *A. niger*, which can be observed growing on moist walls as a significant constituent of mildew (Latgé, 1999).

2.7 Mycotoxins

The term mycotoxin originates from the Greek word ‘mycos’ which means mold, and the Latin word ‘toxicum’ that signifies poison (Smith *et al*., 2016). Mycotoxins are small-sized secondary metabolites synthesized by fungi that have a significant danger to humans and livestock. These metabolites are typically produced after a phase of balanced growth, commonly associated with developmental processes. In recent times, the seed borne fungiion of food, feed, and agricultural products by mycotoxins has evolved into a critical concern due to the potential health risks it presents. These risks range from acute to chronic health issues in animals and humans alike (Hussein and Brasel, 2001).

Toxigenic fungi are responsible for producing these secondary metabolites. They belong to the category of fungi species capable of generating one or more mycotoxins. Multiple fungal species can produce mycotoxins; for instance, aflatoxins and ochratoxins are generated by various fungal species. This contributes to the constant presence of mycotoxins throughout the year (Darwish et al., 2014; Hussein and Brasel, 2001) . mycotoxins have caused major epidemics in humans and animals The most important epidemics were : ergotism which killed hundreds of thousands of people in Europe at 943 AD was caused via the consumption of rye seed borne fungied with the 'ergot alkaloids' , produced by the fungi Claviceps purpurea that was first report of mycotoxin (Pitt *et al.*, 2000).

An outbreak of Beriberi occurred in the Japanese army during the Chinese-Japanese War (1894-1895) due to fungal toxins found in rotting rice. The fungus responsible for this condition was not identified until 1940 when *Penicillium* citro-viride was isolated from the affected rice, and the same symptoms were replicated experimentally. The existence of mycotoxins was initially recognized in the early 1960s after a puzzling "Turkey X disease" resulted in the death of about 100,000 turkey poults in England. The disease was linked to *A. flavus*-seed borne fungied peanut (groundnut) meal, and the toxic substances were named aflatoxins(Tabuc *et al.*, 2011).

Mycotoxins can be found in various types of food and feed, and their presence has been linked to numerous diseases in both humans and animals. Exposure to mycotoxins can lead to acute or chronic toxicity, ranging from fatal outcomes to harmful effects on the central nervous, cardiovascular, pulmonary, and digestive systemsو as well as on the alimentary tract, Mycotoxins can be mutagenic ,cancerous , and immunosuppressive teratogenic (Chen *et al.*, 2015). It is widely accepted that certain mycotoxins compromise the immune response and thus reduce resistance to infectious disease, which is considered the most significant impact of mycotoxins, particularly in developing nations.

The minimum amount of mycotoxins required to cause harmful health effects can differ considerably depending on the type of toxin and an individual's immune system (Okusa Ndjolo *et al.*, 2009).

2.8 Conditions of Fungal Growth and Mycotoxin Production

Mycotoxigenic fungi are prevalent pathogens that exist in agricultural regions globally. They have the ability to seed borne fungie and proliferate on a diverse range of crops, and their adaptability allows them to generate mycotoxins under various circumstances, including different environmental factors (Richard *et al.*, 2003).

The risk of fungal growth and mycotoxin production is escalated by high temperature and humidity in the surroundings. Seed borne fungiion can also be influenced by pH, fungal strain, substrate, and climate-related elements (Daou *et al.*, 2021). Predominant environmental conditions, particularly humidity and temperature on the field, significantly affect the activity and colonization of fungi. These factors affect the development, survival, distribution, frequency of mycotoxigenic fungi, as well as their subsequent accumulation of toxins (Doohan *et al.*, 2003).

Temperature and humidity can additionally affect plant growth, strength, health, as well as the competitiveness of mycotoxigenic fungi (Pitt *et al.*, 2000). Each fungus exhibits a specific optimal range for temperature and water activity necessary for its growth, germination, and production of mycotoxins. Fungal invasion may occur at various stages such as during cultivation in the field or during drying or storage due to favorable humidity and temperature conditions. In certain instances, it might not be feasible to precisely determine the initial occurrence of fungal growth (Perdoncini *et al.*, 2019).

After harvesting, hygrophilic fungi cease to exist. Instead, mesophytic and xerophytic fungal species like *Penicillium* Spp and *Aspergillus* Spp sprout

and develop under relative humidities of 80% to 90%, and 80% or lower respectively (Dix *et al.*, 1995).

The environment's pH plays a crucial role in the growth of fungi and the production of mycotoxins. Fungi can regulate their surroundings' pH by releasing acids or alkalis. For instance, *Penicillium* and *Aspergillus* acidify their environment by discharging gluconic and citric acids (Mannaa and Kim, 2017). The environment's pH plays a crucial role in the growth of fungi and the production of mycotoxins. Fungi can regulate their surroundings' pH by releasing acids or alkalis. For instance, *Penicillium* spp. and *Aspergillus* spp. acidify their environment by discharging gluconic and citric acids (Vylkova, 2017).

The toxicity of fungal species differs, and the production of mycotoxins is occasionally limited to certain types of fungi, and in many cases, it is even restricted to specific isolates within a species (Nicholson, 2004) . Despite the uncertain exact cause for the prevalence of mycotoxigenic fungi on specific food items, these fungi can grow on different substrates because they find essential nutrients like carbon and nitrogen commonly available in food items—especially those with high carbohydrate content (Kokkonen *et al.*, 2005). Various factors present in a substrate, including pH, temperature, composition—particularly the presence of simple sugars—interact to influence mycotoxin production (Özcelik and Özcelik, 1990).

Several factors in a substrate can impede fungal growth, germination, and mycotoxin production. The absence of a single promoting factor can hinder fungal development. Numerous studies have demonstrated the substantial impact of osmotic pressure in the substrate on fungal growth, mycotoxin production, and physiological responses (Duran *et al.*, 2010).

2.9 Aflatoxins

Aflatoxins are secondary metabolites produced naturally by some species of the genus *Aspergillus* , such as *A. flavus*, *A.parasiticus*, and others (Levin, 2012). When grown in various types of food and grain crops (Lee *et al.*, 1992). it pollutes many foodstuffs and fodder. It has carcinogenic effects on the liver (Van Egmond and Jonker, 2004). The discovery of aflatoxins in the 1960s, which represented a historic turning point for the detection of mycotoxins and Davis (Diener *et al.*, 1987).

Aflatoxin is an acronym consisting of the letter "A" indicating the genus *Aspergillus*, the three letters "AFL" indicating the type flavus, and the syllable "TOXIN" meaning poison (Rustom, 1997). Four main types of aflatoxin were found: (AFB_1 and AFB_2 derived from the word blue) because they glow blue when examined under ultraviolet radiation, and AFG_1 and AFG_2 (derived from the word green) they glow green. These four species were spun for the first time by (Hartley *et al.*, 1963) of fungi *A.flavus* and *A. parasiticus* Most human exposure to aflatoxins comes from seed borne fungiion of maize, peanuts and rice and *A.flavus* produces aflatoxins AFB_1 and AFB_2 (Orum *et al.*, 1999) and *A.parasiticus* produces aflatoxins AFB_1 AFB_2 , AFG_1 AFG_2 (Agarwal and Sinclair, 1996; Farag et al., 1987). Aflatoxins vary in their toxicity and presence in food and feed, respectively ($AFG_2 < AFB_2 < AFG_I < AFB_1$) (Turcksess and Wood, 1997) .

Reported that AFB1 is produced in very large quantities by fungi *A.flavas* compared to other fungi, aflatoxin AFB_1 is secondary metabolites of fungal *Aspergillus* growth, harvesting, storage, and transportation (Khanafari *et al.*, 2007). It is the most toxic aflatoxin. It causes hepatocarcinogenic liver cancer (Bennett, 2003). It has been classified under Category 1 (Group 1) of human carcinogens(humans, 2012) .

The biotransformation of aflatoxins AFLB B_1 and AFLB B_2 in many animal species results in the production of aflatoxin M_1 and aflatoxin M_2, respectively. These aflatoxins (AFM_1) and (AFM_2) were first isolated from the milk and urine of animals fed aflatoxins(Allcroft and Carnaghan, 1963).

2.10 Ochratoxins

Ochratoxin A, often abbreviated as OTA, is a toxic compound produced as part of the secondary metabolic processes in several filamentous species that belong to the *Aspergillus* and *Penicillium* genera. ((Bredenkamp et al., 1989; Kuiper-Goodman and Scott, 1989; Trenholm and Miller, 1994).OTA was isolated in 1965 from a culture of *Aspergillus ochraceus* (section Circumdati) , The initial discovery of Ochratoxin A dates back to the year 1965 when it was found present within a culture of *Aspergillus ochraceus* (section Circumdati), marking an important milestone in our understanding of mycotoxins. Despite this initial discovery being limited to one single fungal species, successive research endeavours have expanded our knowledge significantly.) Van der Merwe *et al.*, 1965(who published their findings in 1965, have demonstrated that multiple species within these two crucial fungal genera—*Aspergillus* and *Penicillium*—are capable of producing ochratoxins.

The biosynthetic process through which Ochratoxin A is produced is worth noting as well. This toxin is derived from pentaketides—a family of organic compounds known for their diverse range of biological activities, which originate from another class of compounds known as dihydrocoumarins. Furthermore, these pentaketides are combined with β-phenylalanine—another essential amino acid—in the biosynthetic process leading up to the formation of OTA. To sum up this detailed description with a scientific insight into its chemical structure :

L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl)carbonyl]-(R)-isocoumarin.

The toxicological status of OTA has been investigated multiple times and was the topic of a comprehensive report by the IARC (International Agency for Research on Cancer) in 1993 (Organization and Cancer, 1993). After the identification of spontaneous nephropathies in humans and animals, numerous experimental studies were conducted to demonstrate OTA's involvement in these conditions (Nephrotoxicity, Neurotoxicity, Teratogenicity, Immunotoxicity, Carcinogenesis) (Otteneder and Majerus, 2000; Zimmerli and Dick, 1996).

These investigations indicated that this compound can have various effects such as hepatotoxicity, nephrotoxicity, teratogenicity, immunotoxicity, and neurotoxicity on different animal species. It can induce liver and kidney tumors in mice and rats. However, its toxicity varies based on the species, sex, and cell type of the tested animals (O'Brien *et al.*, 2001). Although findings from microbial and mammalian tests are conflicting, the genotoxic status of OTA remains a subject of debate. Nevertheless, chronic exposure of rats and sub-acute exposure of pigs to OTA have been found to lead to the formation of DNA-adducts (Faucet *et al.*, 2004).

2.11 Fumonisins

Fumonisins are a type of toxin generated by various *Fusarium* spp., which include *Fusarium proliferatum* and *Fusarium verticillioides*. These toxins share a similar structure with sphingosine, the primary backbone of cellular sphingolipids. They have been proven to competitively inhibit sphinganine and sphingosine N-acyltransferase, according to(Marasas, 2001).

Fusarium verticillioides, an economically significant species, can grow within both vegetative and reproductive tissues of corn as an endophyte. This growth often occurs without any apparent signs of disease in the plant. The presence of this species has been detected in nearly all corn samples, including those from Nigeria and South Africa (Ncube *et al.*, 2011). However, depending

on various factors such as weather conditions, insect damage, fungal and plant genotype, it has the potential to result in seedling blight, stalk rot, or ear rot (Czembor *et al.*, 2010).

The production of fumonisin by *F. verticillioides* is associated with adverse effects on different animals. In humans, it has been connected to esophageal cancer (Shephard, 2011), while horses and rabbits may experience illnesses like leukoencephalomalacia due to exposure (Giannitti *et al.*, 2011).

2.12 Mycotoxogenic *Aspergillus* Molecular Detection

The genus *Aspergillus* displays an impressive range of genetic diversity that, if compared to the divergence seen within protostomes, can be equated to the vastness of variation found within the phylum Vertebrata. This suggests a substantial breadth in genetic differentiation and adaptation across the species belonging to this genus.

Despite this, both inter and intra-specific genome structures display significant flexibility. Such flexibility is suggestive of adaptability and evolution over time in response to various environmental pressures or changes. Addressing specific species within the *Aspergillus* genus, it has been observed that some have a richer genomic profile than others do. For example, *A. flavus* and *A. oryzae* demonstrate a higher richness in their genomes, which are approximately 20% larger when compared to other species such as *A. nidulans* and *A. fumigatus*.

This discrepancy in genome size may be attributed to several mechanisms that contribute to changes in genomic structure over time: segmental duplication within the genome which leads to increased genetic material; entire genome duplication which results in significantly larger genomes; and horizontal gene transfer where genes are exchanged between organisms without being inherited directly from a parent organism. These processes work intermittently rather than

continuously, their activity supported by extensive research (Giannitti *et al.*, 2011) (Gibbons and Rokas, 2013).

A closer look at sequenced genomes of certain *Aspergillus* species reveals further differences. The size of the sequenced genomes ranges from approximately 29.3 Mb for *A.fumigatus* up to about 37.1 Mb for A. oryzae. In terms of genes predicted within these genomes, again there is marked variation with numbers ranging from roughly 9926 predicted genes for *A.fumigatus* up to an estimated 12,071 for *A.oryzae*. Moreover, one strain of *A.niger* known for its enzyme-producing capability uniquely possesses a medium-sized genome measuring at around 33.9 Mb (Bennett, 2010).

This finding adds yet another layer to our understanding of the complexity and diversity present within the *Aspergillus* genus.

The DNA sequences of numerous genes involved in the synthesis of these mycotoxins have been published subsequent to their identification. In the meantime, PCR methods for detecting aflatoxigenic isolates of Aspergilli using the gene encoding versicolorin A dehydrogenase (verl), the gene encoding norsolorinic acid reductase (nor1), the regulatory gene aflR, the gene encoding sterigmatocystin O-methyltransferase (omtA), and other genes have been described (Kim *et al.*, 2011).

Several studies have indicated that there is significant variability in the intergenic transcribed spacer (ITS) regions among different species of fungi. To detect fungi that produce mycotoxins, specific primer binding sites can be identified by PCR amplification of either ITS1, ITS2, or both regions in multiple mycotoxin producers and non-producers. The primers are designed to bind to conserved regions of the structural ribosomal RNA genes, as demonstrated in various studies (Grigoriev *et al.*, 2012).

Several mycotoxigenic fungi detection systems have been developed in recent years. There have been reports of PCR methods for detecting aflatoxigenic isolates produced by *Aspergillus, Penicillium* isolates producing

patulin, and *Fusarium* isolates producing trichothecene and fumonisin. Moreover, PCR assays are currently being developed to detect ochratoxin-producing fungi (Razavi *et al.*, 2011).

Prior research into OTA synthesis has predominantly revolved around the deactivation of individual genes or assessing the expression levels within an OTA gene cluster to understand their relevance to OTA production (Frisvad *et al.*, 2004).

Nevertheless, no investigation has so far probed which specific genes within an identifiable OTA gene cluster have been disabled. The particular genes or protein domains which are crucial for the creation of OTA are still uncertain13. PKS genes related to OTA have been discovered across a range of species known to produce OTA, such as *A. niger, P. verrucosum, A. ochraceus*, *P. nordicum, A. steynii* and *A. westerdijkiae* (Egbuta, 2012).

Some PKS and NRPS genes have been functionally characterized via gene deactivation and expression trials in species like *P. nordicum, A. niger*, P. verrucosum, *A. westerdijkiae* and *A. ochraceus* (Akey etal.,2012).

These characterizations have provided useful insight into potential involvement of certain genes in the biosynthesis of OTA.

A (AcOTAnrps) gene10 nonribosomal peptide synthetase, a (AcOTApks) gene polyketide synthase (Gallo *et al.*, 2014) and a halogenase (AcOTAhal) gene(Ferrara *et al.*, 2016) PCR-based methods using the OTA biRibosomal RNA gene have been linked to the diagnosis of OTA-producing fungi. The genes responsible for coding 18S, 5.8S, and 28S ribosomal RNA are separated by two intergenic transcribed spacer (ITS) regions, which are highly useful in molecularly distinguishing between fungal species. In this species, a synthetic gene cluster was identified (O'Callaghan *et al.*, 2013).

Chapter Three

Materials

and

Methods

Chapter Three

3. Materials and methods

3.1 Materials

3.1.1 Equipment and instruments

Table (3.1) The listed equipment and instruments.

No.	Equipment's	**Manufacturing company**
1	Autoclave	HIR Yama -Hve-50 (Japan)
2	Burner	Amal (Turkey)
3	Camera	Alptek (China)
4	Centrifuge	Centrifuge Series (Germany)
5	Compound Microscope	Miji Japan
6	Electrophoresis	Mupid-One Japan
7	Eppendorf	Sigma (England)
8	Hood	Lab Tech Germany
9	Hot Plate	Hana Romania
10	Incubator	Memmert Germany
11	Micro Centrifuge	Hettich (Germany)
12	Micropipette	Eppendorf (Germany)
13	Microscope Slides	Sail Brand (China)
14	Oven	Memmert (Germany)
15	pH meter	Extech (Taiwan)
16	Refrigerator	Vestel Europe
17	Sensitive Balance	Denver Swizer land
18	Thermocycler apparatus	Labnet Germany
19	UV- light	Labnet Germany

20	UV-Transullumnater	Quantum (France)
21	Vortex Mixture	LAB-MX-F (Germany)
22	Water Bath	GFL Germany
23	Water Distillator	GFL(Germany)

3.1.2 Chemicals and Biological materials

The chemicals and biological materials listed in Table (3.2) were utilized in this study.

Table (3.2) The chemicals and biological materials used in the study.

No.	Biological and Chemical materials	Manufacturing company(Origin)
1	Agarose	Intron (Korea)
2	Chloramphenicol	Samarra Pharmaceutical Factory(Iraq)
3	Chloroform	BDH (England)
4	DNA Marker	BDH (England)
5	EDTA	Promega(USA)
6	Ethidium bromide	Promega (USA)
7	Formaline	BDH(England)
8	Lacto phenol-cotton blue stain	Fluka(Switzerland)
9	Ladder 1000-100 bp	Intron (Korea)
10	Primers	Bioneer (Korea)
11	TBE buffer	Promega(USA)
12	TE buffer	Promega(USA)
13	Tris-HCL	BDH (England)

3.1.3 Culture Media

The culture media used in this study were prepared according to the instructions provided by the manufacturer as listed in Table (3.3).

Table (3.3) lists the culture media that were used in this study.

No.	culture media	Manufacturing company(Origin)
1	Potato Dextrose agar	Himedia (India)
2	Potato Dextrose broth	Himedia (India)

3.1.4 DNA Extraction Kit

The contents of the DNA extraction kit (FAVORGEN) used in the laboratory are shown in Table (3.4).

Table (3.4) The components of the FAVORGEN DNA extraction kit used in the laboratory.

No.	Material	Volume
1	Elution Buffer	15 ml
2	FA Buffer	120 ml
3	FB Buffer	65 ml
4	Lyticase solution	550 µl ×10
5	Proteinase K	11 ml
6	TG1 Buffer	45ml
7	TG2 Buffer	30ml
8	W1 Buffer	44 ml
9	Wash Buffer	20 ml

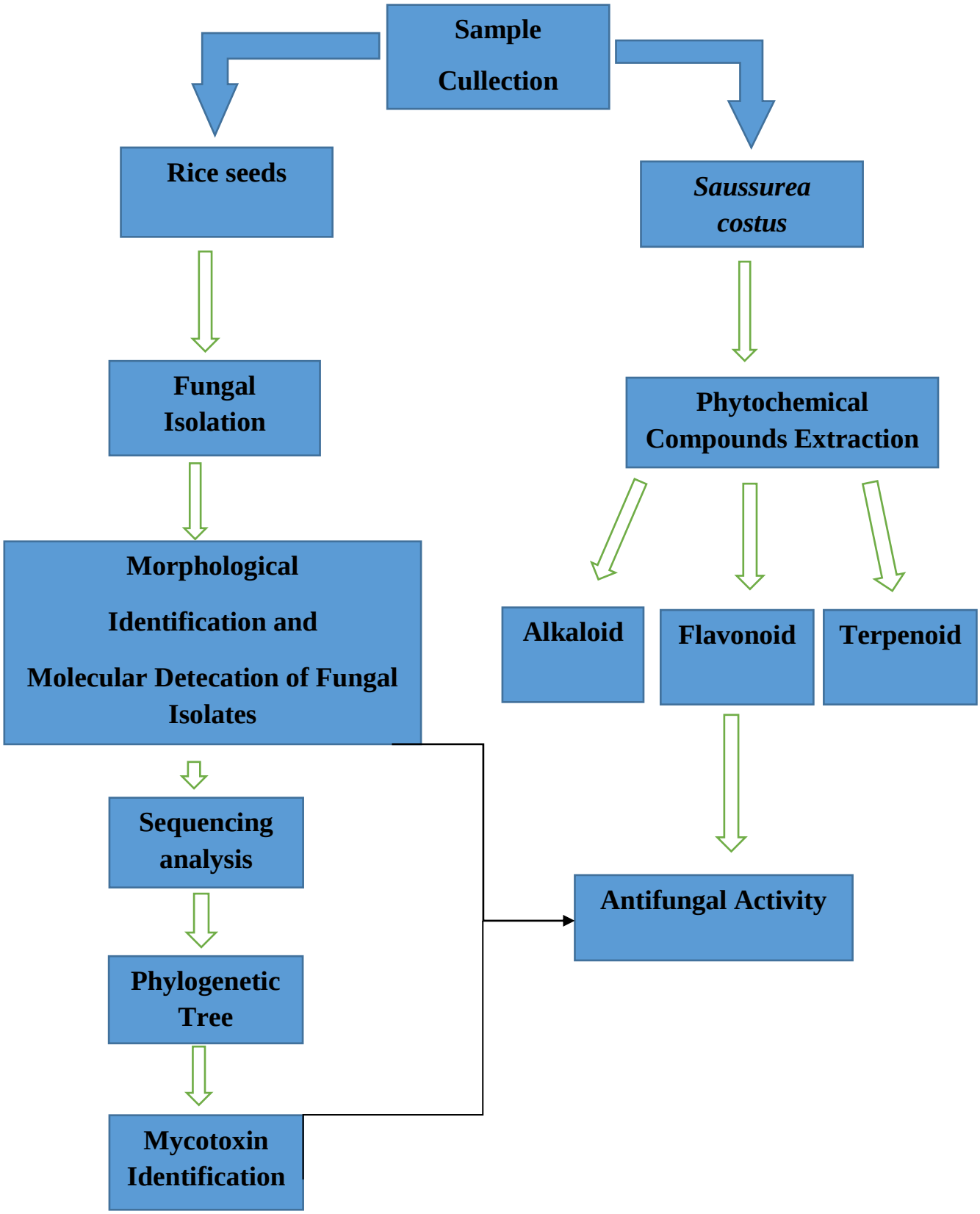
3.1.5 Primers Used in DNA Amplification

Table (3.5) shows the set of primer pairs used in this study to detect the presence or absence of *Aspergillus* and Mycotoxins genes, including Fumonisin (FB), Ochratoxin A (OTA), Ota gene, and Aflatoxin afl. Gene in selected isolates of *A. niger*.

Table (3.5) Set of primer pairs used in this study

Primer name	Sequence	PCR products	Author
fum10 F	F: GTCATTATTCCTCCGGCCCT	651bp	(Susca *et al.*, 2014)
fum10 R	R: TGGGATTCGAAAGCATACCG		
ITS1 F	F: TCCGTAGGTGAACCTGCG	420bp	(Patiño *et al.*, 2005)
NIG R	R: CCGGAGAGAGGGGACGGC nor2 TTGGCCGCCAGCTTCGACACTCCG		
ITS5	ITS5: GGAAGTAAAAGTCGTAACAAGG	600bp	(White *et al.*, 1990)
ITS4	ITS4: TCCTCCGCTTATTGATATGC		

3.2 Study Design



3.3 Methods

3.3.1 Sample collection

In 2022 October, a total of 90 stored rice grain samples were collected from various areas in Babylon Province/Hillh City in Iraq. The samples were purchased from different locations of home and local markets. Each sample, weighing 100 g, was placed in a new paper bag and immediately transferred to the laboratory for fungal determination. The samples were stored at room temperature.

3.3.2 Sterilization:

A. Sterilize by Autoclaving: all media and extraction tools, such as Eppendorf tube tips and PCR tubes, were subjected to autoclaving at 121°C and 1-5 lbs pressure for 15 minutes. Additionally, all glassware was sterilized by autoclaving at 200°C for two hours

B. Sterilization: by Alcohol Sprayer and Heating: The stitch, tweezers, and other instruments were sterilized using heat. The hood surface was sterilized using an alcohol sprayer

C. Sterilization by Formalin: Incubators and refrigerators were sterilized by supplementing with formalin. For this, 15 ml of formalin was added to a petri dish, which was then placed inside the incubator or refrigerator for a period of 7 days to complete the sterilization process.

3.3.3 Prepare of Culture Media:

A. Potato Dextrose Broth: The media preparation was conducted following the instructions provided by the manufacturer in India, Hi-media.

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B. Potato Dextrose agar: The media preparation was conducted following the instructions provided by the manufacturer in India, Hi-media.

3.3.4 Isolation and identification of fungi from stored rice grain:

To isolate the *Aspergillus* spp (rice grains) grain were taken randomly from each of the collected samples. It was sterilized using sodium hypochloride 2% for 2 minutes and then washed with sterile distilled water twice to remove traces of sterile material and dried with sterile filter paper. The grains were transferred with sterile forceps to 9 cm petri dishes containing 20 ml of pre-prepared PDA (chloramphincol) (250 mg / l) to prevent bacterial growth (Pitt and Hocking, 1997), by 5 tablets per dish and three replicates per sample and then incubated in 25 c for 5-7 days as they appear. The fungi associated with the grains were then purified by secondary forms for identification (Pitt and Hocking, 1997).

Fungal isolates were kept in clean, sterile glass containers containing the center of PDA and incubated at 25 °C for 7 days and then placed in the refrigerator at 4°C until it was used isolated fungi were then diagnosed based on the taxonomic keys of (Nelson, 1983)

After incubation and identification the percentages of frequency and appearance of isolated fungi were calculated according to the following equation:-

Percentage of appearance = Number of isolate that appeared in the same type / Total number of samples × 100

Total number of isolates of all species × 100

$$Percentage\ of\ frequency = Number\ of\ isolates\ per\ species$$

3.3.5 Morphological Identification of *Aspergillus* Fungi:

The fungal isolates were transferred to sterilized plates for purification and identification. Identification of different fungi was done with help of slides prepared by direct mount from the culture. The examination under microscope and identified on the basis of their colony morphology and spore characteristics (CMI, 1966; Nelson, *et al.*, 1983; Samson and Hoektra , 1988).

3.3.6 Molecular Identification of Fungal Isolates

Genomic DNA from 2 ml of fungal isolates growing in potota dextrose broth at 25°C for 3-4 days was extracted using the fungal genomic DNA Extraction Kit ,All component and chemicals of kit were listed in Table (3.4), The procedure applied was described in manual instruction as following:

A. Genomic DNA extraction kit (Favrogen) from *Aspergillus* spp. (Mirhendi *et al.*, 2005)

1. At 1.5 ml of *Aspergillus* colony was transferred to a 1.5 ml microcentirfuge tube for each sample under interest.
2. Cells were treated with 200 µl of FATG buffer, and the cells were resuspended by vortexing.
3. The cells were then resuspended in 50 µl of lyticase solution, mixed well by vortexing, and incubated at a temperature of 37 °C for 30 minutes.
4. Next, 200 µl of FATB buffer was added to the cells, and the cells were resuspended again by vortexing.
5. A total of 20 µl of Proteinase K (10 %) was added to the mixture and thoroughly mixed by vortexing. The samples were incubated at a temperature of 60 °C for 15 minutes, with vortexing every 5 minutes during incubation.

6. To the mixture, 200 µl of ethanol (96-100%) was added and mixed well by vortexing for 10 seconds.
7. A spin column was placed in a collection tube, and the sample mixture (including any precipitate) was carefully transferred to the spin column. The column was then centrifuged at a speed of 11,000 rpm for 30 seconds before being moved to a new collection tube.
8. Then, a total of 400 µl of W1 Buffer was added to the spin column. After centrifugation at 11,000 rpm for another 30 seconds, the flowthrough was discarded while keeping the spin column in the collection tube.
9. Subsequently, a volume of 600 µl of Wash Buffer was added to the TG Mini Column. After centrifugation at a speed of 11,000 rpm for 30 seconds, the flowthrough was discarded as well. The spin column was returned to the collection tube and centrifuged at full speed (12,000 rpm) for an additional three minutes to dry the column.
10. The spin column was placed into an elution tube.
11. A volume of 50~100 µl of Elution Buffer was added to the center of the membrane in the spin column. The spin column was then centrifuged at full speed (12,000 rpm) for one minute to elute the total DNA.
12. Finally, the total DNA was stored at a temperature of -20 °C for further use.

B. PCR Assay

1. Preparation of Primers for the Polymerase Chain Reaction Technique (PCR):

The primers were prepared by adding distilled water free of nuclease in the a different volume according to the manufacturing instructions company to obtain a solution of base stock with a concentration of 100 Pico mole / µl, mixed by vortex, then centrifuged for 10sec at 4000 rpm. Then 10 µl of each primer was taken and putted in the micro centrifuge tube with 90 µl of nuclease free distilled water to prepare the working solution (Mirhendi *et al.*, 2005).

2. PCR Mixture:

The PCR mixture for all the primers used in this study was prepared according to the volumes of chemical materials listed in Table (3.6).

Table (3.6) Volumes of chemical materials utilized in PCR assay .

Chemical materials	Volumes	Company
G2 Master Mix	12 µl	(Promega)
DNA	2 µl	
Forward Primer	1 µl	Korea
Reveres Primer	1 µl	Korea
Deionizer D. W	9 µl	Bioneer
Total	25 µl	

C. PCR Gel Electrophoresis:

1. A 250 mL conical flask was filled with 100 mL of T.B.E buffer.
2. In a 250 mL conical flask, 1 g of agarose was added to 100 mL of T.B.E buffer and heated on a hot plate until boiling, ensuring the dissolution of all components.
3. The agarose mixture was subsequently cooled to 50-60˚C.
4. Before solidification for pre-staining, 0.5 µl of Ethidium Bromide dye was added to the agarose-TBE buffer.
5. Placing a comb into one end, the template for the agarose gel was prepared.
6. The agarose was poured carefully into the template to prevent bubble formation and then cooled at room temperature for 30 minutes.
7. A T.B.E buffer solution of 350 ml filled the electrophoresis tray.
8. In the wells of the agarose gel, five µl of DNA product was mixed with one µl of loading stain and loaded.
9. Electrophoresis was conducted at 70 V for 60 minutes.
10. The size of the amplified DNA fragments was determined by comparing them to the molecular size marker DNA (100-bp DNA ladder).

D. PCR Study of Fungi Identification (PCR conditions)

The conditions of PCR reaction for AFU5S used for fungi identification as shown in table (3.7).

Table (3.7) Show the conditions of PCR for *AFU5S* primer Amplication .

AFU5S			
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	30
Annealing	55°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Cooling	4°C	∞	

E. Sequencing Analysis:

In this study, sequencing analysis was conducted on PCR products of 40 *Aspergillus* isolates. For each isolate, the 20 µl PCR product generated from AFU5S primer was directly sequenced by Macrogen Laboratory in Korea. The resulting sequencing data for each isolate were compared to the NCBI Blast nucleotide database to identify the fungal species.

F. Phylogenetic Tree:

Fourty isolates of *Aspergillus* species in this study were analyzed to construct a phylogenetic tree using Mega 6 software program, based on the sequences data amplified by AFU5S primer. The unweighted pair group method with arithmetic mean (UPGMA) tree type was used for the analysis.

- PCR Study of Mycotoxin Identification (PCR conditions):

The conditions of PCR reaction for five primers used for detection under test genes present was shown in Table **(**3.8).

Table (3.8) Show the conditions of PCR for three primer pairs.

Mycotoxins gene	**ITS gene**		
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	30
Annealing	53°C	30 sec	
Extension	72°C	35 sec	
Final extension	72°C	5 min	1
Cooling	4°C	∞	

Mycotoxins gene		**Nor gene**	
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	95°C	5 min	1
Denaturation	94°C	30 sec	30
Annealing	66°C	30Sec.	
Extension	72°C	752Sec.	
Final extension	72°C	3 min	1
Cooling	4°C	∞	

Mycotoxins gene		**Fum10 gene**	
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	95°C	5 min	1
Denaturation	94°C	30 sec	30
Annealing	57.5°C	30Sec.	
Extension	72°C	72Sec.	
Final extension	72°C	3 min	1
Cooling	4°C	∞	

3.4 Plant materials:

The roots of (*Saussurea costus*), had been purchased from local markets, identified and classified according to (Kaur *et al*., 2019) and Dr.Hussein Jebur Hussein , (Table.1). Roots of this plant were cleaned, dried, and kept according to (McClure, 1975), (Figure.1.2).

Table (3.9) Scientific, Local, English name, Family, and active parts.

Scientific name	Local name	English name	Family	Active part used
Saussurea costus	Al-Kost Al-Hindi	Costus	Asteraceae	Roots

Figure (3.1) *Saussurea costus (Falc*.) Lipschitz Plant.

Figure (3.2) *Saussurea costus (Falc*.) Lipschitz Roots.

3.5 Plant materials extraction:

A. Alkaloid determination: 10 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 hrs. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide (NH_4OH) was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute 1% ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed (Harborne, 1973).

B. Flavonoid determination :

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight (Boham and Kocipai-Abyazan, 1974).

C. Extraction of crude terpenoid compounds:

Crude terpenoids compounds were extracted according to(Harborne and Harborne, 1984). 10 gm of plant powder for each leaves and grains were extracted by 200 ml of chloroform . The solvent then evaporated in rotary evaporator. The samples were placed in clean dark vials and kept in refrigerator at 4°C until use. (Al-Jassani, 2017).

3.6 Isolation and diagnosis of *Aspergillus* species: To isolate the *Aspergillus* fungus 100 grains were taken randomly from each of the collected samples. It was sterilized using Sodium Hypochloride 2% for 2 minutes and then washed with sterile distilled water twice to remove traces of sterile material and dried with sterile filter paper. It was transferred with sterile forceps to 9 cm Petri dishes containing 20 ml of pre-prepared Potato dextrose agar (PDA) with (chloramphenicol) 50 mg / l to prevent bacterial growth (Pitt and Hocking, 2009), by 5 grains per dish and three replicates per sample and then incubated in 25C˚ for 5-7 days. The fungi associated with the grains were then purified by secondary cultures for identification. Isolated fungi were then diagnosed based on the taxonomic keys of both (Barnett and Hunter, 1972; Domsch et al., 1980). The fungal isolates were kept in clean, test tube containing the PDA broth. Containers were incubated at 25C˚ for a week and then placed in the refrigerator at 4 ° C until it was used.

Antifungal activity assay of extract: PDA medium was prepared and autoclaved after that a known volume (1, 2, 3ml) of each plant extract is placed in the center of the Petri dishes and complete the volume to 20ml with PDA medium to obtain the required final concentrations (5, 10, and 15) % of the medicinal plant after complete solidification of the medium, 5 mm disc of seven days old culture of the test fungus were placed aseptically in the center of the Petri plates and incubated at 25 ± 2C˚ for seven days, simultaneously 0.02ml of chloramphenicol solution was added to each assay plate to check the bacterial seed borne fungiion as suggested by (Gupta and Banerjee, 1970; Singh and Tripathi, 1999).

Fungicide Carbendazim 500g/l was used as the positive control and 10% dimethyl sulfoxide as the negative control. Observations were recorded on the seventh day. The colony diameter was recorded in terms of millimeters. PDA medium devoid of extract served as control. For each treatment, three replicates were maintained. The fungi toxicity of extracts was calculated in terms of the percent inhibition of mycelia growth by using the formula(Singh and Tripathi, 1999) .

Percent Inhibition = (dc- dt / dc) * 100

Where:

dc = Average increase in mycelia growth in control.

dt = Average increase in mycelia growth in treatment.

3.7 Statistical analysis: All data of treatments were dictated by three replicates. Data were subjected to an analysis of variance by using SPSS 20.0 program, a completely randomized design was used, and the least significant difference (L.S.D) was performed at $P \leq 0.05$.

Chapter Four

Results

and

Discussion

Chapter Four

4. Results and Discussion

4.1 Isolation and diagnosis of fungi associated with rice grains.

In the current study, culture of rice samples showed many types of fungi were 31 isolates of *A. niger* species, which frequency for 25.61% of total isolates and has an appearance of 34.44%. There were 12 isolates of *Aspergillus flavus* species, representing frequency 9.91% of the total isolates with an appearance of 13.33%.

The results in Table (4.1) Show there was 1 *Aspergillus fumigatus* isolate as well as 1 isolate *Bipolarus* frequency for 0.82% of the total isolates with an appearance appearance of 1.11%. There were 7 isolates of *Aspergillus terrus*, frequency 5.78% of the total isolates with an appearance of 7.77%. *Alternaria* species 11 isolates of, frequency for 9.09% of the total isolates with an appearance of 12.22%. There are 23 isolates of *Cladosporium* species, frequency for 19.01% of the total isolates with an appearance of 25.55% and 2 isolates of *Curvularia* species, frequency 1.65% of the total isolates with an appearance of 2.22 while White sterile Mycelium were 10 isolates, frequency for 8.26% *Penicillium* spp. 12 isolates which frequency 9.91%3 isolates of *Rhizopus*, frequency for 2.47% of the total isolates with an appearance of 3.33%.

There was 1 isolate of *Scopulariopsis*, frequency 0.82% of the total isolates with an appearance frequency of 1.11% and 7 isolates of yeast species, frequency for 5.78% of the total isolates with an appearance of 7.77%.Classifiaction according to(Laut et al., 2023), Dr. Zidan Khalif Omran and Abeer Fauzi Al-Rubaye , University of Babylon College of Science for Women.

Table (4.1) Percentage of frequency and appearance of fungal species isolated from different rice sample in Babylon province

No.	Fungi isolates	No. of fungi isolates	Percentage of frequency %	Percentage of appearance
1	*A. niger* spp.	31	25.61	34.44
2	*A.flavus* spp.	12	9.91	13.33
3	*A.fumigatus*	1	0.82	1.11
4	*A.terrus*	7	5.78	7.77
5	*Alternaria* spp.	11	9.09	12.22
6	*Bipolarus*	1	0.82	1.11
7	*Cladosporium* spp.	23	19.01	25.55
8	*Curvularia* spp.	2	1.65	2.22
9	*White sterile mycelium*	10	8.26	11.11
10	*Penicillium* spp.	12	9.91	13.33
11	*Rhizopus*	3	2.47	3.33
12	*Scopulariopsis*	1	0.82	1.11
13	*Yeast* spp.	7	5.78	7.77
Total		121	100	

The results showed fungus *Aspergillus* spp came as the first seed borne fungi of rice grain , followed by the *Cladosporium* spp and the *Penicillium* spp.

at the third rank, reason for the appearance of *Aspergillus* fungus in greater proportions is that it has small, rapidly spreading conidia that tolerate harsh environmental conditions. This result is consistent with the results of the current experiment as showed in Table (1).

With many studies These results coincide in terms of the fact that the fungal species that are included in this study are in the fungi of typical stores that have been monitored in different countries of the world (Proctor *et al.*, 1995; Samson, 1991). The samples yielded 19 isolates in 9 species: *A. tubingensis, A. fumigatus, A. niger, T. radicus, T. purpureogenum, T. pinophilus, T. isl*and*icus, P. citrinum,* and *P. .chermesinum,* the genus *Aspergillus* (80%) was the most common in this refrenses (Laut *et al.*, 2023).

(Sinaga, 1986) indicated that regions between latitudes 26°-35° north and south support the thriving of *Aspergillus* spp., which includes Iraq known for its hot and warm climate. Grains rich favor the growth of *Aspergillus* spp.

In Babylon Province, corn grains were found to be associated with various fungi such as *Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp., *Alternaria* spp., *Fusarium* spp., *Rhizoctonia* spp., and *Mucor* spp. Among these fungi, *Aspergillus* spp. had the highest occurrence (55%) according to a study by (Ali *et al.*, 2018). (El-Shanshoury, 2014) revealed that *Aspergillus* and *Penicillium* were the most common fungi found in grain grain samples (maize and wheat) and peanuts collected from central Delta province in Egypt.

Rice is not as frequently reported to have mycotoxin seed borne fungiion compared to other grain crops (Tanaka *et al.*, 2007). In the study conducted, the main genera of fungi found seed borne fungi rice be present *Trichoderma, Curvularia, Fusarium, Penicillium, Rhizopus, Cladosporium, Aspergillus, Mucor, Helminthosporium,* and *Alternaria*. Other fungal species identified included *Arthrinium, Geotrichum, Syncephalastrum, Rhodotorula, Bipolaris,*

Cryptococcus, Gilocladium and *Nocardia*. The most common fungi species seed borne fungiing rice in Niger State were *A. niger, Alternaria* spp., *Penicillium* spp., *Rhizopus* spp., *A. parasiticus, Mucor* spp., and *A. flavus, fungi Aspergillus* spp, and *Penicillium*. Remain linked to rice grain and what helps its growth and developmentis the availability of appropriate conditions for its growth like availability of storage temperature, moisture and the rate of exposure to insects before and during storage (Haque and Russell, 2005). Reported by (Al-Obaidy, 2015), the most frequently identified fungal genera in grain fields were *Aspergillus* and *Penicillium*. These fungi, along with *Rhizopus*, were found to thrive. Trung and Colleagues (2001) reported that *Aspergillus* sp. accounted for 43.8%, *Penicllium* sp. for 10.9%, *Fusarium sp*. for 21.9%, and other fungi for 23.4%. During periods of drought and insect harm, these events can occur when situations are stored in fields found in sub-tropical and tropical regions (Ominski *et al.*, 1994).

Some references indicate an increasingly widespread presence of fungi that have the ability to produce mycotoxins in hot and dry areas (Cantalejo *et al.*, 1998).The research pinpointed a variety of mycotoxins, such as ochratoxins, aflatoxins, along with over twelve other toxins generated by species of *Aspergillus* (De Scott, 1965). Rice samples collected from local and non-local markets in Iraq as well as other countries were compared regarding fungal isolation. It was found that rice samples from Iraqi markets contained a higher level of fungi compared to non-local samples. Under specific conditions, fungi can grow on rice grain , and some isolates are capable of secreting mycotoxins. This poses a high risk to the population due to the associated health effects, including liver cancer (Ali *et al.*, 2018).

The growth of these fungi can be influenced by factors such as the product's moisture content (Giorni *et al.*, 2008), temperature, duration of storage, and the

level of fungal seed borne fungiion before storage. Insect and mite activity can assist in spreading the fungi (Suleiman and Omafe, 2013).

Various varieties of rice grains were found to be seed borne fungied with five pathogenic fungi: *A. niger, Aspergillus flavus, Alternaria padwickii Rhizopus oryzae,* and *Penicillium citrinum*(Uma and Wesely, 2013).

According to (Uma and Wesely, 2013) *Aspergillus* spp., particularly *A. niger* and *A. flavus*, are frequently present on rice grain and associated with ungerminated ones. These specific *Aspergillus* species as saprophytes may lead to reduced seed germination.

The spread of certain fungal species is indicated in different climatic regions; some thrive in hot and dry areas while others prefer temperate or cold regions (Logrieco *et al.*, 2002; Munkvold, 2003).

The presence of moisture in the product can affect the growth of these molds (Giorni *et al.*, 2008), Factors including temperature, length of storage, and the degree of fungal seed borne fungiion before storage play a significant role, too. Moreover, the actions of insects and mites further facilitate the propagation of fungi (Suleiman and Omafe, 2013). Throughout various stages of its agricultural lifecycle, this important grain can become seed borne fungied. This includes seed borne fungiion during growth, harvest, and various processes related to processing, handling, and shipping in general. Among the microflora found in rice, common species include bacteria that produce endospores from the Pseudomonas genus, yeast, and molds (Haque and Russell, 2005).

Several species belonging to the genera *Aspergillus* and *Penicillium* are known for producing mycotoxins that may pose a health risk (Sekar *et al.*, 2008). Specifically, certain isolates of *Aspergillus flavus* and *Aspergillus parasiticus* (A. parasiticus) are recognized for their production of aflatoxins—a

group of toxic secondary metabolites consisting of aflatoxins AFB_1,AFB_2,AFG_1, and AFG_2—that are part of a wide range of mycotoxins produced by mycotoxigenic fungi. The International Agency for Research on Cancer classified Aflatoxin AFB_1 as Group carcinogen in 1993 based on their research (Organization and Cancer, 1993).

Alongside Samajpati extracted mycotoxin-generating fungi from rice grain tainted and sold in Calcutta's local markets, India. Their research discovered that *A. flavus* and *A. parasiticus* were producers of AFB_1; while *A. flavus* was responsible for aflatoxin AFG_1 production(Begum and Samajpati, 2000). Furthermore, they revealed that ochratoxin and sterigmatocystin were produced by A. ochraceus and A. japonicus respectively; with P. citrinum found to be the producer of citrinum.(Reddy *et al.*, 2008) and his colleagues conducted a study that provided an overview of the primary mycotoxins found in rice from various nations. The mycotoxins mentioned encompassed fumonisins, aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), and deoxynivlenol

4.2 Diagnosis of *Aspergillus* Spp:

4.2.1 Morphological Characterizes

The morphological features were used to identify *Aspergillus* isolates. These included characteristics such as colony appearance, morphology of conidiophore and conidial head, phialides, and conidia. The taxonomic classification of the *Aspergillus* isolates was then categorized into two groups based on these traits.

- *Aspergillus niger* isolates:

Of the 49 *Aspergillus* spp. isolates, 31 were identified as *A. niger*, which typically exhibit a cottony appearance, initially white to yellow, and eventually turning black. Their structure is made up of felt-like conidiophores, and the reverse is white to yellow. In microscopy, the conidial heads are radiate with biseriate conidiogenous cells.

Upon microscopic observation of *A. niger*, it was noted that the fungus possesses smooth-colored conidiophores and conidia. The conidiophores are projections arising from hyaline hyphae. The conidial heads exhibit a radial arrangement and are arranged in columns (biseriate). The conidiophore vesicle produces sterile cells called metulae, which support the phialides on the conidiophores. The phialides, in turn, produce conidia with a rough texture in Figure (4.1).

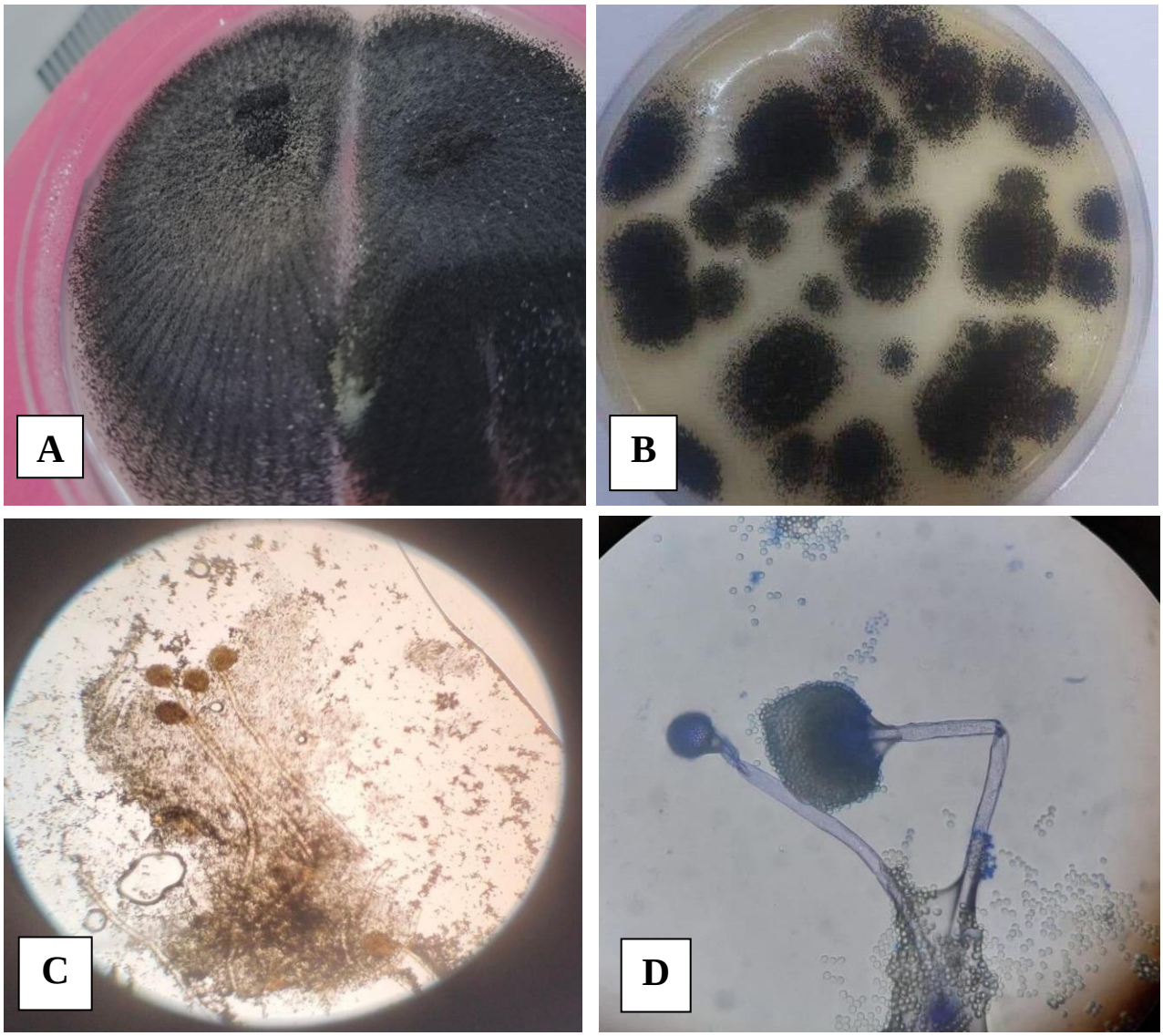

Figure (4.1) (A,B) colony of *Aspergillus niger* fungi on PDA culture, (C,D) Microscopic examination phialides and conidia (40X).

Numerous taxonomists have investigated the taxonomy of *Aspergillus* section Nigri. and was recently reviewed by (Abarca *et al.*, 1994). Mosseray (1934) described 35 species black aspergilli, while (Raper and Fennell, 1965).

In recent years, there have been a reported the seed borne fungiion of rice with mycotoxins such as Aflatoxins and fumonisins, especially with AFs in certain areas of China, Filamentous fungi (often described as molds in this context) and mycotoxins have become the most frequently measured food and

feed contaminants at the global level, with the prevalence of fungal invasion still increasing as a reflection of global climate change. Of particular concern is the fungal and mycotoxin seed borne fungiion of staple foods, such as grain including rice, which is the major dietary product in the Asia-Pacific region. Hence, the present study focused of fungal seed borne fungiion and the presence of mycotoxins in commercial samples of rice taken at random from supermarkets in Bangkok, Thailand. The results showed that *Aspergillus*, *Talaromyces*, and *Penicillium* were the three dominant fungal genera found in the commercial rice samples (Olagunju *et al.*, 2018).

In study done by(Laut *et al.*, 2023) marketed rice, samples showed a high prevalence of *Aspergillus*, *Penicillium*, and *Talaromyces*, but no occurrences of *Cladosporium* and *Alternaria*. *A. niger* (eight isolates) was the most prominent species and our results supported other findings (Reddy *et al.*, 2009).

Some studies have number of reports from various countries on the occurrence of fungal seed borne fungiion in rice with high levels of aflatoxin (Sun *et al.*, 2017)

Fungi of genus *Aspergillus*, mainly *Aspergillus flavus*, *Aspergillus parasiticus* and rarely by *Aspergillus nomius* and *Aspergillus tamari* (Klich 2007; Iqbal *et al* ., 2012) produce aflatoxins (AFs). These fungi are able to grow on different grain and produce AFs before or during harvest, storage, handling, and shipment (Giray *et al.*, 2007; Reddy *et al.*, 2009a). The most important members are AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2).

Reported by EFSA (2006) Ochratoxin A (OTA) is mainly produced by *Aspergillus carbonarius*, *Aspergillus ochraceus*, and *Penicillium verrucosum*. Reported by (JECFA 2001) It is a known nephrotoxic agent and

has been associated with fatal human kidney disease, referred to as Balkan Endemic Nephropathy (BEN) and with an increased incidence of tumors of the upper urinary tract.

4.2.2 Molecular Identification of *Aspergillus* niger Group.

at 31 pure cultures of *Aspergillus niger* group were obtained by isolating 49 different isolates . The initial identification was performed using traditional taxonomy, relying on the morphological features. However, this method has limitations as it provides a restricted number of distinguishing characteristics, making the identification of closely related species difficult. Fortunately, advancements in molecular biology techniques have significantly enhanced taxonomy by enabling highly sensitive and specific genetic differentiation of species. To identify the fungal isolates , 24 isolates from the *Aspergillus niger* group underwent DNA extraction and the ITS region of rDNA was selected as barcoding region.

4.2.2.1 Identification of *Aspergillus* spp. group based on PCR Methods:

The results successfully identified (24) isolates of *A. niger* group based on amplification amplicone included ITS1-5.8S-ITS2 with flanking primer region by primer pair: ITS5: and ITS4: All isolates shown monomorphic Figure (4.2). These results were consonant with results of (Skladny *et al.*, 1999).

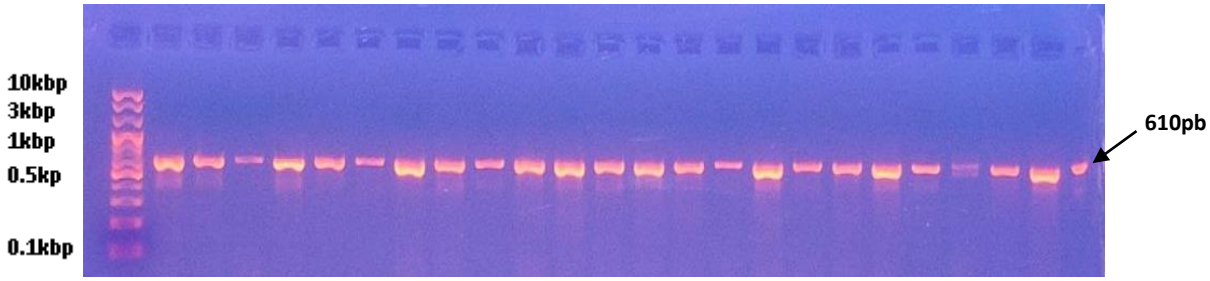

Figure (4.2) Profile Gel electrophoresis of PCR product for amplification IST region with flanking primers, all strain (1-24) of *A. niger* group shown 610 bp. M=molecular marker 100-3000bp.1.5% agarose TBE buffer, 70 volt 30 min.

The PCR products were shown clear bands of ITS1-5.8S-ITS2 and flanking regions of primer pair was illustrated on the agarose gel. These products shown bands were sent for sequencing. This sequence results were identified based on NCBI search and shown 24 different fungal species, most of them 20 species were *A.niger*, others four species were *A.brasillensis* and *tubigensis*, two for each.

4.2.2.2 Identification of *Aspergillus niger* group based on sequence method:

Twenty four isolates of *Aspergillus niger* group were identified, distributed among 20 isolates were *A. niger*, 2 isolates were *A.brasillensis* and *A.tubigensis* for each.

A. Multiple alignment sequence of 24 isolates of *A.niger*.

In order to comprehensive the identity and mutation sites among all isolates sequence charts under interest, a multiple alignments of 24 isolates based on Edit software was performed. The results shown variation among *A.niger* isolates and between other species in figure (4.3).

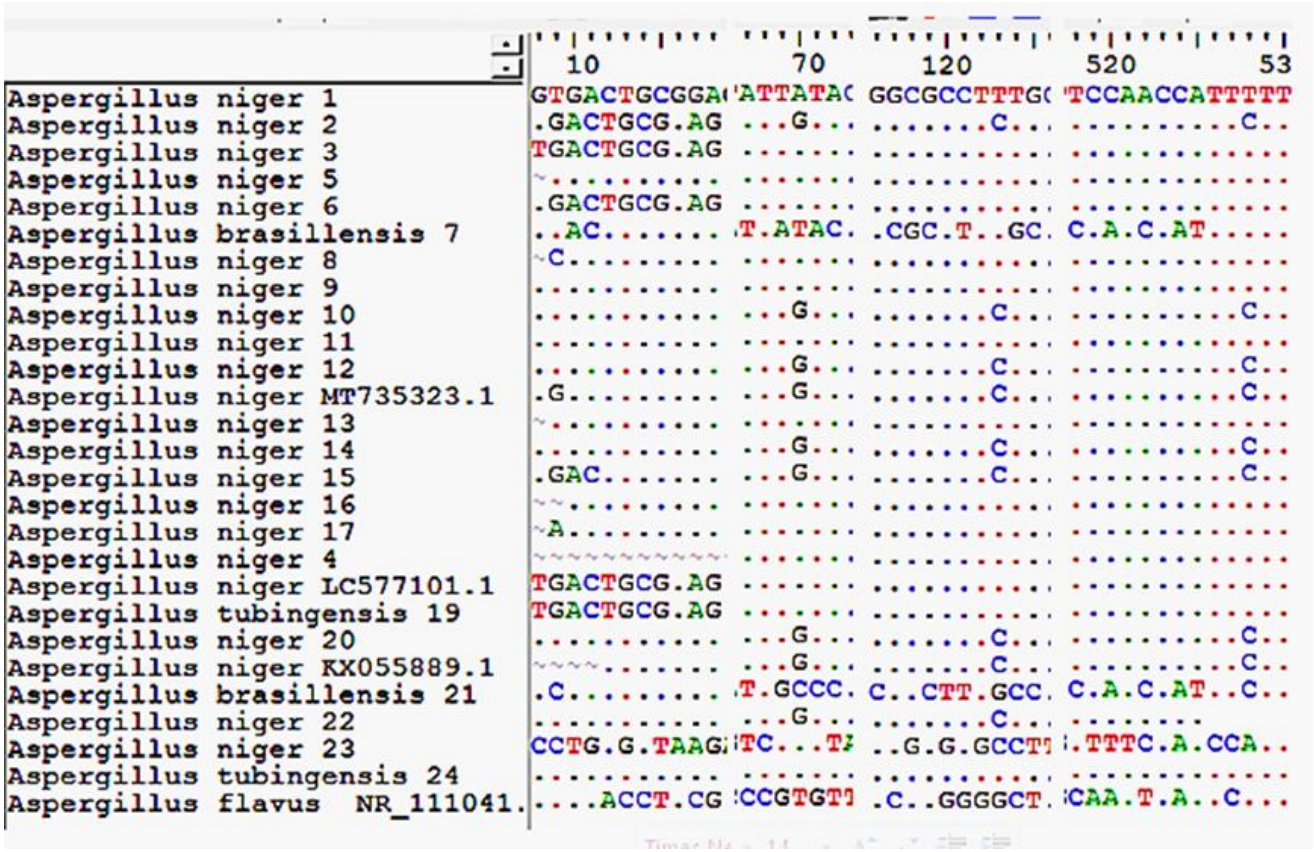

Figure (4.3) Multiple alignment of partial sequence of ITS region for 24 isolates of *Aspergillus niger* and closed related species with reference isolates with accession numbers.

The common mutations sites were oriented not randomly, for example: the Adenine base was subtitled by Guanine base at site 70. In addition, Cytocine in site 123 and 558 respectively Figure 4.5 substituted Thymine.

The isolates of *A.niger*:3-6, 8-9, 11, 13, 16-17, were shown highly identity in their sequences. Also two isolates of *A.brasillensis* (7and21) were shown afar evolution from others *A.niger* isolates. On other hand, two isolates of *A.tubingenesis* were shown trend similar to *A.niger* isolate, this may explained the closes relationship and microevolution from *A.niger* isolates figure (4.3).

A mutation in ITS region may have caused microevolution, which could be credited to the divergent evolution such as the isolates 2,10 and 12 , these results consistent with (Nosil and Feder, 2012). On the other hand, the sequences of ITS region other isolates of *A.niger* showed a high percentage of similarity (>98%).This attributed to convergent evolution, this consistent with (Leander, 2008).

In this study, the DNA sequence of the ITS region was utilized as a barcoding region to identify isolated *Aspergillus niger* groups. This region is characterized by its high level of conservation, frequent repetition, and variation among interspecific and intraspecific species. Often utilized for DNA-centric mycological research at subgeneric stages and species recognition, the nuclear ribosomal repeat unit's internal transcribed spacer (ITS) region is highly esteemed. The sequence of words has been shuffled, ensuring that unchanged terms are located at least four words apart, and punctuation and referencing have been accurately maintained (Horton and Bruns, 2001) (Bridge *et al.*, 2005).

According to the findings of this research (Figure 4.3), it is evident that ITS region displays uneven variability among different *A.niger* species. Moreover, there seems to be no clear correlation between this variation and the organized connection or nutritionary way of these speciesi (Nilsson *et al.*, 2008) .

B. Phylogeny tree:

Many clades and sub clades of *A.niger* (A-E) , *A.tubingensis* (F) and *A.brasillensis* (G) with reference isolates were sprouted from construction phylogeny tree for twenty-four sequences of local isolates of *A.niger* group based on MEGA X software with closes related *A.niger* , *A.tubingensis* and *A.brasillensis* reference isolates figure (4.4)

The isolates of *A.niger* with numbers:22,12,10,14,2,15 were clustered with reference isolates KXO55889.1 in separated clade A, but the isolates 22 and 12 clustered in sub clade A1 while isolates 10 and 14 clustered in sub clade A2 and isolates 2 and 15 away from them figure (4.4).

On the same way, the isolates 3, 4, 11, 13 were clustered in closes clade B, and clade C. figure (4.4).

The two isolates of *A.tubingensis* (clade F) and *A.brasillensis* (clade G) were situating in separated clade for each Figure (4.4). Many isolates of *A.niger* were fare away from these clusters in separated lines.

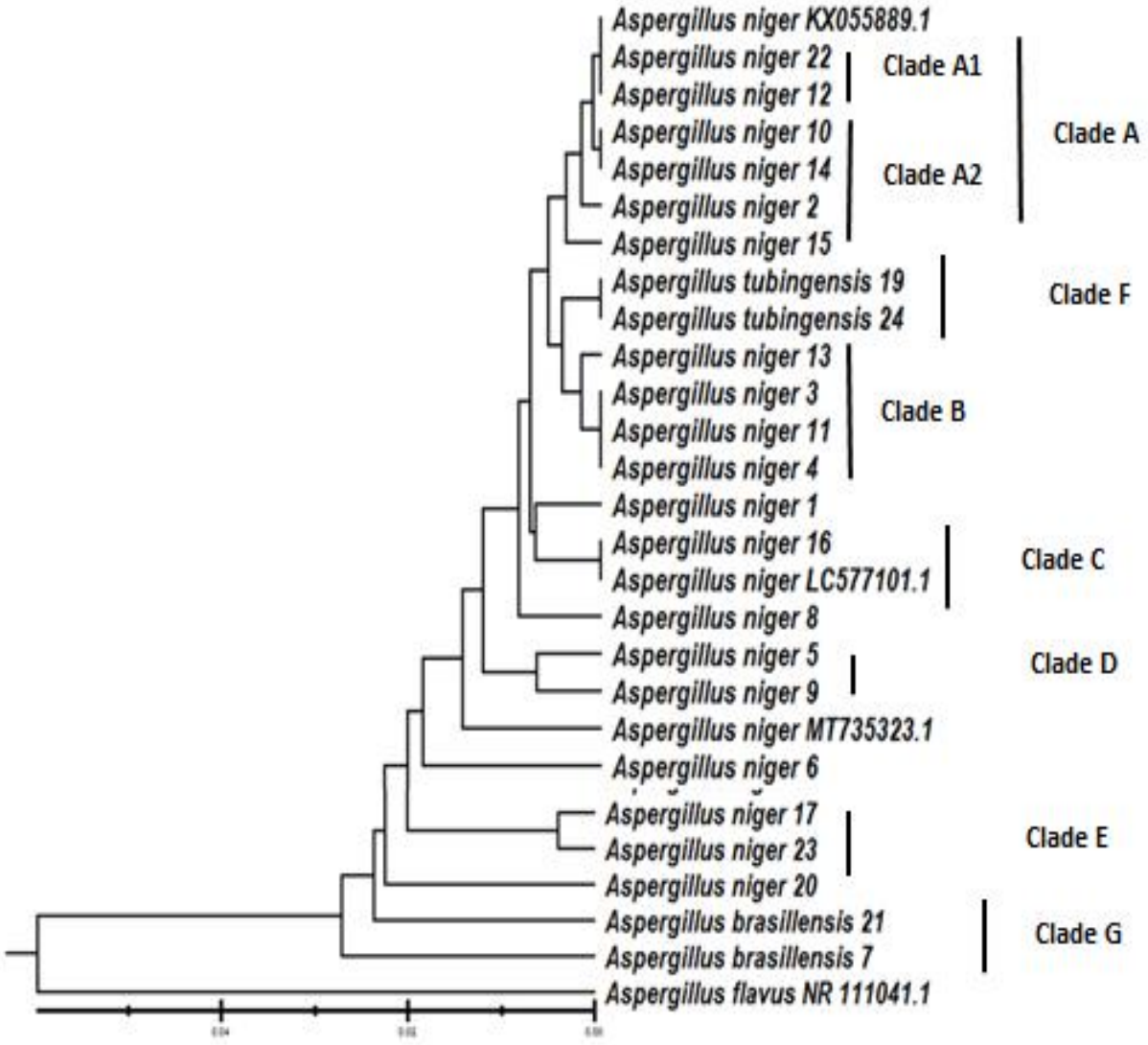

Figure (4.4) Phylogeny tree for 24 isolates of *Aspergillus niger* and closed related species.

The closes isolates in each clades and sub clades of *A. niger* group were explained high or low sequence identity (Leander, 2008; Xie *et al.*, 2015). That means highly identity 98-100% situating in one clade while far away due to low identity 96-97%. The deference in clustering due to micro-evolutionary in some isolates. These results were consistent with results of (Baho, 2022). In addition, the mutation in ITS region may cause this evolution (Nosil and Feder, 2012).

In general, the evolutionary power are mutations, recombination, gen flow, gen draft and hybridization (Ene *et al.*, 2018). Mentioned to the evolution acts on mutations that naturally arise within the genome and are shaped both by intrinsic genomic features and by the cellular environment and he pointed to the role of microevolution of *C. albicans*. In many cases the environmental condition such as sun ray may cause mutations this explanation was agree with (Drott *et al.*, 2021), when they pointed to the role of ecological interactions in micro evolutionary.

4.3 Molecular Detection of *A. niger* Mycotoxins:

4.3.1 Detection Gene of Fumonisin (FB) toxin.

Fifteen isolates of A. nigr were randomly chosen and subjected to genetic analysis to identify the presence of the fumonisin (FB) toxin gene using polymerase chain reaction (PCR). Out of these 15 isolates, only 8 showed distinct bands measuring 651bp on agarose gel electrophoresis (Figure 4.5).

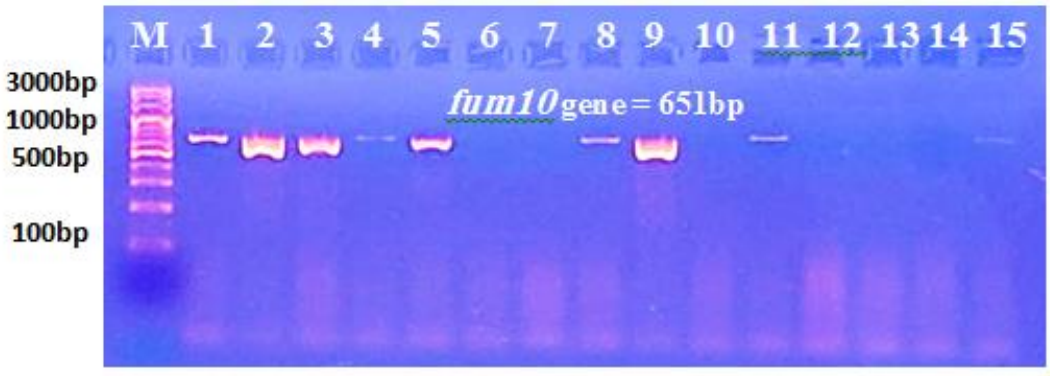

Figure (4.5) Illustration *Aspergillus niger* isolates carrying fum10 gene, 1-5,8-9,11,15 isolates carrying fumonisin mycotoxin gene. M= molecular marker 100-3000bp. 1.5% agarose, TBE buffer, 70 volt 30 min.

4.3.2 Detection gene of ochratoxin A (OTA) toxin:

The aim of this research was to assess the effectiveness of randomly selecting 15 *A.niger* isolates for determining the production of ochratoxigenic toxicity. The polymerase chain reaction (PCR) was utilized as the precise technique for analysis. Out of the total 15 isolaties only 7 exhibited distinct bands (420bp) on agarose gel electrophoresis for ochratoxin A (OTA) toxin (Figure 4.6).

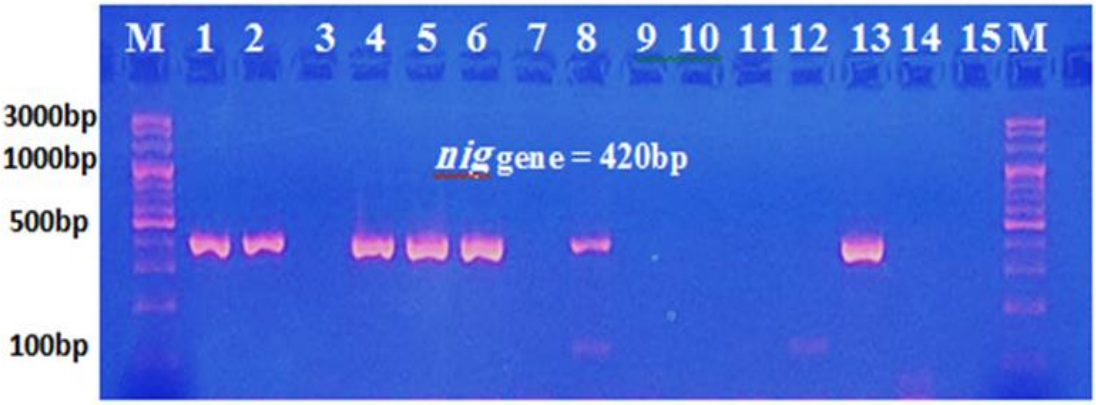

Figure (4.6) Illustration *Aspergillus niger* isolates carrying nig gene ,1,2,4-6, ,8, ,13 isolates carrying nig mycotoxin gene. M=molecular marker 100-3000bp. 1.5% agarose, TBE buffer, 70 volt 30 min.

The findings of the molecular detection of Ochratoxogenic in A.niger isolates grown on rice grains aligned with the outcomes , when they assessed the presence of Ochratoxin A (OTA) in commercially available animal feed in Qatar. In a country like Qatar, grain feeds are primarily imported from tropical climate regions where toxigenic *Aspergilli* and *Penicillium* are commonly found due to favorable weather conditions (Alsalabi *et al.*, 2023).

Our results also corroborate several studies that indicate the presence of mycotoxigenic fungi in marketed food. The major contaminants in the tested

feed samples were *Aspergillus* and *Penicillium*, which is likely attributed to the origin of these samples (Gil-Serna *et al.*, 2009; Hassan *et al.*, 2018).

This study evaluated the potential mycotoxin production, specifically fumonisin (FB) and ochratoxin A (OTA), by 15 isolates of *A. niger*. The genetic analysis revealed that many isolates carried both OTA and FB genes. The presence of these mycotoxin genes raises concerns about the risk associated with growing *Aspergillus* niger on natural products, particularly rice grains, as demonstrated in a (Susca *et al.*,2014).

These findings suggest that molds can infect rice grains, leading to mycotoxin production. Furthermore, *Aspergillus* spp., particularly *A. parasiticus* within the Flavi section, are significant aflatoxin producers, while ochratoxin-producing *Aspergillus* spp. belong to taxonomic groups such as *Aspergillus* section Nigri and section Circumdati. (Bennett and Klich,2003) as well as *Aspergillus* spp. capable of producing ochratoxin belong to the taxonomic groups *Aspergillus* section Nigri and section Circumdati *(Frisvad et al., 2004)*. *P. verrucosum* and *Penicillium nordicum* are the primary OTA producers in the *Penicillium* genera (Logrieco *et al* .,2003). Also *P. chrysogenum, P. polonicum, P. glycyrrhizacola* and *Penicillium* spp have been described as a primary source of OTA seed borne fungiion in liquorice roots (Chen *et al* .,2011; Chen *et al* .,2013).

The presence of mycotoxins, toxic compounds produced by fungi, in naturally occurring products—most notably food and grain items—is a cause for substantial concern due to its potential harm to both human beings and animals. In the worldwide context, it is alarming to note that a significant proportion, ranging from 25% to 40%, of grain crops have been found to be laced with such harmful mycotoxins. This seed borne fungiion with mycotoxins isn’t restricted to one specific phase; rather, it can happen throughout various

stages of the product's lifecycle. This includes during the cultivation period of the crops or during their storage afterwards. It's also important to mention that this seed borne fungiion issue is particularly prevalent in nations with hot and humid environmental conditions. These climates are exceptionally conducive for the proliferation of toxigenic filamentous fungi—the organisms responsible for producing these mycotoxins. Regions such as African countries, South Asia, and South America often experience these climatic conditions. Consequently, staple food items like rice and corn—the primary dietary components for large populations in these regions—frequently suffer from seed borne fungiion by aflatoxins and ochratoxins: two types of highly hazardous mycotoxins (Pittet 1998).

The present investigation successfully identified 8 isolates out of 15 of A.niger using the PCR method with the Fum10 gene. The outcomes of this study emphasized the hazards associated with the synthesis of a toxic substance by *A. niger*, known as fumosin toxin. This mycotoxin adversely affects both human and animal well-being, particularly when *Aspergillus* grows on grain such as rice grains. The risk of fumosin toxin arises from two factors. Firstly, *Aspergillus* is considered a storage fungi that can thrive in low humidity and high temperature conditions. Secondly, rice seed consumption is prevalent among families in Arabic and Iraqi communities as a staple food. The main danger posed by fumosin toxins is their ability to disrupt gat metabolism, posing a significant threat to human and animal health.

The findings of the current investigation involved isolating numerous *Aspergillus* niger isolates from rice grains and evaluating their carrying capacity for ochratoxin genes. These results further highlight the capability of these isolates to produce ochratoxin toxins. Additionally, this study sheds light on the risks associated with *A.niger's* production of mycotoxins in rice grains,

which can cause gastrointestinal issues and other adverse effects. These risks necessitate improvements in rice seed storage conditions and efforts to minimize fungal growth, particularly that of storage fungi, in order to decrease the likelihood of seed spoilage and seed borne fungiion by toxins. Such measures are crucial to safeguard public health, given that rice crops are a primary food source for individuals in Iraq and Arab countries.

4.4 Antifungal activity of the Secondary Metabolites

The results of antifungal activity of the crude Alkaloid compounds extracted from the roots of *Saussurea costus* against *Aspergillus niger* isolated from Rice grains were taken from different local markets Hillh-Iraq is presented in (Table 4.2). The antifungal activity of alkaloid secondary metabolites with three concentrations (5, 10, and 15) % was screened by food poisoning methods. The results revealed that the crude alkaloid compounds extracted from the roots of *S. costus* showed a significant reduction at $P \leq 0.05$ in the growth of *A. niger* and the same effect of the positive control. Antifungal activity was applied at (5, 10, and 15) %. mycelial inhibition ranging from 66.0% in 5 %, 71.6% in 10 mg/ ml, and 100% in 15 % (Figure.7,8,9) compared with negative control represented by 10% DMSO and positive fungicide Carbendazim 500g/l control (Figure: 10) where inhibition percentage was (0.00%) for negative control and 100% for positive fungicide Carbendazim 500g/l control (Figure 11).

Table (4.2) Antifungal activity of the crude alkaloid compounds extracted from the root of Saussurea Costus (*Falc*.) lipschitz against *Aspergillus* niger isolated from Rice grains.

Concentrations (%)	crude alkaloid extracted
	Inhibition percentage %
Negative Control	0± 0.00
5	66± 1.00
10	71.6± 0.57
15	100± .00
Positive Control	100± 0.00
L.S. D(0.05)	0.939
*Mean± standard deviation	

Figure (4.7) Antifungal activity of the crude alkaloid compounds at 5% against *A. niger*
Note: the change in the color of *A. niger* due to the effect of alkaloid compounds

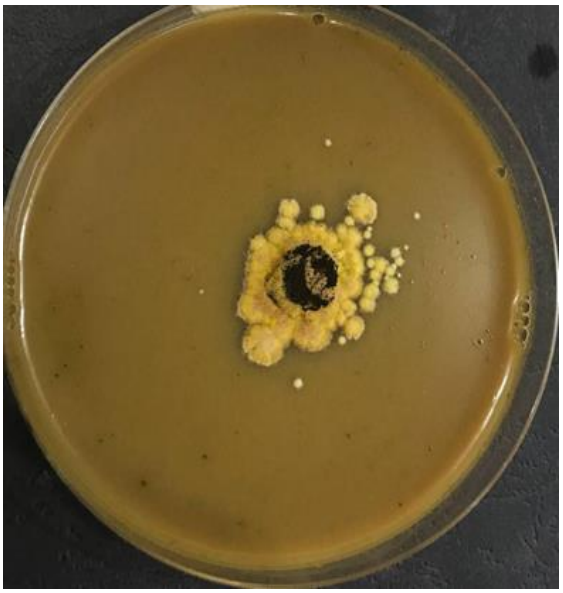

Figure (4.8) Antifungal activity of the crude alkaloid compounds at 10 % against *A. niger*
Note: the change in the color of *A. niger* due to the effect of alkaloid compounds

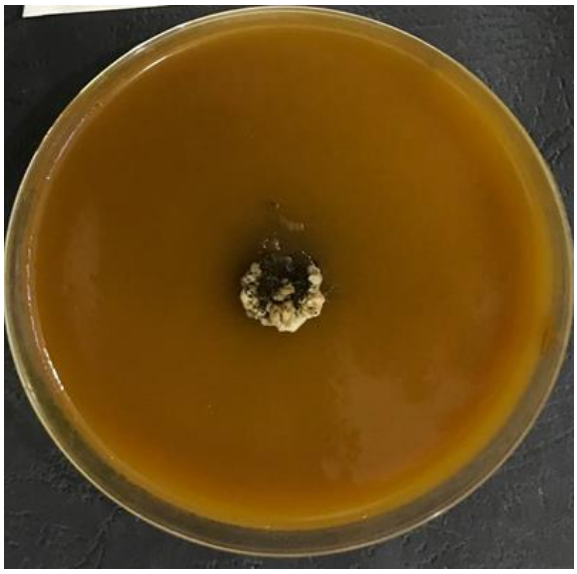

Figure (4.9) Antifungal activity of the crude alkaloid compounds at 15 % against A. niger

Figure (4.10) growth of A. niger in control treatment

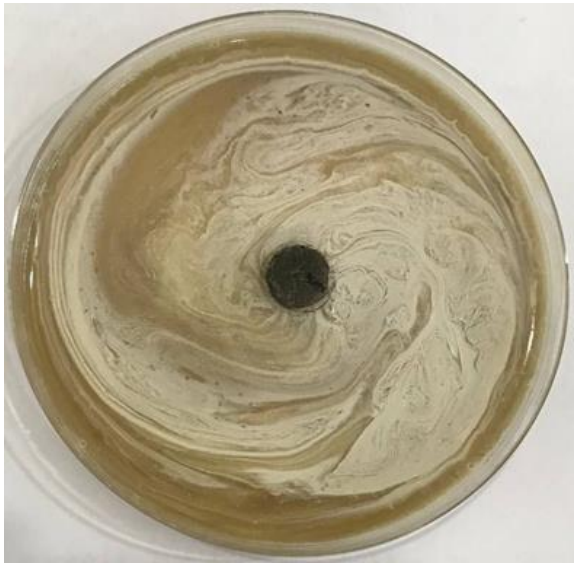

Figure (4.11) growth of A. niger in the fungicide Carbendazim 500g/l treatment

In the same context, the crude Flavonoid compounds showed 62.3% mycelial inhibition at 5 % , 100% at 10 %, and 15 % concentrations respectively (Table 4.3), Thus, it differed significantly compared to the control treatment and the same effect with positive control (Figure:12,13 and 14).

Table (4.3) Antifungal activity of the crude flavonoid compounds extracted from the root of *S. Costus (Falc.)* lipschitz against *A. niger* isolated from Rice grains.

Concentrations (%)	Crude flavonoids extracted
	Inhibition percentage %
Negative Control	0± 0.00
5	62.3± 0.57
10	100± 0.00
15	100± 0.00
Positive Control	100± 0.00
L.S. D (0.05)	0.470
*Mean± standard deviation	

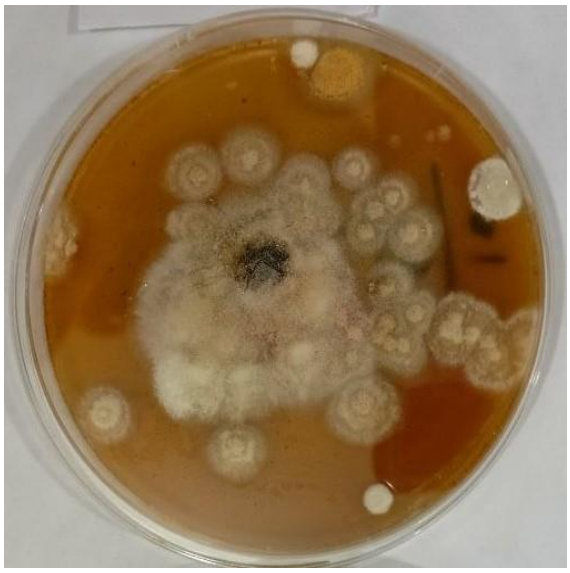

Figure (4.12) Antifungal activity of the crude flavonoid compounds at 5 % against A. niger

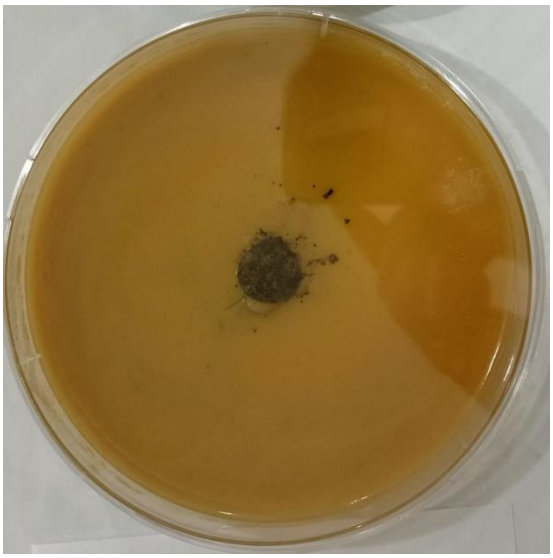

Figure (4.13) Antifungal activity of the crude flavonoid compounds at 10 % against *A. niger*

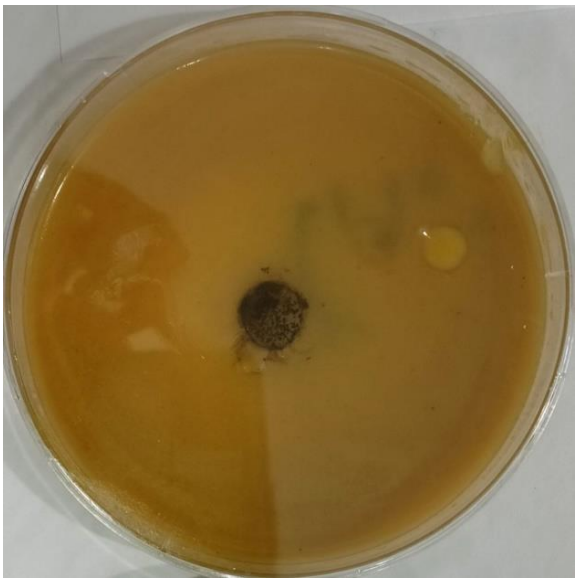

Figure (4.14) Antifungal activity of the crude flavonoid compounds at 15 % against *A. niger*

The crude Terpenoid compounds showed significant activity at three concentrations 5, 10, and 15 % compared with negative control and the same effect of positive control at 10, and 15% (Table 4.4). The inhibition activity was 34.1% at 5 % , 100% at 10 and 15 % respectively (Figure: 15,16,17). Finally, secondary metabolites such as alkaloids, flavonoids, and terpenoids compounds extracted from the root of *S. costus (Falc.) lipschitz* have the same effect compared with the positive fungicide Carbendazim 500g/l control.

Table (4.4) Antifungal activity of the crude Terpenoid compounds extracted from the root of *S. costus(Falc.)*lipschitz against *A. niger* isolated from Rice grains.

Concentrations (%)	Crude terpenoid extracted
	Inhibition percentage %
Negative Control	0 ± 0.00
5	34.1 ± 1.04
10	51.0 ± 1.00
15	100 ± 0.00
Positive Control	100 ± 0.00
L.S. D(0.05)	1.174
*Mean± standard deviation	

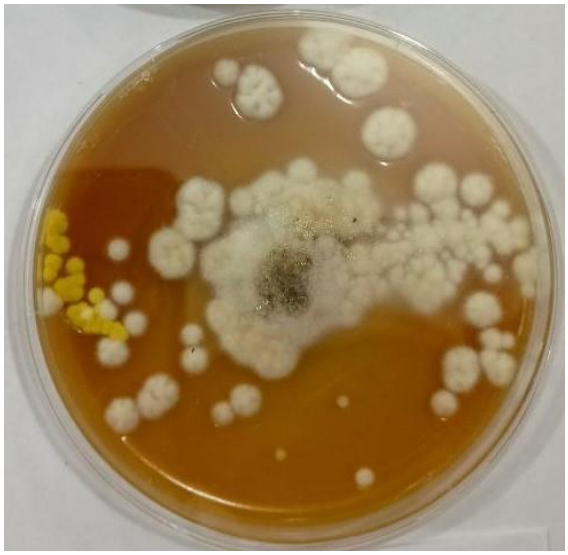

Figure (4.15) Antifungal activity of the crude terpenoid compounds at 5 % against A. niger

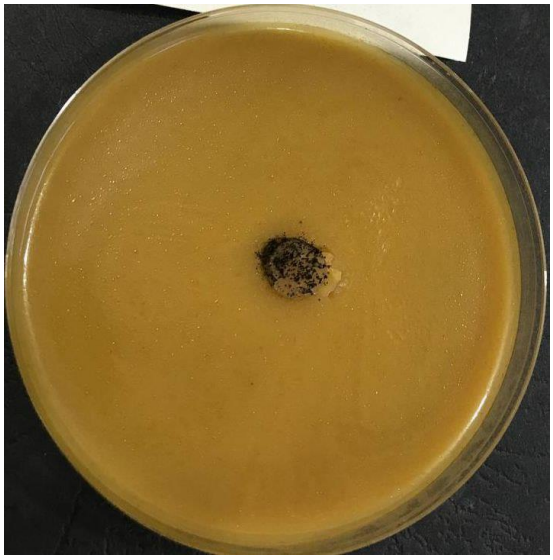

Figure (4.16) Antifungal activity of the crude terpenoid compounds at 10 % against *A. niger*

Figure (4.17) Antifungal activity of the crude terpenoid compounds at 15 % against *A. niger*

Medical plants consider an essential source in treating/preventing various kinds of disease (Rakotoarivelo *et al.*, 2015). Each plant consists of several important ingredients that can be used in the medical field and can be involved in the development of different kinds of drugs (Yuan *et al.*, 2016). The present study proved that the secondary metabolites including alkaloids,

flavonoids, and terpenoids extracted from the roots of (*S. costus*) had antifungal activity against *Aspergillus* species isolated from rice grains taken from different local markets Hillh-Iraq. Secondary metabolites extracted from different active parts of different medicinal plants such as of leaves (Lactuca serriola leaves, Lepidium sativum , Myrtus Communis , Cassia senna , Ricinus communis , Cassia didymobotrya , Melia azedarach , Dianthus caryophyllus flowers bud; and Salvia hispanica grains), possess the ability of antibacterials for controlling several pathogenic microorganisms isolated from different clinical samples (Hussein and Al-Marzoqi, 2020; Hussein et al., 2020; Hussein et al., 2019; Kamil et al., 2020).

Phytochemical compounds extracted from the unicellular primitive plant like Chlorella vulgaris possess ability of antibacterial counter to pathogenic bacteria. (Kamal *et al.*, 2019) Used phytochemical compounds extracted from Hibiscus sabdarifa for controlling *E. coli* and *Proteus* sp. (Kamal *et al.*, 2020)Used phytochemical compounds extracted from *Ficus carica* L. for controlling *E. coli* and *Pseudomonas aeruginosa*. (AL-Masoodi *et al.*, 2020)Used phytochemical compounds extracted from *Boswellia carteri* and Curcuma longa for controlling *Fusarium* sp. isolated from grains of maize. (Hussain *et al.*, 2021a, 2021b)used terpenoid compounds extracted from *Carthamus tinctorius* grains and flavonoid compounds extracted from *M. Communis* leaves against *Aspergillus* species isolated from stored medicinal plant grains. Secondary metabolites represented by Alkaloids and flavonoid compounds extracted from *M. Communis* leaves are respected as a worthy source for controlling pathogenic microorganisms segregated from hemodialysis fluid specimens(Sharara *et al.*, 2021). (Sahi, 2022)Used Callistemon viminalis leaves extracts for controlling isolates of urinary tract infections. *Allium sativum* respected a good source for controlling some bacterial species isolated from infected patients with Corona Virus(Hussein *et*

al., 2023). Secondary metabolite compounds extracted from *D. caryophyllus L.* flower buds such as terpenoid and flavonoid have powerful antifungal activity against *Candida species*(Mohammed Karim *et al.*, 2023).

S. costus has been screened as a medicinally important plant, the various chemical compounds isolated from it possess medicinal properties(Kaur *et al.*, 2019). Alkaloids and Terpenoids extracted from the roots of *S. costus* have powerful antifungal activity against *Candida* species(Hussein *et al.*, 2023). Various compounds were isolated from roots of *S. costus* and tested against the nine fungal isolates i.e. *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus, Aspergillus versicolor, Aspergillus flavus, Penicilium ochrochloron, Penicilium funiculosum, Trichodermaviride, Cladosporium cladosporioides,* and *Alternaria.* The compound showed antifungal effects which were moderate too high (Rao *et al.*, 2007).

Ethanol and ethyl acetate extracts of *S. costus* had the highest levels of polyphenols followed by n-butanol, and then n-hexane extracts. The main phenolic compounds are Naringenin, Chlorogenic acid, Ferulic acid, Ellagic acid, Gallic acid and coffeic acid followed by taxifolin, catechin, syringic acid, methyl gallate, vanillin, kaempferol, cinnamic acid and rutin and this extracts had antibacterial and antifungal agents against a broad range of microorganisms (Deabes *et al.*, 2021). S. costus showed the best antifungal activity against *Aspergillus flavus* followed by Trapa natans and Mangifera indica (Parekh and Chanda, 2008). The methanol extract of roots of S. costus is rich in some bioactive phytochemical compounds such as alkaloids, phenols/polyphenols, flavonoids, terpenoids, tannins, coumarins, quinines, steroids, cardiac glycosides, and resins and had antifungal activity against *Aspergillus niger* ATCC 6275 (Abdallah *et al.*, 2017). On the other hand, the mode of the antifungal action of alkaloids is usually pleiotropic, where protein synthesis is

inhibited, and fungal DNA is intercalated or by boosting development of fungi inhibitors (Arif *et al.*, 2009). Terpenoids reduced the mitochondrial content, thus modifying the level of reactive oxygen species (ROS) and ATP generation. It is also reported that triterpenoid possesses more potent antifungal activity as compared to tetraterpenoid (Haque *et al.*, 2016). Terpenoids and flavonoids make their effects by disrupting microbial membranes (Okusa Ndjolo *et al.*, 2009).

Flavonoids often inhibit fungal growth with various underlying mechanisms, including plasma membrane disruption, the induction of mitochondrial dysfunction, and inhibiting following: cell wall formation, cell division, RNA and protein synthesis, and the efflux-mediated pumping system (Al Aboody and Mickymaray, 2020). Medicinal plants possessed antifungal effects by many mechanisms, they caused membrane disturbance resulting in the loss of membrane integrity, inhibited DNA transcription and reduced the cell populations, inhibited the activity of fungal antioxidant enzymes, and inhibited fungal biofilm formation (Evensen and Braun, 2009; Wu et al., 2013). Finally, the antifungal activity of *S. costus* roots might be belonging to secondary metabolites like alkaloids, flavonoids, and terpenoids and their effect on proteins and DNA synthesis and disruption in membrane permeability or disturbance in metabolic activity.

Chapter Five

Conclusions

and

Recommendations

Chapter Five

5. Conclusions and Recommendations

5.1 Conclusions

A. The efficiency of *Saussurea costus* in inhibiting the fungus was higher at high concentrations, for crude alkaloids, flavonoids, and terpenoids extracts

B. The results and methods of storing rice grain were not efficient, so the isolation results showed seed borne fungi on of these grain with fungi and toxins.

C. Flavonoid compounds extracted from the grains of *Saussurea costus* have powerful antifungal activity against *Aspergillus* spp.

5.2 Recommendations

In order to complete the study and research on all aspects related to *Aspergillus*, we recommend the following:

A. Conducting an extensive study on the pathogenesis of *Aspergillus* fungi.

B. Conducting more studies on diagnosing *Aspergillus* fungi from other plants due to the possibility of the presence of many undiagnosed species.

C. Continuing to conduct studies on the pathology of other fungi accompanying the rice grains and studying the interaction between them and their common pathological effects on this plant.

D. Experiment and seeking for other plant have Antifungal activity against fungi.

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جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية العلوم للبنات

قسم علوم الحياة

الفعالية التثبيطية لمستخلص جذور *Saussurea costus*(*Falc.*) ضد الفطر *Aspergillus niger* المعزول من حبوب الرز المخزونة والكشف عن السموم المنتجة من الفطر

رسالة مقدمة الى

مجلس كلية العلوم للبنات / جامعة بابل / وهي جزء

من متطلبات نيل شهادة الماجستير في علوم الحياة

من قبل

فاطمة سلام جواد النافعي

(بكالوريوس علوم/ علوم حياة / جامعة بابل 2020)

بإشراف

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الخلاصة

تضمن الدراسة الحالي تحديد وتشخيص الفطريات المصاحبة لبذور الأرز التي تم جمعها من الأسواق المحلية المختلفة في مدينة الحلة، الواقعة في محافظة بابل العراقية. تم الحصول على 121 عزلة فطرية من 90 عينة من حبوب الأرز وتم التعرف عليها بناءً على خصائصها الزرعية والمظهرية.

من بين العزلات، تم التعرف على 31 عزلة على أنها *Aspergillus niger* تمثل 25.61٪ من مجموع العزلات وظهرت في 34.44٪ من العينات. اشتملت *Aspergillus flavus* على 12 عزلة تمثل 9.91٪ من مجموع العزلات وظهرت في 13.33٪ من العينات. تم تحديد 7 عزلات من A *Aspergillus.terrus* تشكل 5.78٪ من العزلات بينما 11 عزلة من *Alternaria* spp شكلت 9.09٪ من العزلات.

من 49 عزلة مختلفة تم عزلها، تم الحصول على 31 مزرعة نقية من *Aspergillus* niger. تم إجراء التحديد الأولي باستخدام التصنيف التقليدي بناءً على الصفات المظهرية . بعد ذلك، تم تحديد 24 عزلة من مجموعة *Aspergillus* niger بنجاح باستخدام تضخيم amplicone ITS1-5.8S-ITS2 مع مناطق التمهيدي المرافقة ITS5 و ITS4. أظهرت جميع العزلات خصائص أحادية الشكل، حيث تم تحديد 20 عزلة على أنها *Aspergillus niger*، 2 عزلة على أنها *Aspergillu brasillensis*، و 2 عزلات على أنها *Aspergillus tubigensis*

تم إخضاع خمسة عشر عزلة عشوائية من *Aspergillus niger* للتحليل الوراثي للكشف عن وجود جين fumonsin toxin (FB) باستخدام تفاعل البلمرة المتسلسل (PCR). من بين هذه العزلات الـ 15 ، أظهرت 8 حزم بقياس 651 نقطة أساس في الترحيل الكهربائي لهلام الاغاروز. بالإضافة إلى ذلك، من بين إجمالي 15 عزلة، أظهرت 7 عزلة فقط حزم (420 نقطة أساس) في الترحيل الكهربائي لهلام الاغاروز Ochratoxin (OTA) .

كما تناولت الدراسة الفعالية المضاد للفطريات للمركبات القلوية الخام المستخلصة من جذور نبات القسط الهندي *Saussurea costus* ضد *Aspergillus niger* المعزول من بذور الأرز التي تم الحصول عليها من الأسواق المحلية في الحلة، العراق. تم تقييم فعالية المضاد للفطريات باستخدام ثلاثة تراكيز (5 ، 10 ، 15 %) من خلال تقنية التسمم الغذائي. أظهرت النتائج أن المركبات القلوية الخام من جذور

القسط الهندي تثبط بشكل معنوي نمو *Aspergillus niger* مقارنة بالتحكم السلبي (10٪ DMSO) والتحكم الإيجابي (مبيد الفطريات Carbendazim 500 جم / لتر). تراوحت نسب تثبيط mycelial من 66.0٪ عند 5 مجم / مل ، 71.6٪ عند 10 مجم / مل ، إلى 100٪ عند 15 مجم / مل.

وبالمثل، أظهرت مركبات الفلافونويد الخام فاعلية مضادة للفطريات، مع تثبيط فطري بنسبة 62.3٪ عند 5 ملغ / مل و 100٪ بتركيزات 10 ملغ / مل و 15 ملغ / مل.

ايضا ,اظهرت مركبات التربينات الخام فاعلية مضادة للفطريات , مع تثبيط فطري بنسبة 32.1 % عند 5 ملغ / مل و 51.0٪ عند 10 ملغ / مل و100% عند 15 ملغ / مل اختلفت هذه النتائج بشكل كبير عن معاملة السيطرة وأظهرت تأثيرًا مشابهًا للتحكم الإيجابي.