PRODUCTION AND EXTRACTION OF PHENOLIC COMPOUNDS FROM RICE BRAN, MILLET HUSK, AND GROUNDNUT SHELLS BY SOLID-STATE FERMENTATION USING *Trichorderma harzianum, Aspergillus niger, and Penicillium* sp.

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AUGUST, 2019

DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Kabir Mustapha Umar, and has not been presented elsewhere for the award of a degree or certificate

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CERTIFICATION

This to certify that this research work has been read approved as adequate in scope and content for acceptance in partial fulfilment of the requirement for the award of Master Degree in Applied Biology (Ecology and Environmental).

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DEDICATION

I dedicate this research to my beloved husband Ahmed Hassan Suleiman and my children Aisha, Amani, and Ahmed Jnr.

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LIST OF ABBEVIATIONS

SSF - Solid-State Fermentation

PCs - Phenolic Compounds

G/n - Groundnut

TPC - Total Phenolic Content

TFC - Total Flavonoid Content

DPPH - (2, 2- Diphenyl-1-Picryhydrazyl) Radical Scavenging Assay

TH – *Trichorderma harzianum*

PS – *Penicillium* sp.

AN – *Aspergillus niger*

ABSTRACT

Solid-State Fermentation (SSF) has been employed as a protocol of making available higher content of functional compounds from agro-industrial wastes. In this research, the effect of SSF with fungus on the content of Rice bran, Millet husk and Groundnut shells were examined for production and extraction of Phenolic compounds from these agro-wastes with *Trichorderma harzianum*, *Aspergillus niger*, and *Penicillium* sp. Isolation of fungi from soil samples were made of *T. harzianum*, *A. niger*, and *Penicillium* sp. Free radical scavenging activities at different fractions were measured by 2, 2, *diphenylpicrylhydrazyl* (DPPH) radical scavenging method which was carried out to confirm the antioxidant activity of the compounds. In DPPH assay the concentrations of (µg/ml) 500, 1000, and 1500, extracts of *Penicillium* sp. and on samples of Rice bran showed higher activity. Mortality of *Artemia salina* count was observed at all level of concentration of the extract. Statistically, the result of one way analysis of variance (ANOVA) for each fungal extracts diluted with different concentrations (10ml, 100ml, 1000ml) on the mortality of Brine shrimp larvae revealed a significant difference among fungal extracts of *Aspergillus niger*, *Penicillium* sp. and caffeine on the mortality of Brine shrimp larvae ($P<0.05$). However, there was no significant difference between the concentrations of fungal extract *Trichorderma harzianum* and the control at different concentrations on the mortality of Brine shrimp larvae ($P>0.05$). High mortality value of 8.33±0.58 in all fungal extracts of Rice, Millet and Groundnut was observed in the positive control. There were no significant differences between positive control and all levels of concentrations. But there were significant differences between the negative and positive control in all levels of concentrations. Directly indicates the effectiveness of the extract when compared to the caffeine which is normally recommended as highly toxic to the Brine shrimp larvae. Despite the fact that there were no significant differences between the different levels of concentrations of the extracts and positive control, high mortality value of 8.00±1.00, 8.33±0.58 and 8.00±1.00 were all observed in high levels of concentrations of the fungal extracts. This is an indication of the high toxicity and efficacy of the extracts to the brine shrimp larvae. Phytochemical screening revealed the presense of flavonoids, steroids and phenols in the agro-wastes with groundnut shells extracts having the highest values followed by extracts of Rice bran with *Penicillium* sp. and Millet husk with *T. harzianum*. Hence, the research suggests presence of bioactive compounds in agro-wastes.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Antioxidants are molecules capable of reducing or preventing other molecules from oxidizing, while biological antioxidants may be defined as substances that, when in small concentrations compared to that of an oxidizable substrate, delay or prevent oxidation of that substrate (Moon *et al.,* 2009). Due to risks of consuming synthetic antioxidants, research studies into natural products containing antioxidant activity have increased with the aim of replacing them or applying associations that reduce the toxic effect (Aungulu *et al.,* 2007; Mendiola *et al.,* 2010).

Among the several classes of naturally occurring antioxidant substances, phenolic compounds have drawn particular attention because they inhibit lipid peroxidation (i.e. oxidative degredation of lipid which is a process in which free-radicals steal electrons from the lipid in cell membrane resulting in cell damage, while oxidation is the combination of a substance with oxygen which result in oxidative degredation of lipids, also known as rancidity (Kristinova *et al.,* 2009) and lipo-oxygenation *in vivo*. The inhibition effect is mainly due to the reducing properties of the chemical structures that enable neutralization or sequestration of free radicals, as well as the chelation of transition metals, thus avoiding the phase of inhibiting the spread of oxidative processes. Phenolic compounds, ubiquitous in plants are an essential part of the human diet, and are of considerable interest due to their antioxidant properties. These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer. Flavonoids, which bear the C6–C3–C6 structure, account for more than half of the over eight thousand different phenolic compounds. The antioxidant activity of phenolic compounds depends on the structure, in

particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (Kristinova *et al.,* 2009).

1.2 Sources of Phenolic Compounds

Cereals, fruits, vegetables and beverages are the major sources of phenolic compounds in the human diet. The food and agricultural products processing industries generate substantial quantities of phenolics-rich by-products, which could be valuable natural sources of antioxidants (Samir *et al.,* 2005). Vegetable tissues are good sources of these compounds, often containing simple phenols, phenolic acids (derived from benzoic and cinnamic acid), coumarin, flavonoids, stilbenes, condensed and hydrolysable tannins, lignins, and lignans (de Melo *et al.,* 2002; Moon *et al.,* 2009).

1.3 Importance of Agro-Wastes Used

Cereals play a vital role in human diet as an important source of energy, protein, and micronutrients among others for majority of people in the world (Zhang *et al.,* 2012). Dietary recommendations worldwide emphasize the significance of cereals in a balanced diet. Furthermore, cereals have been proven to provide additional health benefits while satisfying the energy and nutritional needs of humans. Risk of non-communicable diseases (NCDs) is increasing worldwide at an alarming rate in developed as well as developing regions. Several studies found that the regular consumption of whole grains and whole grain products are helpful and they reduce the prevalence of NCDs (Slavin, 2004; Okarter and Liu, 2010).

1.4 Solid-State Fermentation Process

Solid State Fermentation (SSF) is an alternative to liquid or sub-merged fermentation used predominanatly industrial purposes (Zhang *et al.,* 2012). The increased antioxidant potential of several raw materials by fermentative processes has different hydrolytic enzymes and can be produced directly from solid substrate and can, simultaneously, be used to release phenolic compounds.

Solid-state fermentation has been studied, described, and revised by several authors (Slavin, 2004; Okarter and Liu, 2010). Generally, the substrates have a water content oscillating between 30 and 85%. Lower values may induce sporulation of the microorganism, while more elevated levels may reduce porosity of system, which can produce oxygen transfer limitation, and increase risk of bacterial contamination (Pérez-Guerra *et al.,* 2003).

According to Raimbault (1998), the water requirements of microorganism may be better defined in terms of water activity rather than water content in the solid substrate. The establishment of the most suitable conditions for use of the process variables is of relevance to achieve elevated process yields. The use of experimental design statistical methodology may be a useful tool to define such conditions performing a minimal number of experiments. Recently, several works report the use of statistical analysis to maximize the product formation through the establishment of the best SSF operational conditions. Such works include the production of enzymes such as α-amylase (Reddy *et al.,* 2003), inulinase (Xiong *et al.,* 2007), phytase (Singh and Satyanarayana, 2008), protease (Reddy *et al.,* 2008), xylanase (Senthilkumar *et al.,* 2005), laccase (Liu *et al.,* 1989), biosurfactants organic acids such as citric acid (Mukherjee *et al.,* 2008).

1.5 Justification

This dissertation is to provide an overview of the bioactive phenolic compounds extraction and production by fermentation, more specifically by the solid-state fermentation (SSF) technique. The current status of this technology, the microorganisms, substrates and cultivation conditions affecting the phenolic compounds formation are summarized and discussed.

1.6 Aim of the Research

The aim of this research is to determine the production and extraction of Phenolic compounds from agro-wastes mainly rice/bran, millet husk, and groundnut shells by solid-state

fermentation and their antioxidant properties with *Trichorderma harzianum, Aspergillus niger and Penicillium* sp.

1.7 Objectives

1. To isolate and identify endophytic fungi from the soil.
2. To evaluate the availability of compounds with antioxidant properties in solid-state fermented rice/bran, millet husk, and groundnut shells using *T. harzianum, A. niger and Penicillium* sp. using DPPH Radical Scavenging Assay.
3. To determine mortality rate and cytotoxic properties of the fungal extracts.
4. To determine phytochemical screening of the fungal extracts.

CHAPTERTWO

2.0 LITERATURE REVIEW

2.1 Definition of Endophytes

During the past 30 years, the terms endophyte and endophytic fungi have appeared frequently in the mycological literature to describe the internal mycota of living plants. Although the origin of the term "endophyte" can be traced back to the nineteenth century, its contemporary meaning is different from the original one (Moon *et al.,* 1999). Today most commonly used definition is that of Petrini, (1986). In the broadest sense, endophytic fungi are fungi that colonize living plant tissue without causing any immediate, overt negative effects (Hirsch and Kapulnik, 1998). This definition includes virtually the entire spectrum of symbiotic interactions in which fungi and plants participate: parasitism, commensalism, and mutualism. For grass hosts (primarily *Poaceae*), the word endophyte has been used to denote a particular type of systemic, nonpathogenic symbiosis. Grass endophytes provide their hosts with a number of benefits, such as protection against herbivory and pathogens, and thereby increase their fitness (Saikkonen *et al.,* 2002). Taxonomically these fungi are primarily *Neotyphodium* anamorphs of *Balansiae* (*Clavicipitaceae*); they colonize leaf, culms, and root tissues of species of cool-season grasses extensively and are transmitted in their hosts‟ seeds. Sporulation on the host is suppressed completely, and host and fungus function together essentially as a single organism. These symptomless endophytes of *Lolium*, *Festuca*, and other genera of pooid grasses are interspecific hybrid strains derived from *Epichloë* species that cause partial or complete host sterility (Moon *et al.,* 1999). One of the early publications describing an endophytic fungus was by Freeman in 1904, where he has made reference to four other papers on endophytes that were published in 1898. This paper described a fungus in Persian darnel - an annual grass that today is considered a troublesome weed by many

wheat farmers (Schulz *et al.,* 2002; Schardl *et al.,* 2004). Between 1930 – 1990, several discoveries prompted a series of studies in which similar asymptomatic endophytes were recorded in a wide range of grasses (Rodriguez *et al.,* 2009). More recent reports describe European endophytes, endophytes of palms, grasses and woody plants (Petrini and Fisher, 1992; Schulz *et al.,* 2002; Patrcia and John, 2011).

2.2 Endophytes and their Potential in Drug Discovery

Endophytic microbes from medicinal plants are good source of functional metabolites (Weber *et al.,* 2004; Tejesvi *et al.,* 2007; Huang *et al.,* 2008). Endophyte infections are found to alter pattern of gene expression in the host plant (Baily *et al.,* 2006). Interaction between endophyte and plant is mainly controlled by the genes of both organism and host plant modulated by the environment. Endophytes from angiosperms as well as gymnosperms have been studied for presence of novel secondary metabolites. Primary metabolites are common in all living cells and are involved in the formation of biomass and generation of energy, in contrary secondary metabolites are produced by one or few species only. These secondary metabolites are low molecular weight compounds, they are not required for growth in pure culture and are produced as an adaptation for the specific function in nature. Bioprospecting is most frequently used phrase to describe the collection and screening of the biological material for commercial purposes. The importance of natural products in the drug discovery and development has been reported briefly. The natural products produced by endophytes have vast range of bioactivities, representing a vast reservoir offering an enormous potential for exploitation in medicinal and industrial uses (Zhang *et al.,* 2006). Crude extracts from culture broth of endophytes found to show antibacterial, antifungal, antiviral, anti-inflammatory and antitumor activities (Silva *et al.,* 2007). Endophytes open up new areas for the biotechnological exploitations.

2.3 Host Endophyte Relationship and Effect on Metabolites Production

Microorganisms are likely to harbor metabolic pathways that lead to the production of novel secondary metabolites. Endophytes from plant origin are investigated for their secondary metabolite production. Endophytic microbes are nonpathogenic in nature, production of secondary metabolites enable them to survive in the plant interstitial space. Many of the important secondary metabolites have been extracted and characterized from endophytic microbes which includes alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenol. In addition natural products also often serves as lead structures whose activity can be enhanced by exploitation through synthetic chemistry (Strobel and Daisy, 2003).

Endophytes are able to increases host fitness and competitive ability, by increasing nutritional uptake, resistance to seed predators, seed germination success, tolerance to heavy metals, high salinity and good growth rate through biochemical pathways such as phytohormone indole 3 acetic acid (IAA) from fungal endophytes *Acremonium coenophialum, Aureobasidium pullulans, Epicoccumpurpurascens* and *Collectotrichum sp.* along with IAA, cytokinins were also produced by an endophytic *Hypoxylon serpens*.

Plant provides spatial arrangement, shelter, nutrient and distribution to the next generation of microbes (Rudgers *et al.,* 2004). Plant may provide vital compounds for the completion of the life cycle of the endophytes (Strobel, 2002). Current research suggested that endophyte and plant genotypic combinations together with environmental conditions are important source of variation in endophyte host interactions (Faeth and Fagan, 2002). Many factors such as season, age, environment and location these may contributes and influence the biology of endophyte (Strobel and Daisy, 2003). Endophyte derives nutrients from the plant without killing host. Endophytic microbes such as *Phomopsis*, *Phoma*, *Colletotrichum* and *Phyllosticta* have wide host range and found to colonize numerous taxonomically distinct

plants (Pandey *et al.,* 2003; Jeewon *et al.,* 2004; Murali *et al.,* 2006; Sieber, 2007) developing adaptations to overcome different types of host defence, endophyte infection found to affect concentration of abscisic acid in leaves of drought stressed grasses, this helps in the recovery of endophytes infected plant in water deficient conditions, endophytic microbes residing in the host tissue some time turn in to a pathogen in response to some environmental signal (Hendry *et al.,* 2002). Change in the nature of the endophyte would result in its metabolite (Kuldav and Yates, 2000; Suryanarayanan and Murali, 2006), endophytic microbes associated with traditionally used medicinal plants particularly of the tropics could be a rich source of functional metabolites (Tejesvi *et al.,* 2007).

In this regard plant host association could also be exploited in enhancing the production of useful metabolites by the host (Wang *et al.,* 2004). Genomic projects are being performed on some endophytic bacteria such as *Azoarcus* sp., *Herbaspirillum* sp., *Gluconacetobacter diazotrophicus* and *Klebsiella* sp. which will certainly result for further understanding, their molecular interaction with plants may be used as well to study gene expression during endophytic life.

2.4 Protection from Pathogens

Studies indicate that fungi may express different symbiotic lifestyles in respond to host genotypes and environmental factors (Tanaka *et al.,* 2005). Both pathogenic and non-pathogenic fungi isolated from asymptomatic plant tissues, suggestive of that both pathogenic and nonpathogenic microbes express lifestyles or remain dormant in expectation of plant senescence (Schulz *et al.,* 1999). Life style expression is a post colonization phenomenon communicating between the symbiont and host. It is recognized that mutalistic fungi collectively may confer several host fitness benefits, such as growth improvement or tolerance to drought, disease, herbivory, insect and temperature to plant (Azevedo *et al.,* 2000; Kerry, 2000; Redman *et al.,* 2001; Redman *et al.,* 2002). The first verification that

microbial endophytes associations were responsible for anti herbivory in mammals is confirmed by finding that *N. coenophialum* can produce ergot alkaloids with toxicity of cattle and some other livestock (Bacon *et al.,* 1977). Tanaka *et al. (*2005) demonstrated the role of peramine in anti herbivory by *Argentine weevil* stem, *Listronotus bonariensis* is symbiont of perennial rye grasses. It has been also documented that the janthitrems which are found in some strains of *Neotyphodium* in perennial rye grass are found to contribute in insect toxicity. Indole compounds as sesquiterpene and diacetamide along with some unidentified volatile compounds have been connected with *Epichole* species for resistance to leaf spot rust. The endophytes associated plants generally produces some metabolites that induces resistance to pathogens and it was also found that symbiotic plant activates defense system more quickly than non-symbiotic plants after pathogen challenge.

2.5 Diversity and Distribution of Microorganisms Recovered as Endophytes

Endophytes lives, reproduce and forms mycelia that grow between the cells of a plant, more in the leaf sheaths and reproductive structures. When seed production starts, endophyte grows upward in the host plant. After seed formation, endophyte infects outer layers of the seed and endophytes transfers from plants in a seed production field to seed when such seeds germinates and grows, endophytic microbes start infecting the other host. There are some major points represented as (i) Individual endophytes can switch symbiotic lifestyles and the result of symbiosis is influenced by host genotypes (ii) Mutalistic benefits conferred by endophytes are also influenced by plant genotypes (iii) The host range of endophytes is inadequately defined and which includes both monocot and dicot species and (iv) Endophyte host plant describe adaptive symbiosis. Some endophytes have evolved with a high degree of suppleness to enter between genetically distinct plant species so provides endophytes an option to develop habitat range. Endophytic microbes can have intense effects on plant ecology, their fitness and are able to produce number of bioactive agents (Strobel and Daisy,

2003; Young *et al.,* 2012). The fossil proof shows that fungal symbionts have been associated with plants from the Ordovician period of approximately 400 million years ago, when plants first became established on land (Redecker *et al.,*2000), migrating from aquatic to terrestrial habitats. Individual plants represent symbiotic endophytic communities associated within or on tissues below and above ground. Diverse group of this organism are able to produce bioactive agents with broad applications.

There are two major classes of fungal symbionts associated with internal plant tissues such as,

i. Fungal endophytes residing entirely within host plants and associated with roots, stems, leaves and flowers.
ii. Mycorrhizal fungi that are residing only in roots but extend out into the rhizosphere. Fungal endophytes are also divided into two classes:
    a. A comparatively minute number of fastidious species limited to a few monocot host plants (Clay and Schardl, 2002).
    b. A huge number of tractable species with broad host ranges, together with monocots and eudicots (Stone *et al.,* 2000).

Considerable research have been done in class i endophytes corresponding largest group of fungal symbionts. This is because the class ii endophytes have only been elucidated in recent times and shown to be responsible for the adaptation of some plants to high-stress environments (Arnold and Herre, 2003). Endophytic fungi may express different symbiotic lifestyles in response to the host genotypes and environmental factors. Lifestyle expression of endophytes is a post colonization phenomenon which involves biochemical and genetic communications between endophytic microbes and host, grass species have been entirely studied in relation to their endophytic biology (Tran *et al.,* 2010). *Clavicipitaceous* endophytes represents Class i and are small number of phylogenetically related

Clavicipitaceous species which are fastidious in culture and also limited to some cool and warm seasonal grasses (Stone *et al.,*2004). Transmission of these class i endophyte is mostly vertical (Figure 1), with maternal plants passing fungi on to offspring by means of the seed infections (Saikkonen *et al.,* 2002). Class ii endophyte generally comprises diverse species, all of which in general are members of the Dikarya (*Ascomycot*a or *Basidiomycota*) having ability to give habitat specific stress tolerance to host plants. *Clavicipitaceous* endophytes are defensive mutualists of host grasses and play role during their evolution (Faeth and Fagan, 2002; Lane *et al.,* 2003; Leuchtman, 2003; Schardl *et al.,* 2004). Class III endophytes are basically distinguished on the basis of their occurrence and horizontal transmission including vascular, nonvascular plants, some woody and herbaceous angiosperms in tropical forest and antarctic plant communities (Murali *et al.,* 2006). These endophytes are especially known for their huge diversity within individual host tissues, plants and also populations. Class IV endophytes contains darkly melanized septa and they are restricted to plant roots. Generally these are *Ascomycetous* fungi conidial or sterile and forming melanized structures and also founds in non mycorrhizal plants from antarctic, tropical ecosystems and temperate zones.

Fungal endophytes were isolated from healthy stems and pods of the *obromagileri*, many of these species were understood to be *Basidiomycetes.* These endophytes have received far less concentration than ascomycetes and also have potential as biological control agents of the *basidiomycetous* pathogens of *T. cacao, Moniliophthora roreri* (frosty pod rot pathogen) and *M. perniciosa* (witches broomdisease). *Basidiomycetes* are identified to produce a range of bioactive secondary metabolites, which are similar to those produced by *ascomycetes* (Schulz *et al.,* 2002). Mainly successful use of a *basidiomycete* as a biocontrol agent was *Phlebiopsis gigantea* for control of the root-rotting pathogen *Heterobasidion annosum* of *Pinus* sp. Also seven diterpenoid compounds were produced by *Coprinellus heptemerus*, a basidiomycete inhibiting spore germination of the fungal rice blast pathogen, *Magnaporthe grisea*. Their

transmission is either vertically (directly from parent to offspring) or horizontally (from individual to unrelated individual). Fungal endophytes those vertically transmitted are sexual and transmit via fungal hyphae penetrating the hosts seeds for e.g. *Neotyphodium*, these fungi are frequently mutualistic and on the contrary, endophytes transmitting horizontally are sexual and transmit via spores which can be spread by wind and insect vectors also The endophytic microbes possibly adopts the same strategy as that of plant pathogenic fungi in order to get enter the host plant (Sieber, 2007).

Endophytes are very diverse and that only small minorities of all existing endophytes have been characterized as a single leaf of a plant can harbor many different species of endophytes, which may be bacterial or fungal. Tropical and temperate rainforests are the largest part of biologically diverse terrestrial ecosystem on the earth. Bills *et al.* (2002) described that metabolic distinction between tropical and temperate endophytic microbes through statistical data comparing the number of bioactive natural products from endophytes of tropical regions to that of temperate origin. And found that higher number of tropical endophytes produce an enormous number of bioactive secondary metabolites than from other tropical substrata suggesting the importance of the host plants in influencing the metabolism of endophytic microbes.

Research emphasize that endophytes are usually not host specific. Same microbe isolated from different tissues or parts of the same host plant differ in their abilities for utilization of different substances (Carroll, 1986). Endophytic organisms associated with plants are varied and complex for example, fourteen distinct endophytic bacteria were isolated from of *Carica papaya* L. six Gram-negative genera, which are *Pantoea ananatis*, *Enterobacter cloacae*, *Brevundimonas aurantiaca*, *Sphingomonas*, *Methylobacterium rhodesianum*, and *Agrobacterium tumefaciens,* two Gram-positive genera, *Microbacterium esteraromaticum*

and *Bacillus benzoevorans* (Thomas and Clayfuqua, 2007). From last two decades diverse endophytes have been targeted as sources of valuable bioactive compounds.

Several different bacterial endophyte species were isolated from single plant (Zinniel *et al.,* 2002). Eighteen different endophytic fungi were isolated from different tissues of bark, stem and leaf segments of five medicinal plants found within in Kudremukh range of Western Ghats of India, the dominant species isolated were *Curvulana clavata, C. lunata, C. pallescens* and *F. oxysporum*. The highest species richness as well as colonization frequency was found in the leaf segments of the host plant species which confirms tissue specificity of these microbes (Zinniel *et al.,* 2002).

Subsequent identification of potential genes provides evidence of specific pathway for known alkaloids synthesis by endophytes (Panaccione *et al.,*2001; Wang *et al.,*2004; Spiering *et al.,*2005; Tanaka *et al.,*2005; Young *et al.,*2005; Young *et al.,*2006). Consequently if endophyte can produce the same bioactive compounds as their host plants this would reduce the need to harvest slow growing rare plants and also help to preserve the worlds diminishing biodiversity.

Some endophytic *A. niger, A. terreus* and *A. alternata* were organ specific (Khan *et al.,* 2010). A total of four diazotrophic endophytic bacteria were isolated from *Bambusa blumeana*. Showing big diversity with different genera as *Azospirillum*, *Escherichia*, *Pseudomonas* and *Aquaspirillum* (Wei *et al.,* 2007). Diversity of fungi in host tissues like root, stem, leaf and seed of mangrove wild legume *Canavalia cathartica* yields thirty six endophytic fungi with a maximum species in stem which followed root. The species diversity of foliar endophytes assembly changes with the leaf age (Photita *et al.,* 2004; Suryanarayanan and Thennarason, 2004). Indicating that sampling endophytes from a plant for bioprospecting on a single time may not arrest entire variety of endophytes and their metabolites. *Calotropis*

gigantean showed the presence of thirteen endophytic fungal species like *Aspergillus niger*, *Aspergillus flavipes, Alternariaporri, Curvularia lunata, Fusarium oxysporum, Nigrospora sphaerica, Colletotrichum falacatum, Pestalotiopsis sydowiana, Phoma exigua, Phomopsis archeri, Leptosphaerulina chartarum,* and *Mycelia sterilia.* Endophytic diversity is generally manifested with their morphology and also with the types of benefits they offer to the host plant. Fungal diversity is much larger from endophytes of trees than endophytes of grass.

The percent frequency of occurrence of endophytic fungi found to increase between seed, root, stem, leaf segments in host *C. cathartica*. The mean percent frequency of endophytic fungi was highest in pod (11.6%), followed by leaf (9.8%) and root (8.9%). In *C. cathartica*, *Chaetomium globosum* was the dominant endophytic fungus and root showed its dominance as single species (*C. globosum*) (Seena and Sridhar, 2004). In mangrove *C. cathartica*, multispecies dominance was seen. *A. flavus, Fusarium oxysporum* and *Nonsporulating* sp.. *Acremonium, Colletotrichum* and *Fusarium* were common endophytes in halophytes, mangroves and sea grass (Beena *et al.,* 2000; Kumaresan and Suryanarayanan, 2001; Ananda and Sridhar, 2002; Devarajan *et al.,* 2002; Maria and Sridhar, 2003). Tissue specificity of endophytic fungi in whole-stem and xylem has been reported in tree species *Pinus* and *Fagus* (Petrini and Fisher 1992). Some fungi were restricted to specific organ or tissue e.g. leaf: *Cladosporium oxysporum*, *Aspergillus* sp.., pod: *Aspergillus fumigatus*, seed: *Eurotium chevalieri*. *Ascomycetes* usually are the dominant decomposers of plant detritus in mangrove and marine habitats (Kohlmeyer and Volkmann-Kohlmeyer, 1991). However, endophytic fungi were dominated by mitosporic fungi in mangroves and halophytes (Beena *et al.,* 2000; Ananda and Sridhar, 2002; Seena and Sridhar, 2004). Importance of endophytic fungi in grasses has been understood better than non-grass endophytic fungi (Hyde and Soytong, 2009). The endophytic fungi of non-grass are important because of deterring or decreasing insect herbivory, enhancing drought/disease resistance in plants.

2.6 Habitat and Physical Environment

Endophytes have been isolated from the roots and aerial parts of a diverse range of hosts such as bryophytes, pteridophytes, gymnosperms, angiosperms and algae (Wang *et al.,* 2006; Kharwar *et al.,* 2008). Some physical factors, such as temperature, rainfall, edaphic factors and UV radiation also found to affect endophytic communities directly or indirectly. Along with some indirect effect of soil physical and chemical factors including pH, salinity and soil texture. Inside the plant tissue, the density of endophyte is less than pathogen. Both biotic and abiotic effects mainly influence the dynamic patterns of endophytic microbe in relation to plant host (Rosenblueth and Martinez, 2006).

2.7 Endophyte Host Interaction

Endophytes interact with host mutualistically. Endophytic fungi found to produce alkaloids in the plants. Some of the agronomic species infected with endophytes showed resistance to toxic effects on vertebrate and invertebrate herbivores. Endophytic bacteria found within plant tissue as biotrophic symbionts and either obligate or facultative. Endophytic bacteria can colonize thousands of different plant species, while some of them are restricted to selected plant families. Bacterial endophyte produces large number of phytohormones, such as auxins, cytokinins and the gibberellins. Examination indicates that interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environmental conditions (Battistoni *et al.,* 2005; Rosenblueth and Martinez*,* 2006). There is inadequate data on the endophyte host molecular interactions.

2.8 Plant Colonization with Endophytes and Effect of Plant Age on Endophyte

The growth stimulation by the endophytes may also by nitrogen fixation, phosphate solubilization, production of siderophore and also supply of essential vitamins to plants (Sevilla *et al.,* 2001; Verma *et al.,* 2001; Hurek and Reinhold-Hurek, 2003; Iniguez *et al.,* 2004; Pirttila *et al.,* 2004; Wakelin *et al.,* 2004) phytohormones, production of volatile

substances like 2-3 butanediol and aceotin for plant growth (Ryu *et al.,* 2003). Endophyte produces adenine ribosides that found affect nematodes propagation (Sturz *et al.,* 2004) for e.g. endophytic *Herbaspirillum seropedicae* and *Clavibacter xylii* are genetically modified for excretion of deltaendotoxin of *Bacillus thurengensis* to control insect pests, as the future application for agriculture related areas. Bacterial endophytes isolated from leaves of *Ocimumsanctum* shows growth promoting benefits (Tiwari *et al*., 2010). Bacterial endophytes were isolated from the Balloon flower (*Platycodon grandiflorum*) showed that the population of low G+C Gram positive bacteria (LGCGPB) gradually increased 60-80% from 1 to 6 years with maximum hydrolytic enzyme activity and it is presumed that elder balloon flower plants invite more potential antifungal endophytes, therefore plant age is presumed to influence diversity of balloon flower endophytic bacteria (Shah *et al*., 2010). Some bacterial endophytes find their mode of entry through cracks generally formed at the emergence of lateral roots and also at the zone of elongation seed sections carries diversity of endophytes (Coombs *et al.,* 2003). Presence of endophytic organisms were noted in almost all parts of host plant such as roots, stems, leaves, seeds, fruits, tuber, ovules and also inside legume nodules (Benhizia *et al.,* 2004).

2.9 Plant Growth Promoting Endophytes

Management of advantageous microbial communities to favor plant growth could better understand by physiological and molecular interactions between microbe and plant. Endophytes show numerous direct and indirect mechanisms to promote plant growth and health. Consideration of these mechanisms can progress the value of poplar and some other plants as feed stocks for biofuel production and plant growth (Dell Amico *et al.,* 2005). Non reducing disaccharide trehalose is main storage carbohydrate of bacteria, it can be produced in plants to a much lesser extent than sucrose, this sugar thought to play a vital role in plants controlling their partitioning of carbon into cell wall biomass (Ramon and Rolland, 2007).

Activity levels of trehalase enzyme responsible for degrading trehalose sugar strongly induced by infection with the trehalose producing microbes for example *Plasmodiophora brassicase*. Alteration in biosynthesis and metabolism of trehalose also increase tolerance to drought, salt, and cold. Therefore several endophytic bacteria from poplar were able to metabolize trehalose (Taghavi *et al*., 2005; Porteous *et al.,* 2006). Plant-associated bacteria can also indirectly benefit plant growth by preventing the growth of plant pathogens through, antibiosis (Ramos-Gonzalez *et al.,*2005), production of some hydrolytic enzymes along with induction of plant defense mechanisms (Spencer *et al.,*2003; Kloepper *et al.,*2004; Ryu *et al.,* 2004; Zhang *et al.,*2004). Plant growth promoting endophytic bacteria were isolated from *Brachiaria* hybrid CIAT 36062 and introduced into *Brachiaria* hybrid cv. *Mulato,* positive for nif H gene sequence, and inoculated *Mulato* plant showed higher chlorophyll and total nitrogen contents in leaves. DNA sequence analysis demonstrated that the nif H gene found were highly similar to *Klebsiella pneumoniae* and some other N_2-fixing organisms. For this reason plant research area are now diverted to use endophytes in development of agriculture crops and forest regeneration.

2.10 Natural Products of Endophytes

The requirement for new antimicrobial agents generally, comes from the increasing resistance of pathogenic microbes towards antibiotics. Many microorganisms of agricultural concern are also known to acquired resistance to commonly used antimicrobial chemical compounds. So the interest in natural methods of pathogen control through new, eco-friendly agents is increased. The biologically active natural products from endophytes are excellent resources for medicine, agriculture and industry (Guo *et al.,* 2008). Amines and amides are very common metabolite products from endophytes and have shown to be toxic to insect but not to mammals. Bioactive metabolites, such as steroids, terpenoids and diterpenes also are generated by endophytes. Endophyte also produces extracellular hydrolyases to establish a

resistance mechanism against plant invasion which includes some of the extracellular enzymes like cellulases, proteinase, lipases and esterases. The actions of these enzymes found to support the hypothesis of co-evolution between endophytes and their hosts. Number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganisms (Zhang *et al.,* 2006). Endophytic fungi are a promising source of novel compounds. About 51% of biologically active substances from fungal endophytes were previously unknown (Strobel, 2002).

2.10.1 Role of Endophytes in the Discovery of Anticancer Agents

Endophyte hold main position in drug discovery as it has antibiotic, antiviral and anticancer properties, due to their ability to produce chemical which can be used as drug. Pacli taxel was first found in plants and later on reported from fungal endophyte. It is the first major group of anticancer agent which is produced by endophyte and now much research has been conducted on endophytes to determine its anticancer activity. Production of taxol was also done from an endophytic fungus, *Lasiodiplodia theobromae* isolated from *Morinda citrifolia* with its cytotoxicity against human breast cancer cell line.

Subsequently one hundred anticancer compounds which belong to different chemical classes with activity against 45 different cell lines have been isolated from different fungal species belonging to different groups, out of which 57% were novel or analogues of known compounds. Endophytic fungi was isolated and identified from *Juniperus communis, L. horstmann*, as a novel producer of deoxypodophyllotoxin (Strobel, 2002).

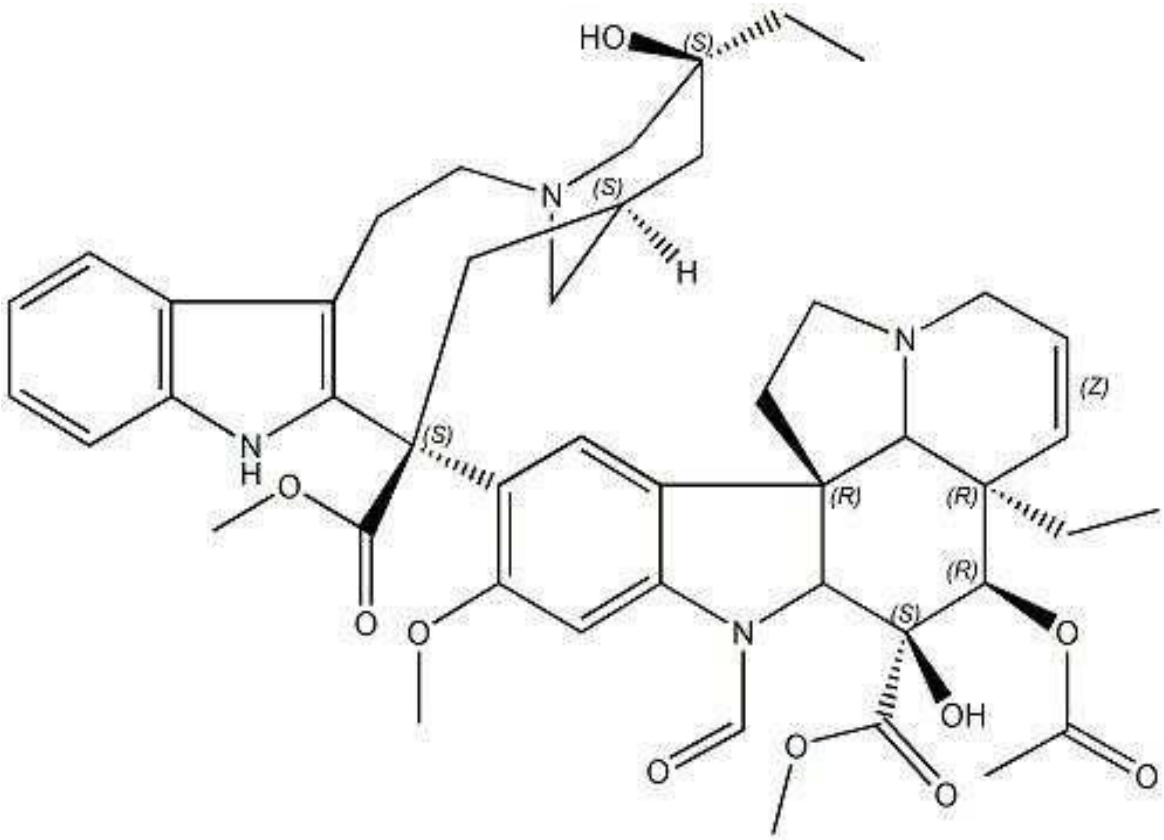


Figure 1.1: Vincristine

Chaetopyranin a benzaldehyde derivative isolated from the endophytic fungus *Chaetomium globosum* associated with the marine red alga *Polysiphonia urceolata*. Chaetopyranin exhibited moderate to weak cytotoxic activities against three human tumor cell lines HMEC (human microvascular endothelial cells), SMMC-7721 (hepatocellular carcinoma cells) and A54 (human lung epithelial cells) (Wang *et al.,* 2004). Some of the most potent plant-derived antitumor alkaloids have also been reported from endophytic fungi. In 2005 camptothecin was isolated from a fungal endophyte *Entrophosphora infrequens* from *Nothapodytes foetida*. Indole alkaloid emindole was isolated from endophyte *Emericella nidulans* var. acristata, from Mediterranean green alga, exhibiting antitumor activity against 36 human tumor cell lines. Anticancer drugs been given in chemotheraphy treatment for several type of NCDs are indole derivatives one of which known as vincristine (Figure 1.1). Vincristine available under trade names Oncovin R was originally obtained from plant *Catharanthus roseus* now Chinese researchers reported evidence that vincristine might be produced by endophytic *Fusarium oxysporum* from the same plant (Zhang *et al*., 2006).

2.10.2 Extracellular Enzymes

Twenty one unique taxa of fungal endophytes were examined for their ability to produce enzymes *In Vitro* from *Brucea javanica*, for production of extracellular enzymes such as amylase, ligninase, pectinase and xylanase, most endophytes were degraders of the simpler sugar and cellulose available in recently dead leaves and wood. Dark septate endophytes (DSE) are widespread in the native tall grass prairie. Species of *Periconia, Fusarium, Microdochium* and *Aspergillus* were isolated. *Periconia* and *Microdochium* were positive for gelatinase, cellulase, amylase, polyphenol oxidases using both of organic and inorganic nitrogen sources (Guo *et al.,* 2008). Aysha, (2006) isolated endophytic bacteria from *Jacaranda decurrens* Cham with enzyme of biotechnological interest. Enzyme activities

ranked as follows proteolytic 60%, amylolytic 60%, lipolytic 40%, esterasic 40%. Production of cellulase, xylanase and pectinase were done by endophytic *Pestalopsis* sp. (Maria *et al.,* 2005). Endophytic fungi were isolated from Plao-yai yielding the highest xylanase-production using 2% rice bran as substrate, and 0.1% ammonium sulfate for xylanase production. Two different *Micromonospora* sp. of endophytic fungi isolated, from Egyptian herbal plant were able to produce alkaline serine protease. Keratinase production was recorded using different keratinous waste as the source of carbon and nitrogen. Both strains grew and produced keratinase. Also activity and properties of keratinase enzymes altered by genetic recombination through protoplast fusion between them, leading to a potent keratinolytic fusant with improved properties such as activity, stability, specificity and tolerance to inhibitors.

Some strains of *Streptomyces* were identified for production of hydrolytic cell wall degrading enzymes such as chitinases, cellulases, hemicellulases, amylases and glucanases, along with enzyme degrading lignin. Endophytic *Streptosporangium* sp. from maize was reported for production of glucoamylase, to improve industrial processes of starch degradation. The cell wall degrading enzyme endogluconase and polygalacturonase seems to be required for the infection of *Vitis vinifera* by *Burkholderia* sp. (Compant *et al.,* 2005). In microbes like fungi enzymes are secreted largely at ambient pH values in corresponds to their optimal activity. This was studied by ambient of pH in the secretion of enzymes such as protease, amylase, cellulase, pectinase and lipase by different species of endophytic *Colletotrichum*. Responsible gene for pH-regulated gene expression was found as *Colletotrichum* pac C homologue gene (Waller *et al.,* 2005). More than three hundred isolates of endophytic actinomycetes were screened for their potential chitinase production. The strain *Streptomyces aureofaciens* (MUAC130) was most effective amongst all these isolates. N acetylglucosamine was found as good inducer. Expression of the enzyme complex was repressed by several mono and

disaccharides, including lactose, mannose, glucose, cellobiose, arabinose, raffinose, sucrose and fructose (Taechowisan and Lumyong, 2003). Endophytes produce extracellular hydrolyases in order to establish a resistance mechanism against plant invasion. The actions of these enzymes support the hypothesis of co-evolution between endophytes and their hosts. Twenty one endophytic isolates from *Brucea javanica* were tested for their ability to produce extracellular amylase, ligninase, pectinase and xylanase (Choi *et al.,* 2005). The enzyme tests indicate that most endophytes are degraders of the simpler sugar and cellulose available in recently dead leaves and possibly wood. Some *Streptomyces* strains are known to produce hydrolytic cell wall-degrading enzymes such as cellulases, hemicellulases, chitinases, amylases and glucanases. Other strains are also known for their ability to produce enzymes that degrade the lignin, cellulose and hemicellulose of higher plants *Streptosporangium* sp. isolated from leaves of maize was reported to produce glucoamylase, which is expected to improve industrial processes of starch degradation.

2.10.3 Secondary Metabolites from Endophytes as Antiviral Agent

Endophytes have also been studied for their antiviral activity, the emergence of multiresistance against existing drugs, and high cost of current therapies as well as the AIDS associated opportunistic infections, such as *Cytomegalo* virus and *Polyoma* virus needs essential antiviral agent. Cytonic acid A and B were recognized as human cytomegalo virus protease inhibitors from endophytic fungus *Cytonaema sp.* isolated from *Quercus* sp. (Guo *et al.,* 2008). A novel quinine related metabolites xanthoviridicatins E and F was also produced by an endophytic *Penicillium chrysogenum* able to inhibit the cleavage reaction of HIV-1 integrase.

2.11 Some Antioxidant Compounds Produced by Endophytes

Free radicals are atoms causing damage to body cells and harmful to our immune system leading to many of degenerative diseases. Antioxidant donates electron to free radicals and

converts them to harmless molecules, protecting cells from oxidative damage aging and various diseases. Antioxidants are habitually produced by many endophytes. Pestacin and isopestacin were produced by *Pestalotiopsis microspora* from host *Terminalia morobensis,* (Strobel *et al.,* 2001).The antioxidant activities *in vitro* of exopolysaccharides (EPS) from endophytic bacteria *Paenibacillus polymyxa* EJS-3 were investigated with sucrose and yeast extract as the carbon and nitrogen source respectively. Purified EPS 1 and EPS -2 were with molecular weight of $1.22x10^6$ and $8.69x10^5$ Da correspondingly showing strong scavenging activities on superoxide and hydroxyl radicals. Broth extracts of the endophytic *Corynespora cassiicola* L36 gives three compounds corynesidones A and B and corynether A, collectively with diaryl ether, corynesidone B, exhibiting compelling radical scavenging activity in the DPPH assay. Also aromatase inhibitory, radical scavenging and antioxidant activities of depsidones and diaryl ehthers from the endophytic fungus *Corynespora cassiicola* L36 were noted. Endophytic fungus *Pestalotiopsis* microspora produces pestacin exhibiting antioxidant activity eleven times greater than the vitamin E derivative troxol. Graphislactone A was also characterized as secondary metabolites with its free radical scavenging and antioxidant activities stronger than those of butylated hydroxytoluene (BHT) and ascorbic acid from endophyte *Ephalosporium sp.* IFB-E001. About 12 endophytes from *Trachelospermum jasminoides* were assayed for more potent free radicals scavenging activities using *1, 1, diphenyl,-2-picrylhyrazyl* (DPPH) and hydroxyl radicals assay. Endophytes from medicinal plants are main resources for antioxidant metabolites helps to study relationship between total antioxidant capacity (TAC) and total phenolic content (TPC).The antioxidant capacities of the endophytes were significantly correlated with their total phenolic contents, suggesting that phenolics are the key antioxidant constituents of endophytic microbes. Metabolites produced by fungal endophyte can be a good source of novel natural antioxidant compounds (Choi *et al.,* 2005).

2.12 Secondary Metabolites from Endophytes as Nematicidal Agents

Endophytic bacterial isolates from, *Euphorbia pulcherima willd*, *Pyrethrumciner ariifolium* Trev founds to have nematicidal activity against *Caenorhabditi selegans* (Maupas) and *Bursaphelenchus xylophilus*. Active strain was identified as *Brevundimonas diminuta* and nematicidal metabolite identified as (R)-(-)-2-ethylhexan-1-01 (Liu *et al.,* 2005). Nematicidal endophytic bacteria were isolated from plants (Zhang *et al.,* 2012). Endophytes also found to produce hydroxypropionic acid as nematicidal agent (Schwartz and Cantor, 2004). 3 Hydroxypropionic acids were isolated from endophytic fungi showing nematicidal activity against the plant-parasitic nematode such as *Meloidogyne incognita* with the LD 5values of 12.5-15ug/ml. This was first report of 3-hydroxypropionic acid from endophytic fungi with the nematicidal activity (Mattioli *et al.,* 2004).

2.13 Ecology

Many of the fungi commonly reported as endophytes are regarded as minor or secondary pathogens by forest pathologists. Their common occurrences in both healthy and diseased tissues underscore the uncertainty of boundaries separating endophytes, facultative pathogens, and latent pathogens. Pathogenic fungi capable of symptomless occupation of their hosts in part of the infection cycle, "quiescent infections" (Semova *et al.,*2006), and strains with impaired virulence can be considered endophytes (Schardl *et al.,* 2004) as well as a variety of commensal saprobic and mutualistic fungi that have cryptic, non-apparent patterns of host colonization. Fungi described as "endophytic" characteristically exhibit a prolonged, inconspicuous period in which growth and colonization cease temporarily, resuming after a physical or maturational change in the host (Arnold, 2007). This episodic growth is a defined feature of endophytes, whether they ultimately are considered commensal saprobes, latent pathogens or protective mutualists. Although such a definition may seem too broad, most fungal biologists agree that the species composition of the internal mycobiota is

distinct for various hosts, organs, and tissues although some species of endophytic infections also may be found in the epiphytic or rhizosphere mycobiota (Saikkonen *et al.,* 2002).

Endophytic fungi are polyphyletic; mostly belonging to ascomycetes and to anamorphic fungi (Arnold, 2007). There are nearly 300,000 plant species on earth and each individual plant is host to one or more endophytes, and many of them may colonize only certain hosts. It has been estimated that there may be as many as one million different endophytic fungal taxa, thus endophytes may be hyper diverse (Petrini and Fisher*,* 1992; Strobel and Daisy, 2003). Endophytes occur in almost all known plants such as algae, mosses, lichens, liverworts, ferns and fern allies (Arnold, 2007), numerous angiosperms and gymnosperms, including tropical palms, broad-leaved trees (Petrini and Fisher, 1992; Clay and Schardl, 2002; Arnold and Herre, 2003), diverse herbaceous annuals, and many deciduous and evergreen perennials, in all known plant-growing regions from xeric to mesic temperate and tropical environments and from extreme arctic to alpine, temperate, tropical and boreal forests (Petrini and Fisher, 1992).

2.14 Biology

Endophytes may be transmitted either vertically (from parent to offspring) or horizontally (from individual to unrelated individual). Vertically transmitted fungal endophytes are asexual and transmit via fungal hyphae penetrating the host's seeds (e.g., *Neotyphodium*). Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutualistic. Conversely, horizontally transmitted fungal endophytes are sexual and transmit via spores that can be spread by wind and/or insect vectors. Since they spread similarly to pathogens, horizontally transmitted endophytes are often closely related to pathogenic fungi, although they are not pathogenic themselves (Selosse *et al.,* 2004). It is generally believed that variations in sexual reproduction and modes of transmission can cause variations in symbiotic traits of plant-fungus interaction. These differences among

endophytes, in concert with biotic and abiotic environmental factors, are likely to have implications for genotypic diversity, generation time, spatial and temporal distribution of endophytes and the nature of host-fungus interplay.

Life history traits, such as the mode of transmission, largely determine the spatial and temporal distribution of endophytes (Saikkonen *et al.,* 2002). Vertically transmitted grass-endophytes usually produce considerable mycelial biomass within the host, sometimes throughout the whole plant and always along the stem to developing flower heads and seeds. The generation time of vertically transmitted grass-endophytes is relatively long, often covering several grass generations. In contrast, abundance and diversity of horizontally transmitted endophytes in plants accumulate throughout the growing season, mostly in foliage (Faeth and Fagan, 2002; Saikkonen *et al.,* 2002). Individual endophyte infections are localized and the mycelial biomass remains very low relative to plant biomass. Spores are usually dispersed from senescent and abscised leaves, and thus the lifespan of foliage limits the lifespan of most endophytes inhabiting woody plants. The spatial and temporal patterns of endophytes differ not only between grasses and trees, but also between evergreen and deciduous trees (Arnold*,* 2007).

2.14.1 Reproduction and Transmission Mode of Endophytic Fungi

Reproductive and transmission modes of endophytic fungi are often used synonymously to refer to their spread within the host and among the population of host plants. They are, however, clearly different processes, whereby reproduction mode specifies the sexual or asexual characteristics of the process, while the mode of transmission describes mechanisms by which fungal infections are distributed. So far, there are two known transmission modes for fungal endophytes: Fungal hyphae may grow clonally into host seeds and are thereby transmitted to offspring of infested plants which is commonly termed as vertical transmission. Alternatively, the fungus may produce spores and promote horizontal

transmission. To fully understand the ecological and evolutionary consequences of these life history strategies, however, it is essential to recognize that fungi may produce either mitotic asexual or meiotic sexual spores. Thus, asexual reproduction of fungi is possible through vertical transmission via host seeds and horizontal transmission by spores, or possibly hyphae, whereas sexual reproduction requires production of sexual spores and is therefore always horizontal (Schroder *et al.,* 2002). The reproductive and transmission mode of the fungus appears to be adapted to the life history of the host, particularly the growth pattern, expected lifetime, and age of sexual maturity of the plant. The vast majority of ecological literature on fungal endophytes associated with grasses has focused on two related fungal genera, *Neotyphodium* and *Epichloë*. Both of them occur as systemic infection (i.e. growing throughout the host plant to developing inflorescence and seeds), and are transmitted vertically from maternal plants to offspring. *Neotyphodium* endophytes are assumed to be strictly vertically transmitted, and thus, considered „trapped" in the host plant (Clay and Schardl, 2002). In contrast, *Epichloë* endophytes can also be transmitted sexually by spores (Clay and Schardl, 2002; Schardl *et al.,* 2004). However, contagious spread should not be ruled out even in *Neotyphodium* endophytes because they produce asexual conidia on growth media and on living plants (White *et al.,* 2010). Recent evidence indicates horizontal transmission in natural grass populations (Schroder *et al.,* 2002). Foliar endophytes of woody plants are non-systemic and transmitted horizontally by spores from plant to plant, usually causing highly restricted local infections. Endophytes of woody plants have also been detected in seeds and acorns (Petrini and Fisher, 1992), but vertical transmission of woody plant endophytes is probably rare (Saikkonen *et al.,* 2002). Many tree-endophytes also produce asexual spores, and horizontal transmission and sexual reproduction of some fungal species is likely to result in relatively higher genotypic diversity in populations of fungal endophytes in trees than in grasses. Reproduction and transmission modes are well

recognized as important factors related to the epidemiology and evolution of virulence in parasite and pathogen interactions (Saikonen *et al.,* 2002). Mode of transmission, pattern of endophyte infections, architecture and lifespan of the host and the fungus likely affect the probability of endophyte-plant interactions occurring along the continuum from antagonistic to mutualistic interactions (Clay and Schardl, 2002). Saikkonen *et al.* (2002) suggested that exclusively vertically transmitted asexual grass endophytes are more likely to fall nearer the mutualistic end of the interaction continuum compared with mixed strategy (both vertically and horizontally) or only horizontally transmitted endophytes. However, strict vertical transmission does not guarantee mutualistic interactions with the host (Clay and Schardl, 2002; Saikkonen *et al.,* 2002).

2.14.2 Partner Fidelity and Evolution of Virulence

Evolutionary theory predicts that vertical transmission should align the interests of partners toward mutualistic associations, whereas horizontal transmission, with increased opportunities for contagious spread, should promote the evolution of increased virulence (Clay and Schardl, 2002). Most empirical literature on endophytes generally supports this theory. Interactions between *Neotyphodium* endophytes and grasses represent an extreme form of partner fidelity, because the fungus spreads only with seeds of infected plants, and thus it is fully dependent on the host plant for survival and reproduction. *Neotyphodium* interactions are often found as mutualistic, lending support to the theory. In contrast, other grass endophytes, such as some *Epichloë* species, with mixed modes of transmission, may incur severe costs to the host by producing fungal sexual structures (stromata) in the plant inflorescences thereby decreasing seed production of the host plant. In general, endophytes that are transmitted horizontally by spores are often either neutral or parasitic (Ahlholm *et al.,* 2002; Saikkonen *et al.,* 2002), even though these endophytes as well were originally proposed as defensive mutualists against rapidly evolving herbivores (Faeth and Fagan,

2002). Although vertically transmitted endophytes appear to be selected for lowered virulence, their interactions with grasses do not necessarily remain mutualistic and evolutionary stable for several reasons. First, costs and benefits of the partners are not symmetric, even in mutualistic plant-endophyte symbioses. The symbiosis is critical for long-term survival and reproduction of the fungus, which has presumably lost the independent phase of its life cycle. Alternatively, the fungus may only minimally increase plant survival and reproduction. Recent empirical evidence suggests in some environments and for some endophyte-host combinations, that endophytes may reduce host growth and reproduction, further skewing the relative cost and benefits of association between partners (Ahlholm *et al.,*2002; Faeth and Fagan, 2002). Another important destabilizing factor is the mismatch between genetic diversity of the host grass and asexual endophytes. Asexual, vertically transmitted endophytes, such as *Neotyphodium*, have greatly reduced genetic diversity and exhibit very low gene flow in natural populations (Faeth and Fagan, 2002). Increased benefits of endophyte are typically manifested through increased production or diversity of endophytic alkaloids (Faeth and Fagan, 2002). The consequence of this strategy is that the majority of vertically transmitted endophytes of native grasses may only be weakly mutualistic, such that genetically limited haplotypes can persist over time in an ever-changing (genetically) host background. Endophyte-host associations that are strongly mutualistic (i.e. great benefits) may also be highly harmful in terms of high or diverse alkaloid production. Faeth and Fagan (2002) reviewed the literature and found far fewer native grass-endophyte associations that were highly toxic to herbivores than expected based upon estimated species of grasses infected with *Neotyphodium*, contrary to prevailing ideas of endophytic mutualisms. The strategy of many seed-borne endophytes may be: do little harm but provide few benefits. In fact, when genetic diversity of the host grass is low, more mutualistic associations are expected because more constant plant genotypic backgrounds appear

generation after generation. This appears exactly the case in agronomic grasses such as tall fescue and perennial ryegrass, well known for high and diverse alkaloid production that inhibits herbivores. Cultivars of these agronomic plants are highly inbred and exhibit much lower genetic diversity than their native counter-parts (Saikkonen *et al.,* 2002).

2.15 Seed Sample Collection, Isolation and Preservation of Fungi

Paddy is one of the most important commercial crops in India for domestic consumption and continues to be an important source of food. One of the main factors to cause a decline in the paddy industry is fungal infection and invasion. Fungi generally invade a cereal grain through the germ (Gupta *et al.,* 2012).

The development of fungi, especially *Aspergillus*, *Fusarium* and *Penicillium* species, is an unresolved problem in storage. They are responsible for quantitative and qualitative losses and under certain conditions these species can develop toxic metabolites (Maina *et al.,* 2007). Redman *et al.* (2002) described the fungi of the genus *Aspergillus* are common contaminants of food and feed stuffs, many species are saprobes and are found in a variety of habitats and are ubiquitous agents of decay. There are 200 species of *Aspergillus*. The fungi in the genus *Aspergillus* are comparatively more widespread than others Redman *et al.* (2002). *Aspergilli* are economically, ecologically, and medically important and constitute a large genus. Several *Aspergillus* species have been reported from rice in processed and stored agricultural commodities and are able to produce mycotoxins (Redman *et al.,* 2002). *A. candidus, A. flavus, A. fumigatus* and *A. versicolor* were reported from rice in Malaysia (Watson and Chambers, 1977). Abel *et al.* (2012) reported the contamination of all rice samples was 93%. The species of *Aspergillus* were the most isolated fungi at 27°C incubation. The most significant rates of *Aspergillus* species contamination were observed in the *A. flavus* (57.9%). *A. flavus* and *A. parasiticus*, the two *Aspergillus* species of most concern in agriculture, are predominant saprotrophs with limited parasitic ability (Abel *et al.,* 2012).

2.16 Agricultural Residues Used

Agricultural wastes are often becoming environmental problems. The use of natural fibers for composite material manufacturing has received great attention. Problems associated with the use of synthetic materials are prompting legislation to protect the environment in many countries. Most natural fibers from these wastes can be used as reinforcements or fillers in polymer composites for creating technology innovative materials as a function of cost (Cruz-Estrada *et al.,* 2007; Treffler, 2007; Hammajam *et al.,* 2014; Carbonell-Verdu *et al.,* 2015). High technology industries are pressured to seek alternative materials to replace the conventional synthetic materials due to environmental and health related issues (Bhattacharyya *et al.,* 2015).

2.16.1 Millet Hull/Husk

Millets are at the 6th place world in cereal production. They are the major food source for people living in economically disadvantaged status in Africa and Asia. Millets are known as the first domesticated cereals that were cultivated at the beginning of human civilization, being recognized as potential future crops due to their nutrient contents similar to other major cereals and non-nutrient compounds having proven health benefits. Studies have shown that millet husks are rich sources of non-nutrients, especially phenolic compounds (Chandrasekara and Shahidi, 2010).

There are evidences to show that phenolic compounds can act as antioxidants within the human body to protect against oxidative stress and to reduce the risk of NCDs (Chandrasekara and Shahidi, 2011). Millet hulls are the by-product of the dehulling of millet grains for human consumption. The millet species is usually unspecified and the hulls may come from any of the major or minor millet species (including proso millet *Panicum miliaceum*, pearl millet *Pennisetum glaucum* and foxtail millet *Setariaitalica*). Millet hulls are a fibrous by-product often used as a filling material (in pillows, for example) but rarely as

a livestock feed. Millet (*Pennisetum glaucum*) is an annual and biannual cereal crop that grows in different climatic conditions and is native to Africa and Asia (Dominique *et al.,* 2012). Millet has become a vital product in sub-Saharan Africa and India, where it is used in several consumables such as food and beverage industries (Hammajam *et al.,* 2014). Millet is a cereal crop that can be produced within a very short period of time.

Several reasons may be responsible for the growing of millet at large scale, but population growth and climate change necessitate the search for drought-resistant crops with high yield as a panacea for food security globally. The husks are left on the farm site after harvest. This resource is costless to whoever needs it and is in good supply for consumption and usage in Nigeria (Abubakar and Ahmad, 2010). When left in the field, it becomes home for microorganisms that shelter diseases. Millet husks are among the millions of tons of agricultural wastes seeking ways of disposal (Rosa *et al.,* 2009; Heuze and Tran, 2012). Attractive attributes of millet husk include renewability, biodegradability, light weight, and cost effectiveness.

2.16.2 Rice Husk

Among the sources of phenolic compounds, rice (*Oryza sativa*) was used because it is one of the most predominant and consumed cereals in the world and plays an important role in the diet-health relation containing distinct phenolic compounds, tocopherols, tocotrienols, and g-oryzanol mainly associated with the pericarp (Ignat *et al.*, 2011). However, grain polishing reduces the concentration of phenolic compounds in the endosperm, which remain in the bran/husk where they can be bounded to carbohydrates, fatty acids or proteins making the hydrolysis process important to obtain maximum yield of the phenolic acids (Zhou *et al.,* 2004).

2.16.3 Groundnut Shells

Groundnut is a self-pollinated; annual and herbaceous legume crop. A complete seed of groundnut is called pod and contains one to negative kernels which develops underground in a needle like structure called peg which grow into the soil and then converts into a pod. Groundnut has taproot system which has many nodules, present in root and lateral roots. These nodules contain *Rhizobium sp.*, which are symbiotic in nature and focus atmospheric nitrogen. Outer layer of groundnut is called groundnut shell: The shell constitute about 25-35% of the pod. The seed accounts for the remaining portion (65-75%).

Nigeria is one of the foremost producers of groundnut in the world, producing up to about 2.699 million metric tonnes in 2002 and 1.55 million metric-tons in 2008. Groundnut shell is found in large quantities as agricultural farm wastes in Northern parts of Nigeria such as Sokoto, Kebbi, Zaria, Borno and Yobe States.

Over the years, groundnut shell constitutes common solid waste especially in the developing part of this world. The utilization of Groundnut shell will promote waste management at little cost, reduce pollution by these wastes and increase the economic base of the farmer when such waste are sold thereby encouraging more production (Zhou *et al.,* 2004).

2.17 Bioactive Phenolic Compounds

Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in plant and food products (Hammajam *et al.,* 2014). Most common bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds (Dominique *et al.,* 2012). Phenolic compounds comprise flavonoids, phenolic acids, and tannins, among others. Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Rosa *et al.,* 2009). With widely variations i.e.

flavones, flavanones, flavanols, isoflavones, and anthocyanidins, these are examples of the most common naturally occurring flavonoids. Similarly to the flavonoids, phenolic acids constitute also an important class of phenolic compounds with bioactive functions, usually found in plant and food products. Phenolic acids can be divided in two sub-groups according to their structure: the hydroxybenzoic and the hydroxycinnamic acids. The most commonly found hydroxybenzoic acids include gallic, phydroxybenzoic, protocatechuic, vanillic and syringic acids, while among the hydroxycinnamic acids, caffeic, ferulic, p-coumaric and sinapic acids can be pointed out (Rosa *et al.,* 2009). In the last few years, great attention has been paid to the bioactive phenolic compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases like cancer and diabetes (Kim *et al.,* 2003), reduction in risk factors of cardiovascular diseases, antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory, and anti-microbial effects (Martins *et al.,* 2011), among others. Due to these countless beneficial characteristics for human health, researches have been intensified aiming to find fruits, vegetables, plants, agricultural and agro-industrial residues as sources of bioactive phenolic compounds.

Usually, bioactive compounds are recovered from natural sources by solid–liquid extraction employing organic solvents in heat-reflux systems (Wang *et al.,* 2004; Martins *et al.,* 2011). However, other techniques have been recently proposed to obtain these compounds including the use of supercritical fluids, high pressure processes, microwave-assisted extraction and ultrasound-assisted extraction (Wang *et al.,* 2004). Extraction/production of bioactive compounds by fermentation is also an interesting alternative that merits attention, which is our main concern here since it is able to provide high quality and high activity extracts while precluding any toxicity associated to the organic solvents.

In this process, bioactive phenolic compounds are obtained as secondary metabolites produced by microorganisms after the microbial growth is completed. Studies on liquid

culture show that the production of these compounds starts when growth is limited by the exhaustion of one key nutrient: carbon, nitrogen or phosphate source (Wang *et al.,* 2004).

2.18 Metabolism of Microorganisms

Solid-state fermentation (SSF), which consists of the microbial growth and product formation on solid particles in the absence (or near absence) of water; however, substrate contains the sufficient moisture to allow the microorganism growth and metabolism (Pandey *et al.*, 2003).

In recent years, SSF has received more interest from researchers since several studies have demonstrated that this process may lead to higher yields and productivities or better product characteristics than other fermentation processes. In addition, due to the utilization of low cost agricultural and agro-industrial residues as substrates, capital and operating costs are lower. The low water volume in SSF has also a large impact on the economy of the process mainly due to smaller fermenter-size, reduced downstream processing, reduced stirring and lower sterilization costs (Pandey *et al.,* 2003).

The main drawback of this type of cultivation concerns the scaling-up of the process, largely due to heat transfer and culture homogeneity problems (Pandey *et al.,* 2003). However, research attention has been directed towards the development of bioreactors that overcome these difficulties. In the last decades, there has been an increasing trend towards the utilization of the SSF technique to produce these compounds (Pandey *et al.,* 2003). Several important factors must be considered for the development of a successful bioprocess under SSF conditions. Some of the most important include the selection of a suitable microorganism strain and the solid support to be used.

A variety of microorganisms, including fungi, yeasts and bacteria may be used in SSF processes; however, due to the low moisture content in the fermentation media, fungi and yeasts are the most commonly used microorganisms due to their ability to growth in

environments with this characteristic. The choice of the microorganism to be used in SSF depends on the desired end-product. Filamentous fungi have great potential to produce bioactive compounds by SSF, and therefore, they are the most commonly used microorganisms for this purpose (Wang *et al.,* 2004). The right selection of the solid substrate is also of great importance for an efficient and economical production of the compound of interest. Mostly the production yields of secondary metabolites can be improved with a suitable choice of substrate or mixture of substrates with appropriate nutrients (Wang *et al.,* 2004). The support material must present characteristic favorable for the microorganism development and be of low cost.

These characteristics are easily found in many residual natural materials proceeding from agricultural and agro-industrial activities. In addition, the use of these residues as carbon sources through SSF provides an important way to reduce the fermentation cost and avoid environmental problems caused by their disposal, being an economical and interesting solution for countries with abundance of these materials.

This study determine the production and extraction of the phenolic compounds fro agro-wastes by SSF using fungal isolates *Trichorderma harzianum, Penicillium* sp.*,* and *Aspergillus niger.* The selection of the most appropriate downstream process for the obtained product is also crucial when SSF processes are performed. The product obtained by SSF may be recovered from the solid fermented mass by extraction with solvents (aqueous or other solvents mixtures). The type of solvent and its concentration, as well as the ratio of solvent to the solid and pH are important variables that influence in the product extraction. In addition, since the metabolites diffuse throughout the solid mass during the culturing, long extraction-times may be required for complete product recovery. The cost of purification depends on the quality of the obtained extract. For example, the presence and concentration of inert compounds in the extract increase the cost of purification and therefore the cost of recovery is

increased. Particularly those secondary metabolites which are used in bulk in the pharmaceutical and health industry and whose purity is governed by stringent regulations need to go through specific purification strategy (Pandey *et al.,* 2003).

Food quality is not only a function of nutritional values but also of the presence of bioactive compounds exerting positive effects on human health. Phenolic compounds, also referred as polyphenols, are considered to be natural antioxidants and represent an important group of bioactive compounds in foods. These compounds are present in all plant foods but their type and levels vary enormously depending on the plant, genetic factors and environmental conditions. In the last years, SSF has been employed to increase the content of phenolic compounds in certain food products, thus enhancing their antioxidant activity. For example, previous study of black beans which are well known for their high nutritional value containing isoflavones, vitamin E, saponins, carotenoids and anthocyanins (Lee *et al.,* 2007).

In a recent study on the bioprocessing of these beans to prepare koji using SSF with different food-grade filamentous fungi (in particular *Aspergillus* sp. and *Rhizopus* sp.), an enhancement of the antioxidant properties of the beans was observed, which might be related to the increase of phenol and anthocyanin contents (Lee *et al.,*2007). Nevertheless, the enhancement of the antioxidant activity of the black bean koji varied to each microorganism used. Similarly, SSF of grass peas cooked seeds using *Rhizopus oligosporus* caused an increase in the phenolic compounds content which significantly improved the antiradical properties of the seeds.Two different filamentous fungi (*Aspergillus oryzae* and *Aspergillus awamori*) used in SSF were very effective for the improvement of phenolic content and antioxidant properties of wheat grains. In this study, fermented rice-bran, millet husk and groundnut shells were considered to be rich antioxidant produce. Previous study of Soybean products fermented by SSF with *Trichorderma harzianum* showed stronger antioxidant activity than unfermented products, which was probably related to the markedly higher

contents of phenolic acids, flavonoids and aglycone isoflavone with more free hydroxyl groups achieved during SSF. Chemical composition and bioactivity of stale rice were also improved by SSF with *Cordyceps sinensis* (Zhang *et al.,* 2006). Besides to increase the antioxidant activity of certain foods, bioconversion of phenolic compounds by SSF may also promote other alterations in the food properties, with influence on human health. An example of this is the SSF of mung beans (also known as green beans) with *Rhizopus oligosporus*. This process has been demonstrated as being able to mobilize the conjugate forms of phenolic precursors naturally found in mung beans and improves their health-linked functionality. According to Wang *et al.,* (2004), SSF of mung beans significantly increased the phenolic content enhancing the antioxidant activity of the beans. This antioxidant activity enhancement contributed to the α-amylase inhibition (which is relevant for the diabetes controlling), as well as for the inhibition of the Helicobacter pylori growth (linked to peptic ulcer management).Utilization and recycling of renewable resources that pose threat to the environment can be systematically carried out to bring about resource productivity needed to make human activity sustainable. Nigeria is an agro-based country that produces large quantities of agro - industrial residues which are rich in nutrients like carbon, nitrogen, minerals, and biomass residues. These agricultural wastes can be used as substrate for enzyme production owing to economically feasibility, as it can help in solving pollution problems which may be caused by their disposal (Wang *et al.,* 2004).

Fungi are mostly referable, because of their extracellular enzymes which facilitate recovery of the lipase from the fermentation broth. Meanwhile fungi have several other advantages over unicellular microorganisms in the colonization of solid- substrates and their good tolerance to low water activity, high osmotic pressure conditions etc. (Yu *et al.,*2002). Filamentous fungi like *Aspergillus* sp*., Trichorderma* sp*. and Penicillium* sp. used are the most ideal and best adapted microorganisms for SSF.

2.19 Antioxidant Activity

Antioxidant Phenolic compounds (PCs) are gaining popularity day by day for their health promoting properties. Rice-bran and millet husk are a very good source of natural antioxidant PCs. In the present study, extraction of PCs was improved by solid-state fermentation (SSF) of the bran, husk and shells by *Trichorderma harzianum, Aspergillus niger and Penicillium* sp. which helped to release the bound compounds from matrix. Different extraction conditions such as solvent composition (water, methanol, 70% methanol, ethanol, 70% ethanol, acetone and 70% acetone), extraction temperature (30–60^{o}C), extraction time (15–90 min) and solid-to-solvent ratio (1:2.5 to 1:20, w/v) have been optimized for the extraction of PCs.

2.19.1 *Trichorderma* sp.

Trichorderma sp. are soil inhabiting filamentous fungi, belongs to the genus *Hypocrea. Trichorderma* sp. are fungi that are present in nearly all soils. In soil, they frequently are the most prevalent culturable fungi. They also exist in many other diverse habitats.

Trichorderma readily colonizes plant roots and some strains are rhizosphere competent i.e. able to grow on roots as they develop. *Trichorderma* sp. also attacks parasitize and otherwise gain nutrition from other fungi. They have evolved numerous mechanisms for both attack of other fungi and for enhancing plant and root growth. Different strains of *Trichoderma* control almost every pathogenic fungus for which control has been sought. However, most *Trichorderma* strains are more efficient for control of some pathogens than others, and may be largely ineffective against some fungi. Characteristics comprising growth rate, colour and colony appearance were examined. These characteristics were regarded as taxonomically useful characteristics for *Trichorderma* (Samuels *et al.,* 2002). *Trichorderma* sp. continue to be a major source of contamination and crop loss for mushroom farmers. *Trichorderma harzianum* is a fungus that is also used as a fungicide. It is used for foliar application, seed

treatment and soil treatment for suppression of various disease causing fungal pathogens. Commercial biotechnological products such as 3Tac have been useful for treatment of *Botrytis,* m *Fusarium* and *Penicillium* sp. It is also used for manufacturing enzymes.

2.19.2 *Aspergillus niger*

Aspergillus niger is a filamentous *ascomycete* fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans (Perfect *et al,* 2001) is most widely known for its role as a citric acid producer (Magnuson and Lasure*,* 2004). With production of citric acid at over one million metric tons annually, citric acid production serves as a model fungal fermentation process. As a common member of the microbial communities found in soils, plays a significant role in the global carbon cycle. This organism is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. A variety of these enzymes are important in the biotechnology industry. is also an important model organism for several important research areas including the study of eukaryotic protein secretion in general, the effects of various environmental factors on suppressing or triggering the export of various biomass degrading enzymes, molecular mechanisms critical to fermentation process development, and mechanisms involved in the control of fungal morphology (Magnuson and Lasure, 2004).

2.19.3 *Penicillium* sp.

Penicillium is also a widespread genus that is important in foods. *Penicillium species* contaminate a wide variety of foods and often spoil refrigerated foods, especially cheese. They are also common on grains, breads, cakes, fruits, preserves, cured and aged hams and sausages, and in the spoilage of certain fruits. The *Penicillia* produce *conidia* from conidiophores that branch near the apex, forming a brush-like structure or *Penicillus* at the apex of the conidiophore are somewhat enlarged cells known as *Metulae*. From the *Metulae* arise the *Sterigma* or *Phialides*. It is in these structures that the conidia are produced and

pushed out in chains (Magnuson and Lasure, 2004). The conidia of the *Penicillia* are coloured, but mostly in shades of gray to blue to blue-green. The colors are not as distinctive for the various species as for the *Aspergilli*, and are therefore not as helpful in the identification of species. Some species form *Ascospores* in *Cleistothecia* and are also placed in the teleomorphic genera of *Talaromyces* or *Eupenicillium.* There are a number of important *Penicillium species. P. verrucosum, P. viridicatum and P. aurantiogriseum* are common in grains and some can also occur on cheese. They can produce a number of mycotoxins, including ochratoxin, penicillic acid, and others. *P. martensii*, a synonym for *P. aurantiogriseum*, has been found growing in high-moisture corn and can produce *Penicillic acid. P. expansum* causes rots of fruits, especially apples, and produces patulin. *P. digitatum*, with green-colored conidia, causes soft rot of citrus fruit, usually at ambient temperatures, whereas *P. italicum*, which has blue spores, causes soft rot of citrus at refrigerated temperatures. *P. roqueforti* has blueish-colored conidia and is used in the ripening of blue-veined cheeses. However, wild types of *P. roqueforti* often occur in dairy environments and also contaminate other types of cheeses, such as Cheddar and Swiss, and grow and cause spoilage under refrigerated storage. *P. camemberti* produces greyish spores, and is used for surface ripening of Camembert and Brie cheeses. Many other *Penicillium species* are known to produce various toxic substances, many of which also have antibiotic properties, but appear to be too toxic for therapeutic use. The most famous antibiotic, penicillin, which has been used to cure countless bacterial infections, is produced by *P. notatu* (Magnuson and Lasure, 2004).

2.20 Free-radical Chelating

Oxidative stress may occur due to an imbalance between oxidants and anti-oxidative defense system of human body. Under this condition excessively produced reactive oxygen species (ROS) and free radicals damage different biological molecules, such as DNA, proteins, lipids

as well as carbohydrates with significant molecular and physiological damages of cells leading to numerous diseased conditions (Halliwell, 1996).

Plant-derived different antioxidant molecules with their reducing, free radical scavenging and metal chelating properties can reduce oxidative stress keeping equilibrium between oxidants and antioxidants in human body (Bhanja *et al.,* 2009). Phenolic compounds are mostly studied diversified group of phytochemicals synthesized from phenylalanine and tyrosine by the enzymatic action of L-phenyloalanine, ammonia-lyase, in secondary metabolic pathway of plants during normal developmental stage or in stressed conditions by ecological and physiological pressures including infection by pathogen or insect, wounding and UV-radiation etc. (Peregrine *et al.*, 1997; Zhou *et al.,* 2004). They have become popular for their potential application in the prevention of various chronic diseases, viz. cardiovascular disease, cancer, osteoporosis, diabetes mellitus, and neurodegenerative diseases etc.

They protect cells by their antioxidant properties. Various natural sources of different antioxidant phenolic compounds have been explored including fruits, vegetables, wines, coffee, tea, pulses and cereals in order to restrict the use of health hazard synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ), in different food products. Without acid/base hydrolysis, extraction of most of the insoluble bound phenolics is difficult by only organic solvents. Extraction of natural phenolics by enzymatic treatment is a useful technique. Several microorganisms are known to produce a variety of enzymes in high titer values preferably under solid state fermentation (SSF) process. Recently, SSF has gained a considerable attention for the production and extraction of antioxidant phenolics from plant materials, mainly pulses and cereals (Martins *et al*., 2011). In this process, different carbohydrases like cellulases, b-glucosidase, xylanase, pectinases, b-xylosidase, b-galactosidase, a-amylases and esteras0e etc., produced by the microorganisms can release

the bound phenolics into soluble form (Bhanja *et al.*, 2009). In the present report, production and extraction of phenolics were improved through SSF of rice-bran, millet husk, and shells of groundnut by filamentous fungi. A single standardized method should not be recommended for the extraction of all types of phenolic compounds. Extraction process has to be optimized depending upon the nature of the sample and purpose of the study (Santos-Buelga, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

Soil Sample was collected and placed into polythene bags and labelled appropriately. This was obtained by digging 5 cm, 15 cm and 20 cm deep. The sample of rice/bran was collected from Dabo rice mill at Kwanar Dawaki in Kano State Nigeria, while Groundnut and Millet Husk were collected from a local farm at Zawaciki in Kano were then taken to the laboratory and stored for processing.

3.2 Isolation and Identification of Fungi from the Soil Samples

Procedure adopted by Watanabe (2010) was employed. Potatoes dextrose agar (PDA) 40 g which was supplemented with 0.04 gm streptomycin (Sigma-Aldrich co., USA) were suspended in 1000 ml of distilled water. It was then autoclaved and plated on Petri-dishes for further use. A control was also prepared without streptomycin using same procedure. PDA poured on Petri-dishes and allowed to cool and solidify. One gram of soil sample was diluted with 5ml distilled water to make microbial suspension. 1ml of the microbial suspension was then added aseptically onto the PDA. Samples were later placed in PDA for isolation of fungi. Pure isolates were cultivated and maintained on potato dextrose agar (PDA) for identification. The Petri-dishes were sealed with paraffin and incubated at room temperature for 15 days under dark condition and monitored. Fungi that grew on PDA were later sub-cultured on separate PDA plates each and kept at room temperature for 7-10 days to obtain pure culture. The pure culture was then taken to Bayero University Kano Central Laboratory for identification.

3.3 Identification of the Soil Fungi

The fungi were identified on the basis of their morphological and cultural characteristics. Fungi were grown on specified media at specified culture condition for identification.

The sub-cultured fungi were inoculated in to a microscopic free-grease clean slide containing a drop of distilled water. The sample was then covered with cover slip and was then examine under microscope (Hundemetzlar 640, Germany) by using x40 objective. Micrographs were taken using camera (Amscope MD900, USA). The observed features were then compared using micrographs and macrographs of Watanabe (2010).

3.4 Cultivation of Fungi for the Production of Metabolites

This procedure was carried out as described by Ghisalberti (2002). The fungi were cultured in appropriate media for the production of metabolites. Small scale cultivation was carried out primarily to perform bioassays for the detection of active metabolites. Both liquid and solid phase were performed. The fungi were cultured in potato dextrose agar (PDA) for the primarily screening. The Czapek dox media was used as primary culture for preparation of inoculums.

3.5 Fermentation in Liquid Media

Fresh mycelia from the fungus grown on PDA in petri dishes were inoculated aseptically in conical flasks containing Czapek dox media for 10 days. The flasks were kept static at room temperature for 30 days and examined periodically for contamination.

3.6 Solid-State Fermentation Process

Grinded Rice bran (100 g), Groundnut Shell (30 g), Millet husk (50 g) were placed in l litre Erlenmeyer flasks each in triplicates, mixed with 100 ml of distilled water. After moistening they were autoclaved (121 ^{o}C, 15 mm) and subsequently cooled to ambient temperature. Fungal spore suspensions (1 x 10^{6} spores/ml) prepared in Czapek dox media at room

temperature using a shaker were used as innoculum. The inoculum was then placed on the surface of prepared rice bran, millet husk, and groundnut shells and allowed to stand for 30days (Emmons and Peterson, 2001).

3.7 Extraction of Metabolites from Liquid Media

Extraction of the metabolites from the liquid media was carried out as described by (Choudhary *et al.,* 2004). The mycelia were separated from liquid broth by decantation. The mycelia were extracted in methanol for 7 days and then with ethyl acetate while the liquid broth was extracted 3 – 4 times. The organic extracts were evaporated using rotary evaporator at 40^{o}C to obtained solid residues, the solid residues were then weighed and the weigh of each extract was recorded.

3.8 Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay was performed using the method described by Jerry *et al.* (1998). One spatula of eggs of *Artemia salina* (about 50 mg) were placed into a hatching chamber containing sea water and kept under a light condition for 48 hours for the eggs to hatch into shrimp larvae. Meanwhile twenty milligram of each fungal extracts were weighed and transferred into clean vials and dissolved in 3 ml of absolute ethanol. Five hundred, fifty and five microliter of each of these solutions were transferred into empty vials corresponding to one thousand, one hundred and ten µm/ml concentrations respectively. All vials containing the dosage were kept overnight to vaporise, leaving only fungal extracts as residue. 3 ml of sea water containing approximately 10 larvae were added to the vial and kept for one day. Positive control was prepared by weighing 0.6 g of caffeine and dissolved in 3 ml of sea water while negative control consists of 10% methanol. Approximately 10 larvae were added to each vial for twenty four hours, light sensitive was considered. The mortality of the shrimp larvae at each vial was counted and recorded.

3.9 DPPH (2, 2 – diphenyl –1 – Picrylhydrazyl) Radical Scavenging Assay

The free radical scavenging activities of different fractions were measured by the DPPH radical scavenging method according to Brand – Williams *et al.* (1995). DPPH (Sigma Aldrich Chemie, Steinheim, Germany) solution of 0.1mM concentration in methanol was added to 40ml of different concentration of extracts(1000, 100, 10mg/l). The change in absorbance at 515 nm was measured after 30 mins of incubation and calculated according to the following equation.

% of DPPH radical scavenging activity

$$= \frac{(Abc - Abs)}{Abc} \; x \; 100$$

where, AbC was the absorbance of the control and AbS was the absorbance in the presence of the test compound. A standard curve was prepared by using different concentrations of Trolox. The DPPH scavenging activities of phenolic extracts were expressed as μmol Trolox equivalent (TE) g−1 grain.

3.10 Phytochemical Screening for the Presence of Metabolites

Phytochemical examinations were carried out on the extract with highest mortality rate as per the standard methods.

3.10.1 Detection of Flavonoids

Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colorless upon the addition of diluted HCl indicate the presence of flavonoids.

3.10.2 Detection of Steroids

Detection of steroid was carried out using Salkowski test. To 2 ml of extract 1.0 ml of concentrated sulfuric acid was added carefully along side of the test tube. A red colour was produced.

3.10.3 Detection of Phenol

Extract was treated with 3 – 4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenol.

3.10.4 Statistical Analysis

Data obtained were analysed using destriptive statistics. Variation between extracts were analysed using two-way ANOVA with the aids of SPSS version 20.0 statistical software. Probit anlaysis was used to determine the mortality rate between the fungal extracts.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

During the study period, *Trichorderma harzianum, Aspergillus niger* and *Penicillium* sp. were extracted from soil dilution plate method (plate 1, 2 and 3). The extracted fungi were identified. The isolates were then inoculated on sample of residues (Rice husk, Millet husk Groundnut shells) obtained and processed in aseptic conditions.

Trichorderma which was identified through its dark green conidiation towards the margins with a single cottony concentric ring which was found around the inoculums on PDA. As shown in Plate I. Macroscopically, this fungus can be identified growing on substrates producing colonies or felt like yellow to white *hyphae*, which turns black with the formation of conidia. The black conidia and spores confirm the specie to be. Microscopically were identified by its hyaline with conidiophores being long and globose at the tip each ejecting its own spore.

Microscopy

Identified fungal organisms *Penicillium* sp*., Trichorderma harzianum and Aspergillus niger.*

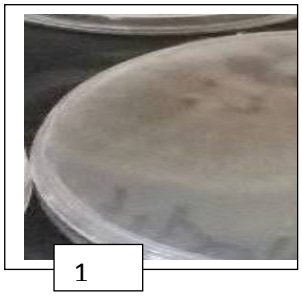
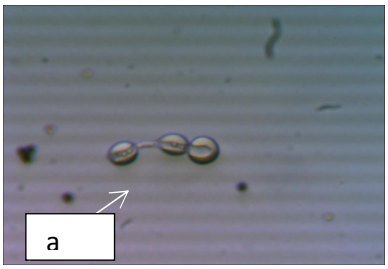
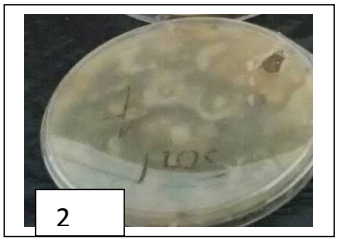
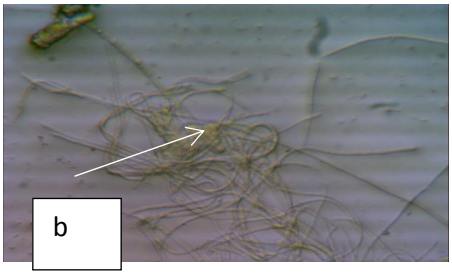
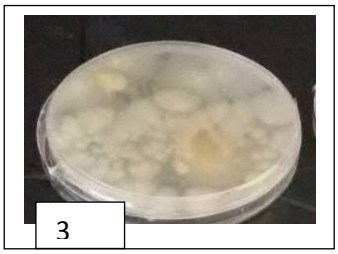
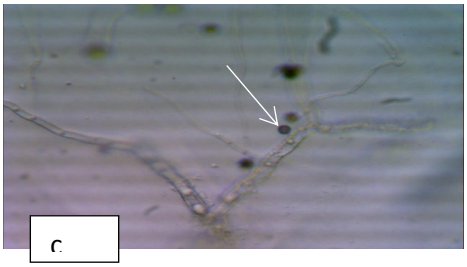
	Macrograph	**Micrograph (x400)**
T. harzianum *mg×400*		
Penicillium sp. *mg×400*		
Aspergillus niger *mg×400*		

Plate I: Identified fungal organisms

During the study period, the isolates were inoculated 0.5mg in each flasks of the agro-wastes and was put in a shaker for 5days, the result were kept in aseptic condition for 30days to ferment. After the fermentation process has taken place, extraction was made with methanol and Ethyl acetate and was put in a rotary evaporator. The result was then weighed. As seen in Table 4.1, the weight of extracts of fungi in millet husk was found to be higher (2.955 – 4.60 mg) in all than in rice bran and groundnut shells. Extract of *Penicillium* in millet husk produced the highest quantity of extract 4.60 mg, whereas *Aspergillus* in groundnut shells gave the least quantity of extract 2.621 mg. (Appendix I).

Table 4.1: Weight of Extracts in Different Agricultural-residues Fermented in Fungal Isolates

Agro-wastes/ mg	***T. harzianum***	***A. niger***	***Penicillium* sp.**
Rice bran	2.63	2.93	2.639
Groundnut Shell	2.648	2.621	2.629
Millet husk	2.955	2.94	4.60

There was a graded increase in antioxidant activity in all extracts. Extracts of fungi cultivated in rice bran (R3 and R2) had higher antioxidant activity than that of millet (M1 and M2) and ground nut shells (G2 and G3). In all agro-waste, extracts of *Pennicilium* sp. (R3, M3) was found to show higher activity followed by *Trichoderma* (R2 and M2) in millet (R3 and M3 and G3) (figure 4.1).

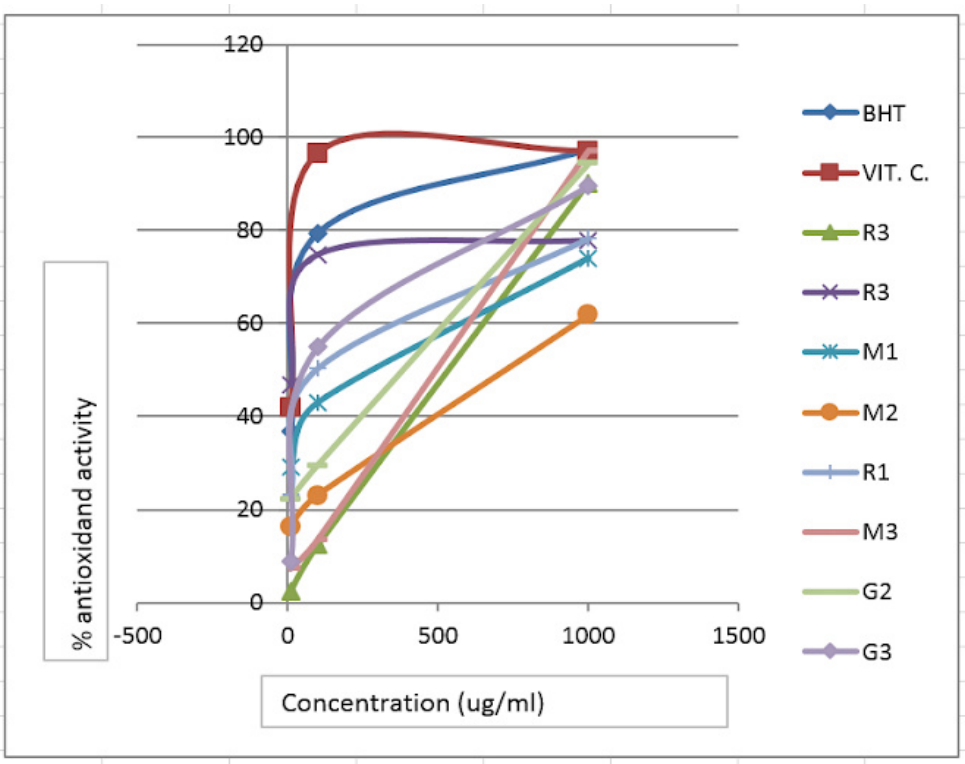


Figure 4.1: Percentage Antioxidant Activity of an Extracts

4.1.1 Mortality Count of Brine shrimp Larvae against Fungal Extracts

The result for the analysis (Table 4.4) revealed significant difference within the mean level of values of fungal extracts (*Trichoderma harzianum* TH, *Penicillium specie* PS, *Aspergillus niger* AN) on the mortality of Brine shrimp larvae ($P<0.05$). However the result revealed no significant different within the mean levels of agro-waste and the interaction combination effect of fungal extract and agro-waste ($P>0.05$), (Figure 4.2), (Table 4.4). The result of multiple comparisons of means turkey test (Figure 4.2), within the levels of different fungi extracts revealed a significant different in means levels between Control and AN, control and caffeine, control and PS, and control and PS ($P<0.05$) (Figure 4.2). However, the result showed no significant means different between the levels of other fungal extracts combination Caffeine and AN, PS and AN, TH and AN, PS and caffeine, TH and Caffeine, and TH and PS ($P>0.05$), (Table 4.4, Figure 4.2).

The result of one way analysis of variance (ANOVA) for each fungal extracts diluted with different concentrations (10ml, 100ml,1000ml) on the mortality of Brine shrimp larvae revealed a significant difference in fungal extracts AN, PS, and caffeine on the mortality of Brine shrimp larvae at different concentrations ($p<0.05$) (Table 4.5). However, one way analysis of variance revealed no significant difference between the concentrations of fungal extract TH and control at different concentrations on the mortality of Brine shrimp ($P>0.05$) (Table 4.5). A further multiple comparison of means pairwise test between different concentrations of fungal extract AN on mortality of Brine shrimp revealed a significant difference between the concentrations at 10 -1000 ($P<0.05$), and no significance difference was observed between concentrations of AN fungal extracts at 10-100,100-1000 ($P>0.05$) (Figure 4.3). multiple comparison of means pairwise test between different concentrations of fungal extract PS on mortality of Brine shrimp revealed a significant difference between the

concentrations at 10 -1000, and 100-1000 ($P<0.05$), (Figure 4.4) and no significance difference was observed between concentrations of PS fungal extracts at 10-100, ($P>0.05$) (Figure 4.5). However, multiple comparison of means pairwise test between different concentrations of fungal extract caffeine on mortality of Brine shrimp revealed no significant between difference concentrations at 10-100,10-1000,100-1000 ($P>0.05$) (Figure 4.6).

Table 4.2: Analysis of Variance Two-way Anova Result of Fungal Extracts, Agro Waste on Mortality

S/n	Variables	Df	Mean Square	F.Value	P.Value
1.	Fungal Extracts	4	667.3	166.83	**<2e-16*** **
2.	Agro waste	2	6.0	2.99	0.273
3.	Fungal extract and Agro waste	8	14.7	2.28	0.598

Note: P. value is significant at P <0.005*** are bold and underline

Table 4.3: One-way Analysis of Variance Result for Each Fungal Extract against Concentrations

Concentrations Fungal Extracts	F. Value	P. Value
TH	2.1485	0.1503
AN	7.642	**0.004842***
PS	8.0999	**0.0039***
Caffeine	3.8794	**0.04509***
Control	0.0679	0.5208

Note: P. value is significant at P <0.005*** are bold and underline

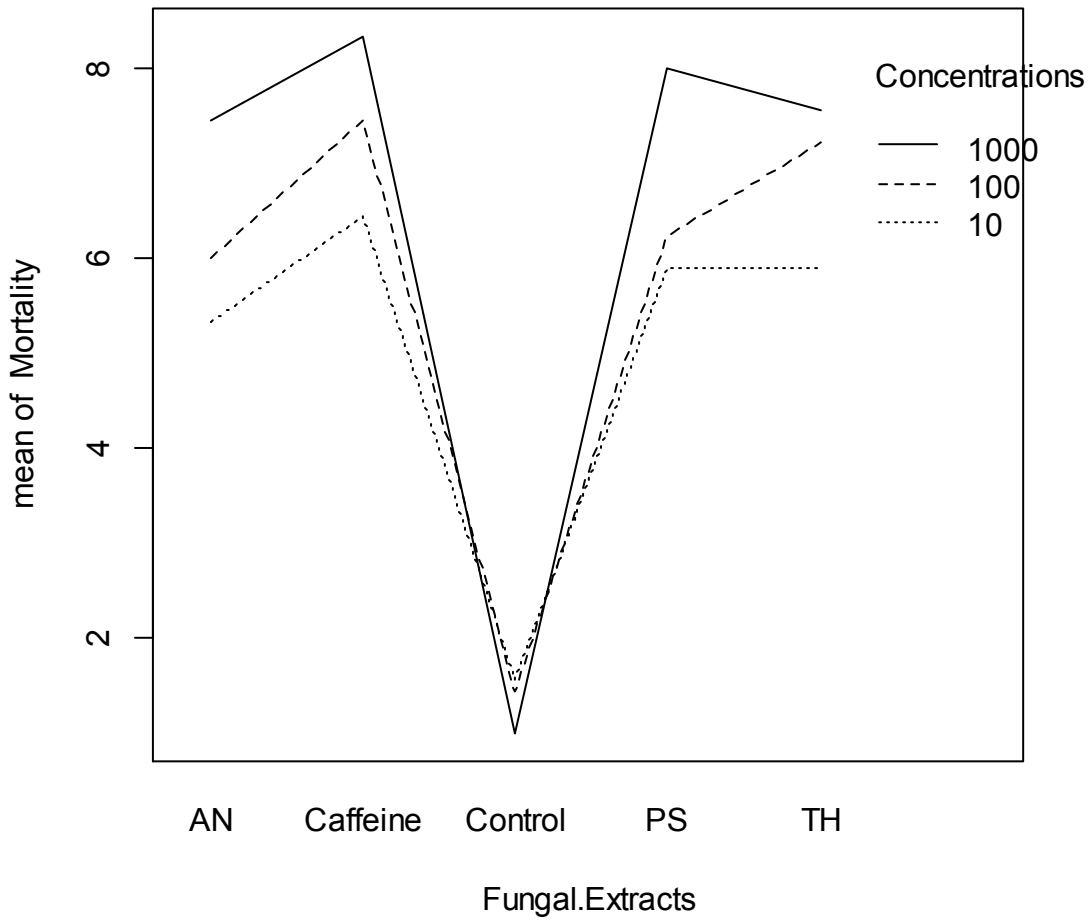


Figure 4.2: Comparison of the Mean Mortality of Different Fungal Extracts at Different Concentrations

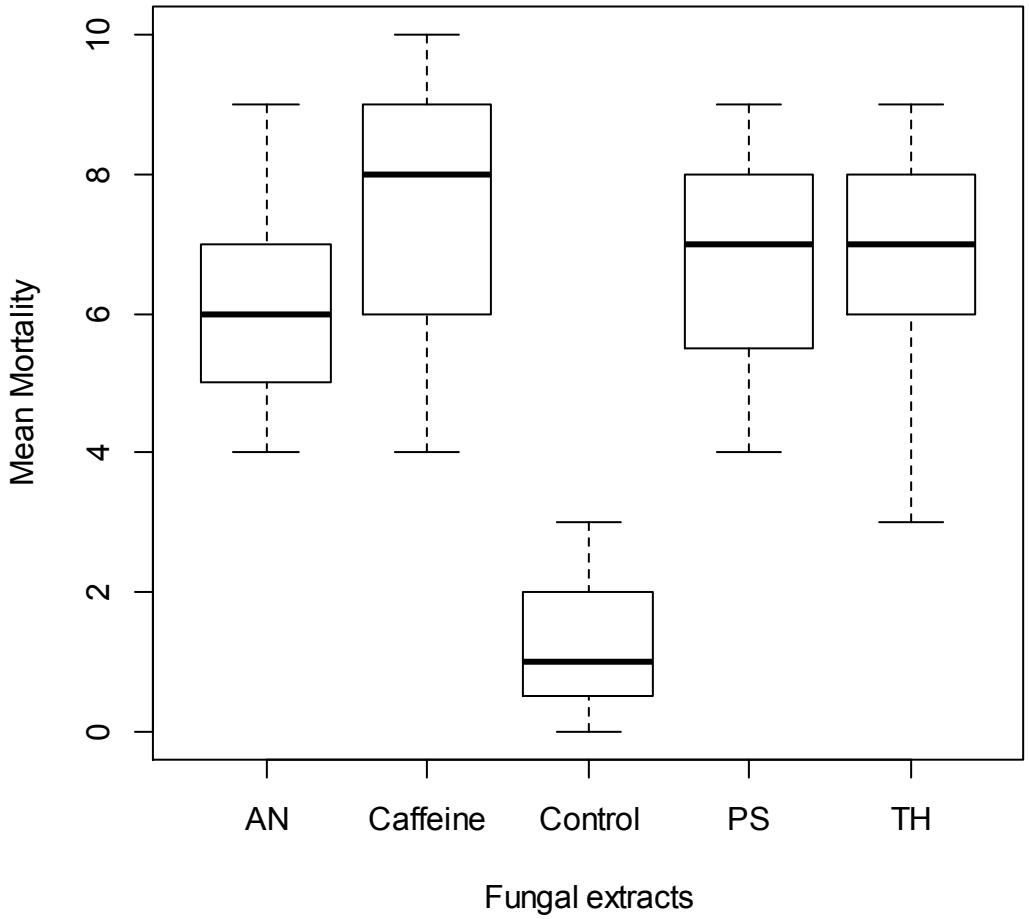


Figure 4.3: Post Hoc Multiple Comparisons of Mean Mortality of Brine Shrimp between Fungi Extracts

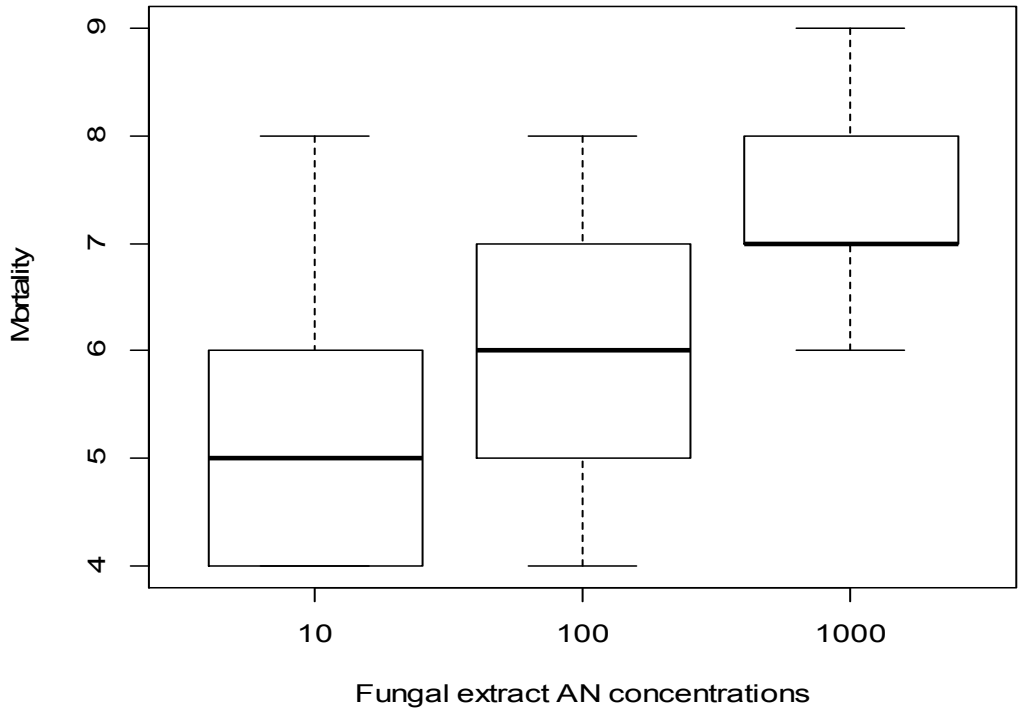


Figure 4.4: Comparison of Mean Mortality between Different Concentrations of Fungal Extract

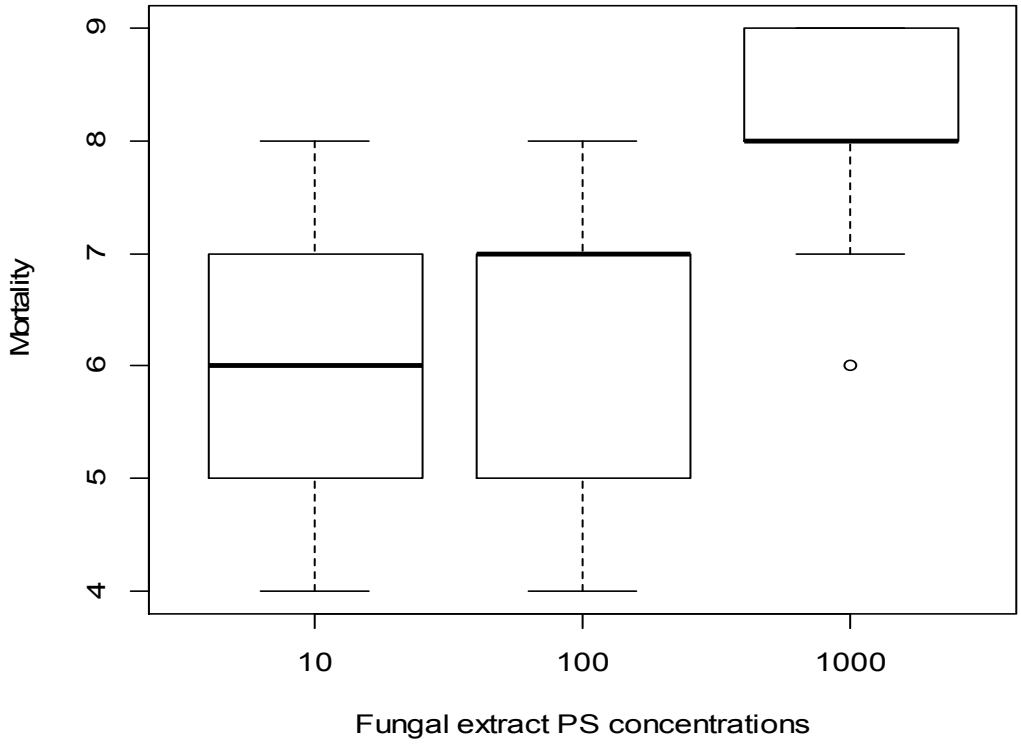


Figure 4.5: Comparison of Mean Mortality between Different Concentrations of Fungal Extract.

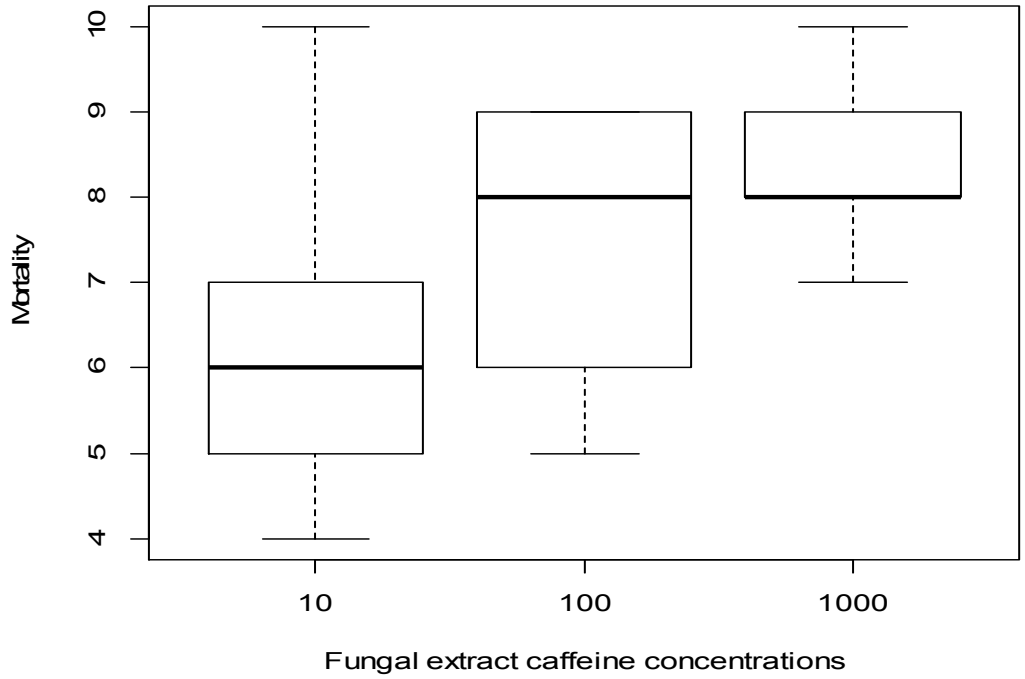


Figure 4.6: Comparison of Mean Mortality between Different Concentrations of Caffeine Fungal Extract

Table 4.4: Table Showing Mean Mortality Count at Various Level of Concentration of Extracts

Sample	First Concentration (10)	Second Concentration (100)	Third Concentration (1000)
(Rice)*Trichorderma harzianum*	7.00±1.73[b]	6.67±1.53[b]	7.33±1.53[b]
(Rice) *A. niger*	6.00±2.00[b]	6.67±1.53[b]	7.67±1.15[b]
(Rice) *Penicillium sp.*	5.67±2.08[b]	5.33±1.53[b]	8.00±1.00[b]
0.6 g Caffeine	8.33±2.08[b]	7.67±1.53[b]	8.33±1.53[b]
Control Distle water	1.67±1.53[a]	1.67±1.53[a]	1.33±0.58[a]

n = 3. Values in each column having the same superscript are not significantly different ($p > 0.05$).

Table 4.5: Table Showing Mean Mortality Count at Various Level of Concentration of Extracts

Sample	First Concentration (10)	Second Concentration (100)	Third Concentration (1000)
(Mlt)*Trichorderma harzianum*	6.67±2.08[b]	8.00±1.00[b]	7.33±1.53[b]
(Mlt) *A. niger*	5.00±1.00[b]	6.33±1.53[b]	7.33±1.53[b]
(Mlt) *Penicillium sp.*	5.00±1.00[b]	6.67±0.53[b]	8.33±0.58[b]
0.6 g Caffeine	6.33±0.58[b]	7.33±2.08[b]	8.33±0.58[b]
Control Distle water	1.00±1.00[a]	1.33±1.53[a]	1.33±1.53[a]

n = 3. Values in each column having the same superscript are not significantly different ($p > 0.05$).

Table 4.6: Tables Showing Mean Mortality Count at Various Level of Concentration of Extracts

Sample	First Concentration (10)	Second Concentration (100)	Third Concentration (1000)
(GN)*Trichorderma harzianum*	4.00 ± 1.00^{b}	7.00 ± 1.00^{bc}	8.00 ± 1.00^{b}
(GN) *A. niger*	5.00 ± 1.00^{b}	5.00 ± 1.00^{b}	7.33 ± 0.58^{b}
(GN) *Penicillium sp.*	7.00 ± 1.00^{c}	6.67 ± 1.53^{bc}	7.66 ± 1.53^{b}
0.6 g Caffeine	4.67 ± 0.58^{b}	7.33 ± 1.53^{c}	8.33 ± 0.58^{b}
Control Distle water	2.00 ± 1.00^{a}	1.33 ± 0.58^{a}	0.33 ± 0.58^{a}

n = 3. Values in each column having the same superscript are not significantly different ($p > 0.05$).

Phytochemical screening was carried out and the results of Rice bran (R_1 R_2 R_3) containing *T. harzianum* (S_1), (S_2), and *Penicillium* (S_3) respectively contains Phenols, Flavonoids and Steroids which however, (S_2 and R_2) contained the least content of phytochemicals, and (S_3 and R_3) were found to contain all phytochemicals as well as that of *T. harzianium* in Millet husk M_1 (S_1 and M_1) as showed in Table 4.2.

Components of Groundnut shells (G/N_1, G/N_2 G/N_3) with respective fungi (S_1, S_2 and S_3) have all phytochemicals. Samples of Millet husk (M_2 and M_3) with *(*S_2) and *Penicillium sp.* (S_3) were found to contain all phytochemicals with the exception of flavonoids, as well as that of *Penicillium sp. with* Rice bran (R_3), while steroids were found in all samples.

Table 4.7: Phytochemical Screening of Extracts from Agro-waste Fermented in Different Fungi

Extract (s)	Agro-waste sample	Phenols	Steroids	Flavonoids
S1	R1	-	+	+
S2	R2	-	+	-
S3	R3	+	+	+
S1	M1	+	+	+
S2	M2	+	+	-
S3	M3	+	+	-
S1	G/N1	+	+	+
S2	G/N2	+	+	+
S3	G/N3	+	+	+

Key: S1= *T. harzianum,* S2= *Aspergillus niger,* S3= *Penicillium sp.*. R= Rice bran, M= Millet husk, G/N= Groundnut shells. += Presence of compound, - = Absence of compound.

4.2 Discussion

This study demonstrates that SSF of rice bran, millet husk, and groundnut shells with *T. harzianum, Aspergillus niger and Penicillum* sp. is a very fruitful method for the enhancement of Phenolic content and antioxidant potential. To maximize the possible health benefits of these agro- wastes, SSF is a great option for the improvement of bioavailability of the phenolics in the residues by increasing their solubility. Consumption of fermented bran, husk and shells might give more health protection against oxidative damages. Moreover, fermented extract can be served as powerful sources of natural antioxidants over the synthetic antioxidant compounds used very often in food and pharmaceutical industry. Additionally, along with PCs some other bioactive compounds might be produced during SSF, which were contributing antioxidant property. SSF process could be an innovative technology in cereal science research to develop nutrition rich and more healthy cereal products. Fermented agro-wastes extract contains a complex mixture of phenolics. The colonization of the entophytic fungi is ubiquitous yet selective in nature. This selective colonization of the endophytes may lead to the production of special compounds within the host plant (Huang *et al.,* 2008). Fungi have been widely known as a source of bioactive compounds. An excellent example for this is the anticancer drug taxol, which was previously supposed to occur only in the plant tissues (Strobel and Daisy, 2003). Endophytic fungi from soil have recently gained importance in biological control of plant diseases and also as a source of pharmacologically active compounds (Strobel and Daisy, 2003). Therefore, any information and/or research on endophytic fungal extract activity, such as in this study is of value. Effective extracts could provide potential leads towards the development of novel and environmental friendly biologically active agents.

There was a graded increase in antioxidant activity in all extracts. Extracts of fungi cultivated in rice bran had higher antioxidant activity than that of millet and groundnut shells. In all agro- waste, extracts of *Penicilliun sp.* was found to show higher activity followed by *Trichoderma harzianum* in millet.

According to the statistical data above, mortality count was observed at all level of concentration of extracts including the positive control which is caffeine. Although, the high mortality values of fungal extract of Rice, Millet and Groundnut (agro-wastes) were observed in positive control, there were no significant differences between positive control and all level of concentration. But there were significant differences between the negative control and all level of concentration as well as positive control. This is directly indicates the effectiveness of the extract when compared to the caffeine which is normally recommended as highly toxic to brine shrimp larvae. However, despite the fact that there were significant differences between the different level of concentration of the extracts and positive control but the high mortality value of 0.04509, 0.0039 and 0.004842 at *Aspergillus niger, Penicillium* sp. and Caffeine respectively were all observed in high level of concentration of the fungal extracts of the agro-wastes. Whereas, *Trichoderma harzianum* 0.1503 has no significance diference at $P<0.005$. This is an indication that, the high concentration of the extracts has the high toxicity and efficacy to brine shrimp larvae.

High mortality value of 8.33±0.58in all fungal extracts of Rice, Millet and Groundnut was observed in positive control, there are no significant differences between positive control and all level of concentration. But there are significant differences between the negative control and all level of concentration as well as positive control. This directly indicates the effectiveness of the extract when compared to the caffeine which is normally recommended as highly toxic to brine shrimp larvae. However, despite the fact that, there are no significant differences between the different level of concentration of the extracts and positive control

but the high mortality value of 8.00±1.00, 8.33±0.58and 8.00±1.00 were all observed in high level of concentration of the fungal extracts of the agro-wastes. This is an indication that, the high concentration of the extracts has the high toxicity and efficacy to brine shrimp larvae. Test for presence of compounds such as the Flavonoids, steroids and phenols by phytochemical screening was carried out also in which it showed presence of all compounds in all the agro-wastes with G/N shells extracts having the highest availability of the compounds followed by extracts of Rice bran with *Penicillium* sp. and Millet husk with *T. harzianum*. Hence, the research suggests presence of bioactive compounds in the agro-wastes with remarkable antioxidative activity.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

The study investigated the production and extraction of phenolic compound through solid-state fermentation using *Trichorderma Harzianum, A. niger* and *Penicillium* sp. The result indicates extract of *A. niger* and *Penicillium* sp. to have higher graded antioxidant activity due to different metabolites present. The result however explains and supports the claim by researchers that the species are capable of homogenizing the residues to produce the bioactive phenolic compounds. The free radicals such as superoxide anion radical, hydroxyl radical and H_2O are continuously generated inside the human body can cause oxidative damage of DNA, proteins, lipids and small cellular molecules. Increasing evidence has suggested that many human diseases, such as cancer, cardiovascular disease, neurodegenerative disorders and also the process of aging, are the results of oxidative damage by free radicals hence this study shows antioxidative activity that will inhibit free radicals.

5.2 Conclusion

The present study demonstrated potential of soil fungi to have antioxidant activity similar to plants and mushrooms, thus further highlighting their significance as new sources of natural antioxidants and thus endorse the future prospects for the commercial production of natural and safer antioxidant compounds from such fungi. These fungi may provide easier set up for production and purification of natural antioxidants as compared to higher plants. The high toxicity exerted by the extract of the bran in brine shrimp lethality bioassay suggests bioactive compounds. The bran exhibited potential antioxidant activity in which is remarkable cytotoxic and antioxidative activity. Vast diversity of microbes still remains untapped for structurally diverse metabolites possessing highly valuable bioactivities

including antioxidant activity. Easier downstream processing of the fungal compounds as compared to phytochemicals offers a ray of hope for further development of chemotherapeutic agents as antioxidants are used as protective measure in various diseases. There are potentials of converting rice bran, millet husk and groundnut shells to useful products in industry especially for the production of antioxidants.

5.3 Recommendations

- More research is needed to scale up the fermentation process and improve the extraction procedures to maximize the production of extracts.
- Further study is needed to identify other unknown phenolic compounds.

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APPENDICES

Appendix I

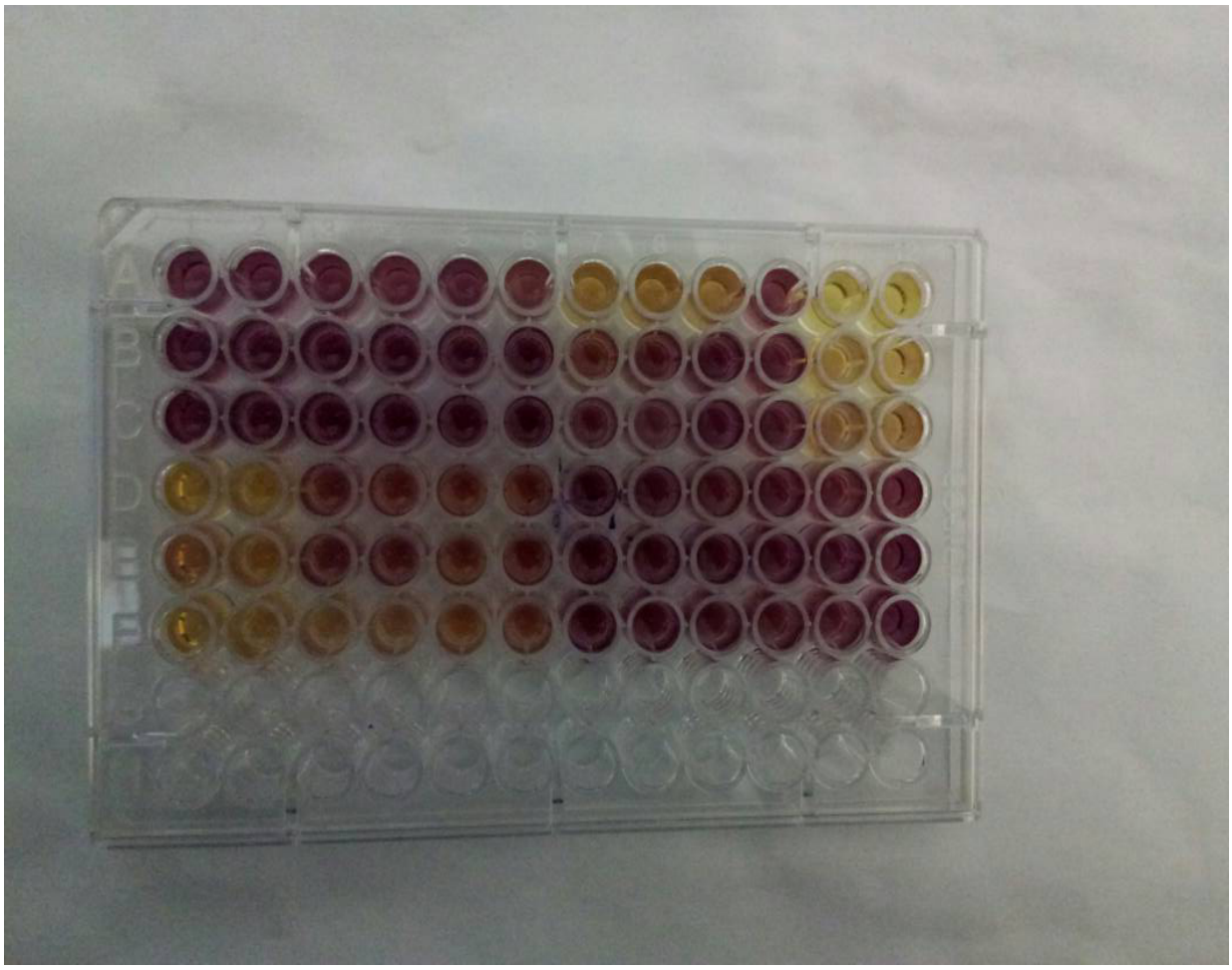
Plate I: Solid State Fermentation Set up

Appendix II

Plate II: Image showing methanol extracts

Appendix III

Plate III: Image showing methanol extracts after evaporation



Appendix IV

Plate IV: DPPH Assay of Extracts of Agro/wastes in Different Fungi

Appendix V

Plate V: Fungal growth on Czapex dox media

Appendix VI

Plate VI: Fungal Isolates on PDA

Appendix VII

Plate VII: Slides containing Fungi ready for microscopy

Appendix VIII

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Type 'demo()' for some demos, 'help()' for on-line help, or
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

>  my_data<-read.table(file=file.choose(),header=T,sep="\t")
> attach(my_data)
> str(my_data)
'data.frame':   27 obs. of  4 variables:
 $ Concentrations : int  10 10 10 100 100 100 1000 1000 1000 10 ...
 $ Agro.wastes    : Factor w/ 3 levels "Gnut","Millet",..: 3 3 3 3 3 3 3 3 3 2 ...
 $ Mortality      : int  6 9 6 5 7 8 7 6 9 9 ...
 $ Fungal.Extracts: Factor w/ 1 level "TH": 1 1 1 1 1 1 1 1 1 1 ...
> oneway.test(Mortality~as.factor(Concentrations))

        One-way analysis of means (not assuming equal variances)

data:  Mortality and as.factor(Concentrations)
F = 2.1485, num df = 2.000, denom df = 15.432, p-value = 0.1503

> boxplot(Mortality ~as.factor(Concentrations))
> detach(my_data)
>  my_data<-read.table(file=file.choose(),header=T,sep="\t")
> attach(my_data)
> str(my_data)
'data.frame':   27 obs. of  4 variables:
 $ Concentrations : int  10 10 10 100 100 100 1000 1000 1000 10 ...
 $ Agro.wastes    : Factor w/ 3 levels "Gnut","Millet",..: 3 3 3 3 3 3 3 3 3 2 ...
 $ Mortality      : int  4 8 6 8 7 5 7 7 9 4 ...
 $ Fungal.Extracts: Factor w/ 1 level "AN": 1 1 1 1 1 1 1 1 1 1 ...
> oneway.test(Mortality~as.factor(Concentrations))

        One-way analysis of means (not assuming equal variances)
```

```
data:  Mortality and as.factor(Concentrations)
F = 7.642, num df = 2.000, denom df = 15.635, p-value = 0.004842

> pairwise.t.test(Mortality,Concentrations,pool.sd=FALSE)

        Pairwise comparisons using t tests with non-pooled SD

data:  Mortality and Concentrations

     10     100
100  0.3171 -
1000 0.0052 0.0509

P value adjustment method: holm
> boxplot(Mortality ~as.factor(Concentrations))
> detach(my_data)
>  my_data<-read.table(file=file.choose(),header=T,sep="\t")
> attach(my_data)
> str(my_data)
'data.frame':   27 obs. of  4 variables:
 $ Concentrations : int  10 10 10 100 100 100 1000 1000 1000 10 ...
 $ Agro.wastes    : Factor w/ 3 levels "Gnut","Millet",..: 3 3 3 3 3 3 3 3 3 2 ...
 $ Mortality      : int  5 8 4 4 7 5 8 9 7 5 ...
 $ Fungal.Extracts: Factor w/ 1 level "PS": 1 1 1 1 1 1 1 1 1 1 ...
> oneway.test(Mortality~as.factor(Concentrations))

        One-way analysis of means (not assuming equal variances)

data:  Mortality and as.factor(Concentrations)
F = 8.0999, num df = 2.000, denom df = 15.496, p-value = 0.003909

> pairwise.t.test(Mortality,Concentrations,pool.sd=FALSE)

        Pairwise comparisons using t tests with non-pooled SD

data:  Mortality and Concentrations

     10    100
100  0.626 -
1000 0.012 0.012

P value adjustment method: holm
> boxplot(Mortality ~as.factor(Concentrations))
> detach(my_data)
>  my_data<-read.table(file=file.choose(),header=T,sep="\t")
> attach(my_data)
> str(my_data)
'data.frame':   27 obs. of  4 variables:
 $ Concentrations : int  10 10 10 100 100 100 1000 1000 1000 10 ...
 $ Agro.wastes    : Factor w/ 3 levels "Gnut","Millet",..: 3 3 3 3 3 3 3 3 3 2 ...
```

```
 $ Mortality      : int  10 9 6 8 6 9 8 7 10 6 ...
 $ Fungal.Extracts: Factor w/ 1 level "Caffeine": 1 1 1 1 1 1 1 1 1 1 ...
> oneway.test(Mortality~as.factor(Concentrations))

        One-way analysis of means (not assuming equal variances)

data:  Mortality and as.factor(Concentrations)
F = 3.8794, num df = 2.000, denom df = 14.301, p-value = 0.04509

> pairwise.t.test(Mortality,Concentrations,pool.sd=FALSE)

        Pairwise comparisons using t tests with non-pooled SD

data:  Mortality and Concentrations

     10    100
100  0.300 -
1000 0.066 0.300

P value adjustment method: holm
> detach(my_data)
>  my_data<-read.table(file=file.choose(),header=T,sep="\t")
> attach(my_data)
> str(my_data)
'data.frame':   27 obs. of  4 variables:
 $ Concentrations : int  10 10 10 100 100 100 1000 1000 1000 10 ...
 $ Agro.wastes    : Factor w/ 3 levels "Gnut","Millet",..: 3 3 3 3 3 3 3 3 3 2 ...
 $ Mortality      : int  3 0 2 2 3 0 1 1 2 2 ...
 $ Fungal.Extracts: Factor w/ 1 level "Control": 1 1 1 1 1 1 1 1 1 1 ...
> oneway.test(Mortality~as.factor(Concentrations))

        One-way analysis of means (not assuming equal variances)

data:  Mortality and as.factor(Concentrations)
F = 0.67975, num df = 2.000, denom df = 15.943, p-value = 0.5208

>
```

Appendix IX

```
R version 3.4.0 (2017-04-21) --
Copyright (C) 2017 The R Foundation for Statistical Computing
Platform: x86_64-w64-mingw32/x64 (64-bit)

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Type 'demo()' for some demos, 'help()' for on-line help, or
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

> my_data<-read.table(file=file.choose(),header=T,sep="\t")
> attach(my_data)
> str(my_data)
'data.frame':   135 obs. of  4 variables:
 $ Concentrations : int  10 10 10 100 100 100 1000 1000 1000 10 ...
 $ Agro.wastes    : Factor w/ 3 levels "Gnut","Millet",..: 3 3 3 3 3 3 3 3 3 2 ...
 $ Mortality      : int  6 9 6 5 7 8 7 6 9 9 ...
 $ Fungal.Extracts: Factor w/ 5 levels "AN","Caffeine",..: 5 5 5 5 5 5 5 5 5 5 ...
> Mortality_aov<-aov(Mortality~as.factor(Agro.wastes)*as.factor(Fungal.Extracts))
> summary(Mortality_aov)
                                    Df Sum Sq Mean Sq F value
as.factor(Agro.wastes)               2    6.0    2.99   1.311
as.factor(Fungal.Extracts)           4  667.3  166.83  73.241
as.factor(Agro.wastes):as.factor(Fungal.Extracts)   8   14.7    1.84   0.807
Residuals                          120  273.3    2.28
                                   Pr(>F)
as.factor(Agro.wastes)              0.273
as.factor(Fungal.Extracts)          <2e-16 ***
as.factor(Agro.wastes):as.factor(Fungal.Extracts)  0.598
Residuals
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> Mortality_aov<-aov(Mortality~as.factor(Fungal.Extracts)*as.factor(Agro.wastes))
> summary(Mortality_aov)
                                    Df Sum Sq Mean Sq F value
as.factor(Fungal.Extracts)           4  667.3  166.83  73.241
as.factor(Agro.wastes)               2    6.0    2.99   1.311
as.factor(Fungal.Extracts):as.factor(Agro.wastes)   8   14.7    1.84   0.807
```

```
Residuals                                   120  273.3    2.28
                                          Pr(>F)
as.factor(Fungal.Extracts)                 <2e-16 ***
as.factor(Agro.wastes)                     0.273
as.factor(Fungal.Extracts):as.factor(Agro.wastes)  0.598
Residuals
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> Tukey_Fungal.Extracts<-TukeyHSD(Mortality_aov,as.factor("Fungal.Extracts"))
> Tukey_Fungal.Extracts
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = Mortality ~ as.factor(Fungal.Extracts) * as.factor(Agro.wastes))

$`as.factor(Fungal.Extracts)`
                    diff        lwr        upr     p adj
Caffeine-AN       1.1481481  0.01046727  2.2858290 0.0467662
Control-AN       -4.9259259 -6.06360680 -3.7882451 0.0000000
PS-AN             0.4444444 -0.69323643  1.5821253 0.8154725
TH-AN             0.6296296 -0.50805124  1.7673105 0.5433422
Control-Caffeine -6.0740741 -7.21175495 -4.9363932 0.0000000
PS-Caffeine      -0.7037037 -1.84138458  0.4339772 0.4298354
TH-Caffeine      -0.5185185 -1.65619939  0.6191624 0.7146555
PS-Control        5.3703704  4.23268950  6.5080512 0.0000000
TH-Control        5.5555556  4.41787468  6.6932364 0.0000000
TH-PS             0.1851852 -0.95249569  1.3228661 0.9913656

> interaction.plot(Fungi.Extracts,Agro.wastes,Mortality)
Error in tapply(response, list(x.factor, trace.factor), fun):
  object 'Fungi.Extracts' not found
> interaction.plot(Fungal.Extracts,Agro.wastes,Mortality)
> boxplot(Mortality ~as.factor(Fungal.Extracts))
> boxplot(Mortality ~as.factor(Agro.wastes)>
```