DETERMINATION OF FUNGAL AND AFLATOXIN CONTAMINATION OF CIGARETTE TOBACCO AND TOXICITY SCREENING OF CRUDE EXTRACTS OF THE FUNGI IN MICE

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DECEMBER 2003

DECLARATION

I hereby declare that this thesis is my original work and to the best of my knowledge, has not been presented in any form for the award of degree or diploma at any other institution.

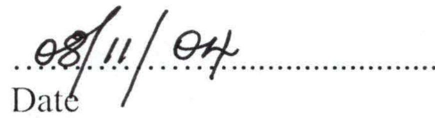
All information derived from published and unpublished works of others have been well acknowledged in this thesis.

Musa, Achimugu Dickson

Date

CERTIFICATION

This project entitled **"Determination of Fungal and Aflatoxin contamination of Cigarette Tobacco and toxicity Screening of Crude Extracts of the Fungi in Mice".** was carried out by **MUSA, ACHIMUGU DICKSON** under my supervision and has been examined, read and found to meet the regulations governing the award of the Degree of Masters of Technology in Biochemistry of Federal University of Technology, Minna and is approved for its contribution to knowledge and literary presentation.



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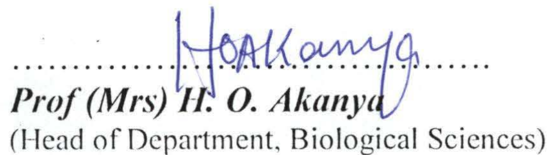

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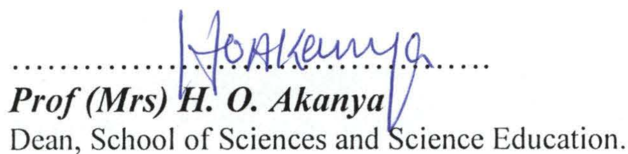
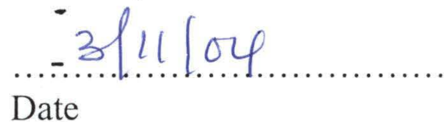

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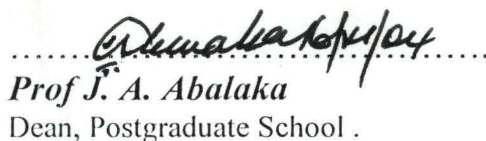

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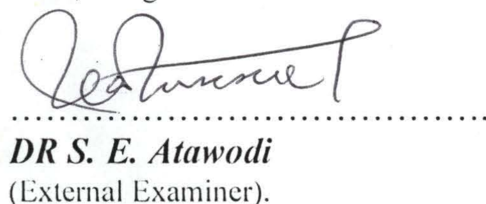
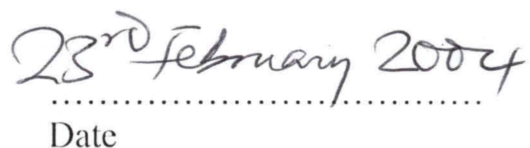

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DEDICATION

To my exuberant little niece, Ufedo and to her mother, my dear sister, Christie, who made me an uncle without seeking my consent. Also to the memory of my late brother and nephew; Wilson and Dickson, and to all those who have had the courage to quit smoking.

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ABSTRACT

Tobacco from 32 commercial brands of cigarettes were analyzed for the presence of fungal and aflatoxin contamination; thirty of the 32 brands analyzed were contaminated with up to 17 different types of Fungi species producing a total of 115 isolates on culture. Fifty Nine (51%) of the isolates elaborated mycotoxins on rice medium. A dose of 5mg/kg(body weight) of the secondary metabolites produced were administered unto healthy young male mice weighing about 300g through intraperitoneal route and they exhibited varying degree of acute toxicity (lethality) on the test animals. Some isolates elaborated secondary metabolites that induced 100% mortality, while others produced metabolites that had 0% lethality on the test animals. Between them, there was a broad spectrum percentage of mortality induced by the test substance. On direct screening for aflatoxin in the cigarette tobacco, six of the cigarettes samples, representing 18% were found to have aflatoxin contamination. These results suggest that cigarettes marketed in Nigeria may also produce mycotoxin – related toxicity in smokers due to fungal contamination.

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CHAPTER ONE

1.1 INTRODUCTION

Tobacco is a plant grown commercially for its leaves and stems, which are rolled into cigars, shredded for use in cigarettes and pipes, processed for chewing, or ground into snuff, a fine powder that is inhaled through the nose. Tobacco is the source of nicotine, an addictive drug that is also the basis for many insecticides (Hynes, 2000)

Smoking is the inhalation and exhalation of the fumes of burning tobacco. Cigarettes is the most popular method of smoking (Peterson, 2000) and it consists of finely shredded tobacco rolled into lightweight paper usually with filter at the mouth end, making it look deceptively simple. However it is a highly engineered product designed to deliver a steady dose of nicotine. Cigarette tobacco is blended from two main leaf varieties: yellowish bright, also known as virginia where it was originally grown, and burley. Some US blends also contain oriental tobacco which is more aromatic (Milne, 1998). In addition to leaf blend, cigarettes contain "fillers" which are made from the stems and other bits of the tobacco which would otherwise be waste products. These are mixed with water and various flavourings and additives. The ratio of fillers varies among brands of cigarettes (ASH, 2001) .

Additives are used to make cigarettes more acceptable to consumer. There are over 600 approved additives by the European Union and they include humectants (moisturizers) to prolong shelf life; sugars to make the smoke seem milder and easier to inhale; and flavourings such as chocolate, menthol and vanilla. (Bates *et al.,* 1999) While some of these may appear harmless in their natural forms, they may become toxic in combination with other substances.

Also when additives are burned, new products of combustion are formed and these may also be toxic.

Until the 1940s, smoking was considered harmless, but laboratory and clinical research has since confirmed that tobacco smoke presents hazard to health (Peterson, 2000). In response to this, cigarette companies added filters to cigarette in the 1950s. (ASH, 2001). Filters are made up of cellulose acetate and they trap some of the particles of the inhaled smoke. They also cool the smoke slightly, making it easier to inhale.

1.1.1 Constituent Of Cigarette Smoke

Phillip Morris (a major player in the tobacco industry) in 1972 explains that "The cigarette should be conceived not as a product but as a package. The product is nicotine. Think of the cigarette pack as a storage container for a day's supply of nicotine......Think of the cigarette as the dispenser for a dose unit of nicotine and the cigarette the most optimized dispensers of smoke " (Bates *et al.*, 1999).

Although most smokers are aware of the harmful effects of smoking, this may not be enough to overcome the reasons why they are smoking. Probing into what makes cigarette so irresistible, it was discovered that much of the recent researches corroborates earlier claims: It is the nicotine in cigarette that makes smokers smoke (Ginzel, 1990). In contrast to other drugs, nicotine delivery from tobacco carries an ominous burden of chemical poisons and cancer producing substances that boggle the mind. Many toxic agents are in cigarette. However, additional toxicants are manufactured during the smoking process by the chemical reactions occurring in the glowing tip of the cigarette.

Cigarette smoke is made up of "side stream smoke" from the burning tip of the cigarette and "mainstream smoke" from the filter or mouth end. Smoke from cigarette contains around 4000 chemicals (Peterson, 2000;Ginzel, 1990) with more than 500 known poisons (The Analyst, 2003), at least 60 of which causes cancer (Peterson, 2000; ASH, 2001).The hazardous constituents are present in higher concentrations in side stream smoke than in mainstream smoke. (ASH, 2001). The particulate phase includes nicotine, an alkaloid; tar also known as total particulate matter is itself composed of many chemicals and benzo[a]pyrene which constitute about 5-8% of the total output of cigarette (ASH, 2001;Peterson, 2000). The remaining bulk makes up the vapour or gas phase of cigarette smoke and includes carbon monoxide, ammonia, nitrosamines, formaldehyde, hydrogen cyanide, acrolein and nitrogen oxides (ASH, 2001). Smokers efficiently extract about 90% of the particulate as well as gaseous constituents (about 50% in the case of carbon monoxide) from the mainstream smoke (Peterson, 2000).

Table 1.1 Some identified carcinogens in cigarette smoke

Gas phase
Dimethylnitrosamine, methymethylnitrosamine, N-nitrosopyrrolidine, N- nitrosopiperidine, Tobacco-specific Nitrosamines

Particulate phase
Benzo(a)pyrene, methylbenzo(a)pyrene, dibenzo(a,h)acridine, Dibenz(a,j)acridine, Dibenz(c)carbazole, naphtalamine, Benzo(b)fluoranthene, Benzo(./')fluoranthene, chrysene methylfluoranthene, Benzo(a)anthracene, , methchrysenes, Benzo(c)phenantheren

Source:http://tobaccodocuments.org/pm/206362 8499-8502.html

Tobacco smoking is an important causative factor in cancer of the lung, as well as cancers of other organs such as larynx, oral cavity and bladder (Atawodi *et al.,* 1998) Table 1.1 shows these carcinogens present in cigarette smoke and Table 1.2 shows how various factors affects the amount of these carcinogens obtained by a smoker.

Table 1.2: Factors affecting the composition of cigarette smoke and exposure to smoke constituents

Nature of the Tobacco	**Cigarette Design**	**The Way the cigarette is smoked**	**Inhalation**
Strain of plant soil	Tobacco amount	Puff number	Depth of inhalation
Climate	Packing and cut	Puff duration	Duration of inhalation
Time of harvesting	Moisture and content	Puff volume	
Location of leaf	Tobacco rod length	Puff pressure	
Method of curing	Draw resistance		
Aging and fermentation	Nature of filter		
Storage	Efficiency of filter		
	Porosity of paper		
	Degree of ventilation		
	Additives		
	Butt length		

Source: http://tobaccodouments.org /pm/2063628863-8873.html (11)

1.2 STATEMENT OF PROBLEM

In many modern day societies, smoking of cigarettes is an accepted habit with adults of all age groups and both sexes being able to smoke. In Nigeria, although cigarette smoking is not socially acceptable, the practice is wide spread. Micro organisms including fungi are involved at one stage or the other in production process of cigarette from tobacco. There are no known conscious efforts taken by manufacturers to exclude mycotoxin producing moulds.

If such moulds infest or attack the tobacco plants after harvesting, they may produce spores, and although the moulds themselves may not survive the manufacturing process of cigarette production from tobacco leaves, the spores may; such spores may elaborate mycotoxins. During preliminary investigation, it was discovered that cigarette packs do not contain information like date of manufacture and expiry date, that means, cigarette could still get to consumers after a very long time in storage, within which time, the mould spores could have elaborated much mycotoxins in the cigarette.

During smoking, the tobacco leaves are combusted. The contaminating moulds are also combusted, leaving no trace of itself (NewScientist, 2002). However, mycotoxins that such fungi would have elaborated are not combusted, being highly heat stable (Smith and Moss, 1985). The mycotoxins are then inhaled along with the total particulate matter, and then synergistically together with other known carcinogens in cigarette to elicit its toxicity and carcinogenicity in (active smokers and passive smoking –those exposed to ETS). No wonder then that tar has been implicated in smoking induced carcinogenesis. Researches have shown that aflatoxin (a mycotoxin) raises levels of the AIDS virus in the blood by 400% when these cells are exposed to this mycotoxin in the laboratory (TobaccoNews, 2003). This is especially disconcerting, because benzopyrene, which is already an acknowledged carcinogenic component of tobacco smoke,

stimulates cells to form the active metabolite of aflatoxin, the epoxide. Furthermore, aflatoxin causes mutation in the p53 tumour suppressor gene at codon 249 in liver cells, the same site often mutated in lung cancer (TobaccoNews, 2003). It is unlikely that this is a mere coincidence. There is thus a need to invest resources in elucidating the veracity or otherwise of the foregoing, because of the high toxicity and carcinogenicity of aflatoxins.

Unfortunately though, there has been dearth of research in this area. Studies has shown that smoking is the most preventable cause of death in America today (Peterson, 2000), more the 2.5million people die each year in developing countries from illness related to cigarette smoking and deaths through cigarette and tobacco has outpaced those caused by Acquired Immune Deficiency Syndrome [AIDS] (Oyegbile, 2003). What is most needful now therefore is conscious efforts in research to elucidate on the role of fungi and their secondary metabolites in toxicity of cigarette smoke.

1.3 PURPOSE OF STUDY

This research has been designed to achieve the followings in a bid to shedding more light on the possible role of fungi and mycotoxins in toxicity of cigarette:

1. To determine if there are fungal spores in packaged cigarette sold in the Nigerian market
2. To determine if cigarette sold in Minna environ are contaminated with aflatoxin.
3. To determine the number of isolates of fungi from each of the 32 brands of cigarette to be studied
4. To determine if each of the isolates elaborates mycotoxins on natural media
5. To determine the lethality of each of the mycotoxin elaborated on mice

6. To give informed recommendation from results generated from the study.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 TOBACCO

The tobacco plant is one of the most important commercial non food crops grown in the world today. Tobacco is a member of the nightshade family, *Solanaceae*. There are more than 70 species, common tobacco (*Nicotiana tabacum)* and wild tobacco (*Nicotiana rustica*), are annuals-they survive only one growing season. Common tobacco is 1-3m tall and has a thick woody stem with few side branches. One plant typically produces 10-20 broad leaves that branch alternately from the central stalk (fig 2:1). The leaf size depends on the strain. The narrow, trumpet shaped flowers are dark pink to almost white. Wild tobacco is about 0.6m tall and has a stem that is more slender and less woody than common tobacco. The leaves have short stalk that attach to the stem. The flowers are pale yellow with five separate lobes (Hynes, 2000) .

Fig 2.1: Tobacco plant
Source: Microsoft Encarta Encyclopaedia 2003 (CD ROM).

2.1.1 History

Huron Indian myth has it that in ancient times, when the land was barren and the people were starving, the "Great Spirit" sent forth a woman to save humanity. As she travelled over the world, everywhere her right hand touched the soil, there grew potatoes. And everywhere her left hand touched the soil, there grew corn. And when the world was rich and fertile, she sat down and rested when she arose, there grew tobacco (Borio, 2001).

Although small amount of nicotine may be found in some old world plants, including belladonna and *Nicotiana africana,* there is no indication of their habitual use or that of tobacco on any continent, save the Americas. As early as 2000 years ago, natives of the Americas used tobacco as medicine, as a hallucinogen in religious ceremonies, and as offering to the spirits they worshiped (Hynes, 2000). When Italian Spanish explorer, Christopher Columbus traveled to the Americas in 1492, he observed the indigenous Arawak people of the Caribbean smoking tobacco loosely rolled in a large tobacco leaf. They also smoked tobacco through a tube they called a 'Tobago' from which the name tobacco originated. (Hynes, 2000). The first pictorial record of smoking is a pottery vessel found in Uaxactun, Guatemala dating back to the 11th century. On it a Maya is depicted smoking a roll of tobacco leaves tied with a string. The Mayan term for smoking is 'Si Kar', (Borio, 2001) from where cigar and cigarette where probably derived. Columbus crew introduced tobacco growing and use to Spain.

During the next 50 years, sailors, explorers and diplomats helped spread pipe and cigar smoking throughout Europe. At first it was used medicinally as a purported treatment for such disease and disorders as bubonic plague, migraines, labour pains, asthma, and cancer. Within the next 100 years however, smoking

for pleasure became common. So much so that in 1603, physicians in England upset that tobacco was being used by people without a physician's prescription complained to King James I, who then increased import tax on tobacco by 4,000% to discourage tobacco use. In 1614 however, King Philip III of Spain established Seville as tobacco centre of the world. Seville thus became the centre for production of cigars. European cigarette use also began there, as beggars patch together tobacco from used cigars and roll them in paper. However cigarette did not become popular for about two and half centuries; snuff, cigars and pipes remained the popular means of using tobacco. In 1880, James A. Bonsack an American inventor patented a machine to roll cigarettes and the price fell drastically and by 1919 cigarettes were more popular than cigars.

2.1.2 Growing and Harvesting

Tobacco grows both in tropical and temperate regions, and it can be grown as far north as Canada and Norway. It thrives best in area with frost-free growing seasons of 120 to 170 days, depending on the type of tobacco. Good quality tobacco requires fertile well drained moist soil and warm temperatures. Most types of tobacco are grown in full sun. Environmental factors influence the plant's characteristics. Soil, for example, can affect leaf size, texture and colour.

Several strains of tobacco are grown for use primarily in different tobacco products. In the United States, Virginia tobacco is the main tobacco used in cigarettes. Burley tobacco is used in cigarettes and pipe. Several countries including Cuba and Turkey grow cigar tobacco.

Tobacco plants are susceptible to attack from a wide range of insects and bacterial, fungal and viral disease. To counteract these problems, tobacco farmers grow strain of tobacco that resist disease and insects. Before planting,

farmers may also work fungicides into the soil to control fungal diseases among other strategies. The annual tobacco cultivation cycle begins with the planting of seeds. Tobacco seeds are so small that they need special care to keep them from drying once they start sprouting. To keep young plants watered and weeded, growers sow their seeds in specially prepared seedbeds of fertile loose soil, rather than directly on the soil.

One to two months after planting, the growers transplant the seedlings into the field-a labour intensive process called setting the tobacco. As flowers form on the plants, growers remove them in a process called topping, which encourage more leaf growth. Tobacco is harvested 70 to 130 days after setting. The harvesting method used depends on the type of tobacco. For some tobacco, farmers cut whole plants off at the ground and spear them onto a stick about 1m long, called tobacco stick. Each stick holds about six plants. For other tobaccos, farmers remove the mature leaves and string them on wires, leaving the rest of the plant to grow. With the exception of countries such as the United States (where crop is mechanically harvested), the farmer will typically harvest by hand, sequentially taking off 2-4 leaves per plant as the leaves ripen from bottom to top, which stretches the harvest period from over 2 to 4 months (BAT, 2003).

Fig 2.2: A young farmer harvesting tobacco
Source: Microsoft Encarta 2003 (CD ROM)

2.1.3 Curing and Aging

After tobacco is harvested, it is cured, or dried and then aged to improve its flavour. Curing is a carefully controlled process to achieve the texture, colour and overall quality of a specific tobacco type. During the cure, leaf starch is converted into sugar, the green colour vanishes and the tobacco goes through colour changes from lemon to yellow to orange to brown (CIRL, 1999) all these albeit by microorganisms. There are four common curing methods, and the method used depends on the type of tobacco and its intended use.

1. Flue Curing: This is the most popular method of curing. The tobacco sticks are loaded in bulk into containers. The containers are then put in a curing barn. A curing barn is a closed building that has a heat source. It uses a flue to let smoke in and out. The process usually takes about one week. According to BAT (2003) the steps involved are:

    i. Firing up: this goal of this step is to bring the plant to a brilliant lemon-orange colour. This is done by slowly raising the temperature.

    ii. Yellowing: In this step moisture are removed. This creates the "yellowing' of the tobacco. It also prepares the tobacco for drying in the next step.

    iii. Leaf drying: This is the most important step in curing process. Much time is needed for tobacco to dry. Air flow is increased in this step to help the drying.

    iv. Stem drying: The drying process continues.

    This method produces cigarette that is high in sugar and has medium to high level of nicotine. It is the fastest method of curing and most Virginia cigarette are flue cured.

2. Air Curing: Air cured tobacco, is hung unheated and sheltered from wind and sun in a well ventilated barn to dry naturally for about six to eight weeks until the leaves reaches a light, sweet flavour, and high nicotine cigar and burley tobaccos are air cured.

3. Fire Curing: Here, smoke from a low-burning fire on the barn floor permeates the leaves, drying the leaves and producing a distinctive smoky fragrance. Fire curing takes 3 to 10 weeks and produces a tobacco low in sugar and high in nicotine. Pipe tobacco, chewing tobacco, and snuff are fire cured.

4. Sun Curing: In sun curing, the leaves are strung out on racks and exposed to the sun. The whole process takes form 12 to 30 days. The sun's direct that fixes the leaves at a yellow to orange colour. This method is used in Greece, turkey and other Mediterranean countries to produce oriental tobacco which is low in sugar content and nicotine.

After curing, workers tie the tobacco into small bundles of about 20 leaves, called hands and carefully age it for one to three years to improve flavour and reduce bitterness. The farmer then grades the leaves into different lead positions, qualities and colours and packs his grades into what is known as bales of 30-50kg. He then takes them to a buying centre or auction for sales.

2.1.4 Processing and Manufacturing

The cured and aged tobacco leaf is then processed through a Green leaf threshing plant. The main aim of the processing is to:

- Remove sand, dust, scraps and foreign matter
- Separate the lamina from the stem (threshing)
- Drive down the tobacco to a safe "keeping moisture" content.

- Processed tobaccos are packed into 200kg cardboard boxes, for shipping to manufacturing sites.

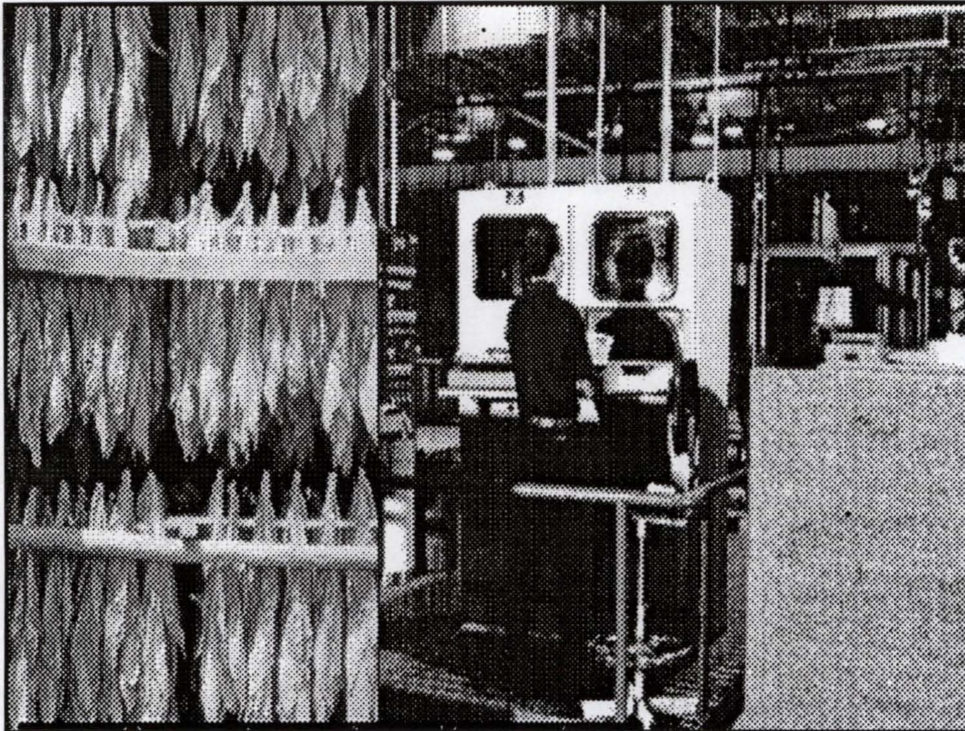

Fig 2.3: Tobacco leaf processing plant
Source: Microsoft Encarta 2003 (CD ROM)

At the factory, various strains of tobacco are then blended in rotating drums and with other ingredients which the brand recipe may call for, such as flavourings or pre-processed tobacco (CIRL, 1999). It is customary in the tobacco industry to improve the organaleptic quality of tobacco products by applying several kinds of additives to the tobacco. Thus casing liquids such as licorice extract, fruit juices or other natural extracts, and humectants such as glycerol and solutions of sugar or sorbitol are sprayed over the tobacco. Also, water soluble flavourings are sometimes added (European Patent Application, 1990). Keeping tracks of the various types of tobacco and blend components is key and computers are increasingly used to track production runs.

Moisture is crucial. Too dry and the tobacco leaf will crumble, too moist and it may spoil during storage. The blended tobacco is treated with just the right

amount of steam and water to make it supple, and then cut into the form in which it appears in the cigarette. Excess moisture is then removed so that the cut tobacco can be given final blending.

Cut tobacco, cigarette paper and filters are continuously fed into cigarette making machine which roll tobacco in the paper and then add the filter. Finally, packing machines put them into familiar brand packs and wrap them in protective films and then group them into cartons and cases, ready for distribution.

2.1.5 Tobacco Industry

Approximately 7 million tons of commercial tobacco are grown each year, with a value of $39 billion (Hynes, 2000) leading tobacco-growing countries are China, the United States, India, Brazil, Turkey and Zimbabwe. Brazil leads in tobacco exports and the United States export the most cigarettes. There are two main manufacturing companies in Nigeria, both of which are affiliates of multinationals.

2.2 CIGARETTE SMOKING

2.2.1 Adverse Health Effects of Cigarette Smoke

According to the American cancer society, smoking is the most preventable cause of death in America today (Peterson, 2000). The debilitating effect of cigarette smoke on major body organs like skin, heart, lungs, brain and liver is well documented (Napier, 1996). Some of the diseases associated with smoking are bronchitis, emphysema, coronary heart disease, cirrhosis of the liver, smoker's face, retarded fetal growth, decreased birth weight, peripheral vascular disease, lip and tongue cancer, gum cancer, larynx cancer, lung cancer, peptic ulcer, bladder cancer and Buerger's disease (Ashton, 1999).

The evidence available suggests that the diseases associated with smoking relate to intake of smoke constituents in a dose-dependent way; the greater the exposure to smoke constituents, the greater risks than smokers of lower yielding filter brands; and smokers of many years standing are more threatened than those with a shorter history (Ashton, 1999). Starting to smoke while young significantly increases the risk of developing cancer. Also breathing second hand smoke also known as Environmental Tobacco Smoke (ETS) increases the risk of children and spouses of smokers and in non smokers who work in smoky places like restaurants, and bar (Peterson, 2000). Tobacco-specific nitrosamines 4-(methylnitrosamino)-1(3-pyridyl)-1-butanone (NNK) is considered to play a major role in the induction of lung cancer in cigarette smokers (Atawodi and Richter, 1996). Besides its reduction product which is a procarcinogen, 4-(methylnitrosamino)-1(3-pyridyl)-1-butanal (NNAL) have been detected in the urine of smokers and non-smoker heavily exposed to sidestream cigarette smoke (Atawodi *et al.,* 1994). This tobacco-specific nitrosamines are also present in Nigerian cigarettes and are formed in tobacco during nitrosation of nicotine and other related tobacco alkaloids (Hecht *et al.,* 1978; Hoffmann and Hecht , 1985)

A United States patent application has linked grain toxins to tobacco (TobaccoNews, 2003) and this has worsened the carcinogenic effect of tobacco consumption, hence cigarette smoking. This is corroborated by reports by some Dutch scientists that cigarette was found to contain fungal contamination (NewScientist, 2002).

2.2.2 Smoking in Nigeria

In most modern societies, like in Nigeria, cigarette smoking is more or less perceived as glamorous by young people. A recent survey conducted by the United States Centre for Disease Control in Cross Rivers State in Nigeria (CDC 2001), found that as many as 45% of the people surveyed think boys who smoke

and 28% think girls who smoke have more friends. Again 17% think boys who smoke and 16% thinks girls who smoke have more friends. Again 17% think boys who smoke and 16% thinks girls who smoke look more attractive. Little wonder then that as many as 22% of the sampled population currently use tobacco with as many as 20% of these who had never smoked likely to start smoking in the next year. Smoking is seen as something attractive probably because of the way young people see their role models-film stars, musicians and celebrities smoking or portraying smoking as something glamorous. Also the average Nigeria youth tends to want to be as westernized as possible and because smoking cigarettes is common in western societies, he or she feels smoking should be cultivated to make him or her belong.

Table 2.1: Smoking prevalence in Nigeria

Youth (13-15)*, 2001	Adults (15 +)*, 1990	Senior executives
Males 23.9%	Males 15.4%	Males 17.4%
Females 17.0%	Females 1.7%	Female 0.02%
Overall 18.1%	Overall 8.9%	Overall 13.9%

Sources:

Youth :CDC 2001

Adult: Federal Ministry of Health

Senior Executives: Survey in Benin City reported by Isah E.C and Okoro, E. (2000)

*years

Table 2.1 above shows a pattern of smoking among Nigerian youths, adults and Business executives. In the early 1990s adult per capita consumption of cigarettes averaged 370 annually, a 32% increase from the early 1970s (WHO, 1997). However due to reports of widespread smuggling consumption may actually be higher. On June 18, 2003, the British American Tobacco (BAT)

commissioned an 'ultra modern' factory valued at N22billion ($180 million) covering a 2.5 hectares land in Ibadan (Oyegbile, 2003).

Table2.2: Nigerian Annual Tobacco and Agriculture Statistics

	Unit of Measurement	1970	1980	1990	1995	2000
Cigarette imports	Sticks in million	20	60	198	846	2966
Cigarette exports	Stick in million	-	-	-	-	-
Tobacco leaf imports	Metric tons	523	2700	9608	6459	2668
Tobacco leaf exports	Metric tons	4	-	101	155	-
Cigarette production	Stick in millions	-	-	10380	9413	-
Tobacco leaf production	Metric tons	11177	13000	900	9200	9200
Land devoted to tobacco growing	Hectares	21043	20680	22000	22000	22000

Source: CDC2001

Experts have observed that while tobacco consumption in developed countries has fallen sharply due to governmental regulation and health education, the problem continues to grow in many developing countries. (Oyegbile, 2003). Table 2.2 shows an increase in most of the parameters considered for Nigeria. In Nigeria there is no age restriction on buying cigarettes and also cigarette companies advertise unrestricted (Odigwe, 2003). They sponsor major sporting tournaments and stage promotional shows with little or no restriction. They are only obliged to issue health warnings after each adverts and to print warnings beneath their bill boards . Table 2.3 shows the National Tobacco control provisions, some of which are not adequate in tackling the problem of smoking in Nigeria

Table 2.3: Infrastructure for Tobacco Control (National Tobacco Control Provisions)

(a) Tobacco Bans and Restrictions	Banned	Restricted	Not Regulated	Unknown
Advertising in certain media		X		
Advertising to certain audiences				X
Advertising in content locations		X		
Advertising content and design			X	
Sponsorship promotion for certain audiences			X	
Sponsorship advertising of events			X	
Brand stretching			X	
Sales to minors			X	
Sales by minors			X	
Place of sale		X		
Single cigarette sales			X	
Misleading information on packaging			X	
Smoking in government building	X			
Smoking in private worksites	X			
Smoking in educational facilities	X			
Smoking in health care facilities	X			
Smoking on buses	X			
Smoking in trains	X			
Smoking in taxis	X			
Smoking in ferries	X			
Smoking in domestic air flights			X	
Smoking on international air flights			X	
Smoking in restaurants, bars and nightclub			X	
Smoking in other public places		X		

Fig 2.3: continued

(b) Tobacco Requirements and Regulations	Required	Regulated	Not regulated	unknown
Advertising health warnings and messages	X			
Age verification for sale			X	
Manufacturing licensure				X
Package health warning/messages	X			
Label design or packaging		X		
Ingredient/constituent information on package label	X			
Amount of tar			X	
Amount of nicotine			X	
Amount of other ingredients/constituent			X	
Product constituents as confidential information			X	
Product constituents as public information			X	
Constituent disclosure by brands			X	
Constituent disclosure in the aggregate			X	

Sources: CDC 2001

Tar which is the total particulate matter of cigarette smoke has been shown to contain a lot of dangerous chemicals. This has led the EU to phase out cigarettes with a tar yield of 15mg or more by the end of 1992. Also from 1st January 1998, an upper limit of 12mg per cigarette was applied and then 10mg per cigarettes, effective from September 2002 (ASH, 2001). However in Nigerian, studies

shows that only 78% of the manufactured cigarettes were filter tipped and the most popular brand contained 19mg of tar (WHO, 1997). According to another study, cigarette and tobacco related deaths have outpaced those caused by AIDS (Oyegbile, 2003), thereby placing a heavy burden on a country like Nigeria where public health facilities are scarce and the few that are available are overstretched, as yet no schemes to expand health care are in place and appreciable improvements cannot be reasonably expected within the near future.

2.3 FUNGI

Fungi are non-photosynthesizing, heterotrophic organisms that derive their energy from saprophytic or parasitic existence. They are unicellular, amoeboid, or filamentous, never having leaves, stems, and roots characteristic of higher plants (BoDD, 2002). They range from tiny, single-celled organisms invisible to the naked eye to some of the largest living multicellular organisms. Some experts estimate that they are 1.5 million fungus species, of which approximately 100,000 have been identified.

2.3.1 Morphology

With the exception of one-called species, most fungi, like plant and animals, are eukaryotic multicellular organisms, unlike these groups however, fungi are composed of threadlike tabular filaments called hyphae, connected end to end (Hawksworth, 1983). Hyphae that are partitioned by dividing cross walls are called septate hyphae, and hyphae that are without cross walls are called non septate hyphae. The hyphae grow by elongation at the tips and by branching to form an interwoven mat known as mycelium, a term which is applied to the whole body of any fungus. As the mycelium develops, it may produce large fruiting bodies or other structures that can contain reproductive spores-these are the most visible structures of a fungus, usually growing above the surface of its

habitat in contrast with the mycelium which is hidden beneath the surface of the material it is decomposing. In addition to being filamentous, fungal cells often have multiple nuclei. Most fungi also lack flagella,

2.3.2 Classification

Increasingly, it is becoming evident that mycologist regard the fungi as being distinct from plants, and accordingly classified then within their own kingdom, namely Fungi or Myceteae (Hawksworth, 1983). Two divisions of the fungi are recognized: the Myxomycota (the slime moulds or slime fungi) and the Eumycota (the true fungi). The Eumycota consists of the so-called lower fungi, or moulds and the higher fungi amongst which are included the mushrooms and toadstools. The lower fungi are classified into two phyla: Zygomycota and Chitridiomycota, the higher fungi into Ascomycota and Basidomycota (BoDD, 2002).

Fungi are classified primarily by the type of spores and fruiting bodies produced. A fifth phylum of the Eumycetes division, Deuteromycota, or Fungi imperfecti, is used by some taxonomists for fungi that typically produce only asexual spores (Seidi and Ammirati, 2000).

2.3.3 Reproduction

The wide varieties of fungi demonstrate many reproductive methods. In general, most fungi as part of their life cycle, produces spores that are carried on a club like sporangium (Mykoweb, 2003). When environmental conditions are favourable, the spores germinate and develop into a mycelium that produces fruiting bodies with enormous numbers of sexual spores, which repeat the reproductive cycle (Hynes, 2000).

The sexual phase is begun when haploid hyphae from two different fungal organisms meet and fuse. When this occurs, the cytoplasm from the two cells fuses, but the nuclei remain separate and distinct. The single hyphae produced by fusion typically have two nuclei per cell and it is known as a dikaryon. The dikaryon may live for years and eventually form sexual sporangia in which the nuclei fuse into one, which then undergoes meiosis to form haploid spores, and the cycle, is repeated (Mykoweb, 2003).

Some fungi have lost the capacity for sexual reproduction, and reproduced by asexual spores or by vegetative growth only. These fungi are referred to as Fungi imperfecti (Mykoweb, 2003).Other fungi such as yeast reproduce primarily by asexual fission or fragmentation of their hyphae. Each fragment develops into a new individual organism (Hynes, 2000).

2.3.4 Uses of Fungi

Fungi have been used as food source since the beginning of recorded history. Mushrooms and flavour, texture, and nutritional values to many dishes. Other fungi are used in the manufacture of foods. Yeast, for example is added to fruit juice which ferments to produce wine, they are also used in brewery process and in bakery. Certain moulds are used to ripe cheese. Many fungi also produce biologically active compounds, that are useful in manufacturing. These compounds include alcohols-such as ethanol and glycerol-and plant regulators such as giberellic acid.

Fungi are extremely important in the production of antibiotics; for example, penicillin, griseofulvin, cyclosporine and cephalosposin. Fungi are also increasingly becoming important tools in the cleaning the environment. A number of fungi are used in bioremediation, where they decompose the organic

materials in pollutants and in the process detoxify them. Fungi have also been used successfully to control insects, and other organisms that cause damage and disease to agricultural crops.

2.3.5 Harmful Fungi

Fungi cause about 100,000 diseases of plants. A number of fungi cause diseases in humans and other vertebrates(BoDD, 2002). In general, these fungal infections, or mycoses, develop slowly, recur more frequently than bacterial infections and do not produce a lasting immunity in the body (Mykoweb, 2003).

A mycosis is classified in one of two groups, depending on the part of the body that is infected. A dermatomycosis is an infection of the skin, nails or hair. These infections rarely progress to the internal organs and most respond well to treatments through treatment may take several weeks. A systemic mycosis, which is an infection of the entire body, is typically more serious and can be fatal for individuals whose immune system has been weakened. Only a few drugs are effective in treating systemic infections, but because treatment may last for several months to years to prevent relapse, of infections, these drugs may often cause toxic side effects(Hynes, 2000).

Fungi are ubiquitous to the environment and are primarily saprophytes, production. During the digestion process, fungi secrete enzymes into the which are taken up by fungi and digested. The digested nutrients are classified into two categories, primary and secondary metabolites. The primary metabolites consists by cellulose and other compounds that are used for energy to grow and reproduce. The secondary metabolites includes antibiotics and mycotoxins (Mold-Help, 2003). More than 100 species of fungi produces poisons called

mycotoxins during their growth. The most common toxin is aflatoxin, produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin is the most potent carcinogen yet discovered (Christensen and Tuthill, 1985).

The specificity of the negative impacts of fungi on human health depends on the nature of species, the amount and duration of individuals exposure to the actual fungi, the metabolic or by product s produced by the species, and the specific susceptibility or state of health of the person exposed. The health effects generally fall into seven categories.

1. Type-1 allergy or immediate type hypersensitivity
2. delayed type hypersensitivity reaction
3. infection
4. Mucous membrane and trigeminal nerve irritation
5. Adverse reactions to odour or pseudoallergy
6. Toxicity or neurotoxicity by fungi and mycotoxins
7. Immunotoxicity induced by fungi and mycotoxins.

2.4 MYCOTOXINS

Mycotoxins are non volatile compounds which may be sequestered in spores and vegetative mycelium or secreted into growth substrate (Mykoweb, 2003). They are products of secondary metabolism of fungi (Mold-Help, 2003; Hudler, 1998). They are not essential to maintaining the life of the fungal cell in a primary way, such as obtaining energy or synthesizing structural components, informational molecules or enzymes (Mold-Help, 2003) They are products whose function seems to be to give fungi a competitive advantage over other fungi and bacteria. Mycotoxins are nearly all cytotoxic, disrupting various cellular structure such as membranes, interfering with vital cellular processes such as protein, RNA, and DNA synthesis (Jacobsen *et al.,* 1993). Of course they are also toxic to the cells of higher plants and animals , including humans

potentially (Frazer and Westhoff, 1998; Wannemacher and Wiener, 2000). The toxic effect of mycotoxins on animals and human health is referred to as mycotoxicosis (Peraica *et al.,* 1999; Jacobsen *et al*., 1993; Hawaii, 2001). Not all fungi produce mycotoxins, but numerous species do. Toxigenic fungi vary in their mycotoxin production depending on the substrate on which they grow (Kendrick, 1992). There are over 200 recognized mycotoxin (Mykoweb, 2003). Mycotoxins vary in their specificity and potency for target cells, cell structures or cell processes by species and strain of fungi that produced them (Smith and Moss, 1985). Higher organisms are not specifically targeted by mycotoxins, but seems to be caught in the crossfire of the biochemical warfare among fungal species and fungi and bacteria vying for the same ecological niche. The study of mycotoxins and their health effects on humans are however still in their infancy, and many more are still yet to be discovered.

2.4.1 Formation and Chemistry

Each mycotoxin is formed or produced by one or more very specific fungal species (Lawley, 2000) and their production is influenced by both the genotype of the organism and the physicochemical environment in which it is growing (Smith and Moss, 1985). In some cases, one species can form more than one mycotoxin (Lawley, 2000), and it is also important to appreciate that the production of any particular mycotoxin depends on the strain and not only the species. Thus, although aflatoxins are only known to be produced by *Aspergillus flavus* and *Aspergillus parasiticus*, there are strain of both species which are non aflatoxigenic (Smith and Moss, 1985). Hence, the presence of a recognized toxin-producing fungus does not automatically imply the presence of the associated toxin and conversely, the absence of any visible fungi does not guarantee the freedom from toxins as the fungi may have already died out while leaving the toxin intact (Lawley, 2000) . Even if a strain of fungi has genetic

potential to produce a particular mycotoxin, the level of production would be influenced by the nutrients available. Very little aflatoxin is produced during the phase of vigorous growth and it is only when some nutritional factors runs out that toxin biosynthesis occurs rapidly (Smith and Moss, 1985). Even when the nutritional requirements are suitable for mycotoxin biosynthesis, physical parameters such as temperature and water activity will influence production (Kendrick, 1992).

Although mycotoxins do not form a neat and recognizable group of organic structures, they can be classified in terms of the biosynthetic pathway leading to their formation. Primary and secondary metabolism are also linked by a relatively small number of simple intermediates which are used to classify the mycotoxins.

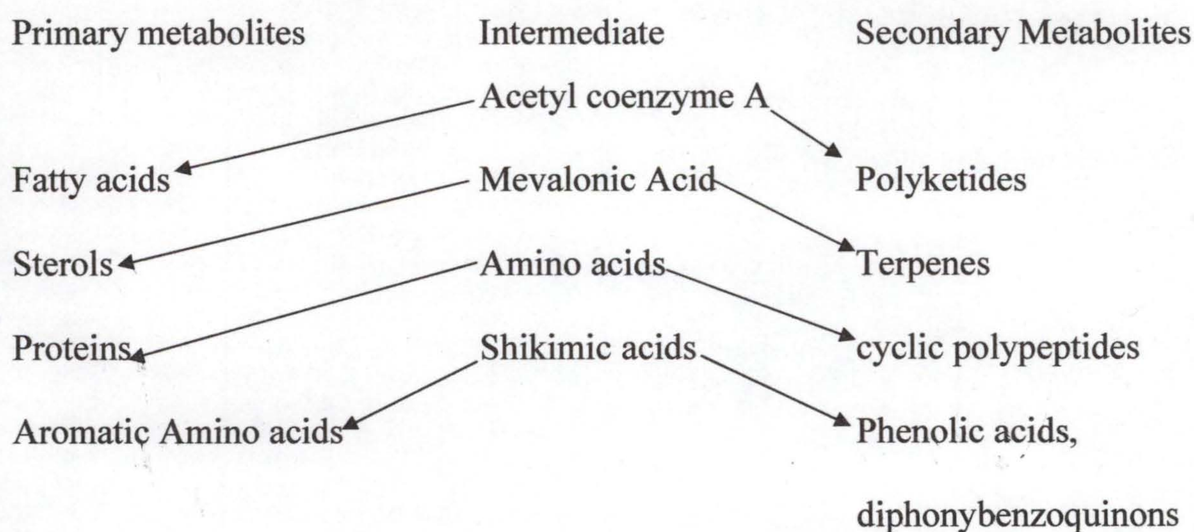

Fig2.4: Intermediates linking primary and secondary metabolism

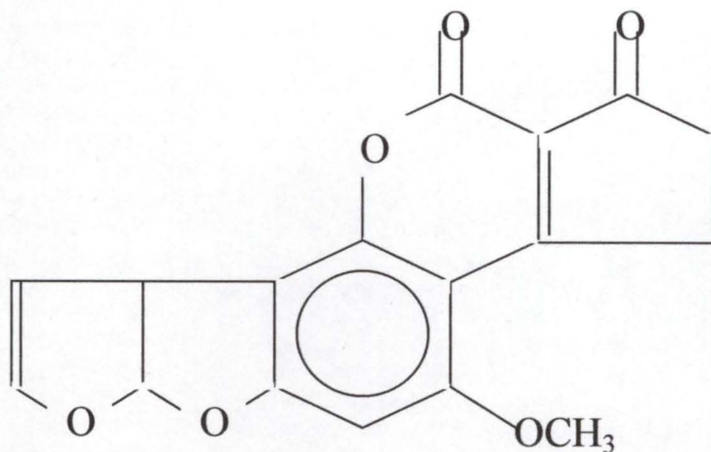

Fig 2.5 Structure of Aflatoxin B_1

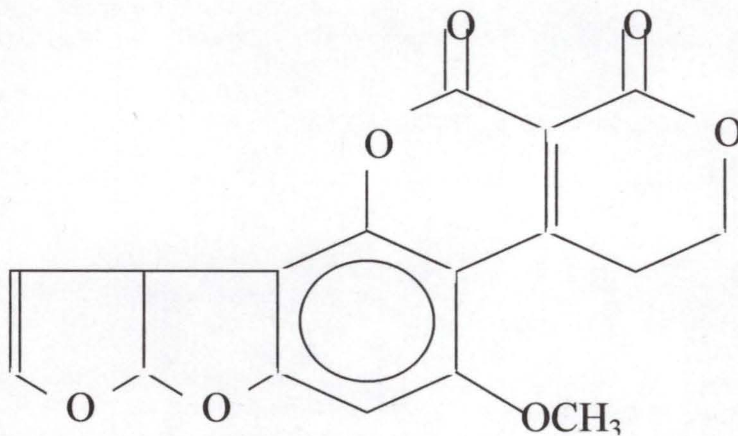

Fig 2.6: Structure of Aflatoxin G_1

It is commonly observed that the more steps there are leading to the formation of mycotoxins, the more limited is the number of species that can produce them (Smith and Moss, 1985).

Most mycotoxins are chemically stable and so they tend to survive storage and processing, even when cooked to very high temperature, such as that reached during baking bread (Smith and Moss, 1985). Also, mycotoxins are relatively small and complex organic compounds.

2.4.2 Adverse Effects of Mycotoxins

The effect of mycotoxins can be broadly categorized into three identifiable forms (Pier et al 1980).:

1. Acute primary mycotoxicosis
2. Chronic primary mycotoxicosis
3. Secondary mycotoxin disease

Acute Primary Mycotoxicoses

These are effect produced when high to moderate concentrations of mycotoxins are consumed causing a specific observable, acute disease syndrome such as hepatitis, heamorrhage, nephritis, necrosis of oral and enteric epithelia or death. A substance can be considered to be toxic to an animal when a single high dose of a series of small dose leads to death. The quantitative expression of toxicity, the LD_{50}, is the dosage expressed per unity body weight necessary to kill 50 percent of a statistically valid group of animals. Levels of toxins below the LD50 value will produce variable degrees of sickness and chronic malfunction of the animal (Wallace, 1982).

Chronic Primary Mycotoxicoses

In these types of mycotoxicoses, there is a relative lack of macroscopically visible changes in the infected animals and thus prevents an easy diagnosis based on symptoms.

Secondary Mycotoxin Diseases

The effect of secondary mycotoxin disease is simply enhancing the infectious process of which the host animals is naturally predisposed.

2.4.2.1 Biochemical Effect of Mycotoxins

The primary effect of mycotoxins on animals will occur at the cell or molecular levels.

Energy Metabolism: Several mycotoxins including aflatoxin B1, G1 andM1, rubratoxin B, patulin and ochratoxin A inhibit oxygen uptake in whole tissue homogenates from several animal species. Electron Transport chain system and Adenosine triphosphatase activity is also inhibited (Smith and Moss, 1985).

Carbohydrate and Lipid metabolism: Inhibition of enzymes of glycogenesis, depression of glucose transport into hepatocytes, accumulation of hepatic lipids and other problems has been observed and associated with Mycotoxicoses.

Nucleic Acid and Protein Synthesis: Some mycotoxins has been reported to have pronounced effects on nucleic acid and protein synthesis. Effects such as disruption of protein synthesis at the level of DNA-dependent RNA polymerase, of DNA transcription and reducing enzyme activity by forming Schiff bases with the functional groups.

2.4.2.2 **Biological Effects of Mycotoxins**

Mycotoxins exhibits a wide array of biological effects because of their diversity of chemical structures. They include:

Carcinogenesis: Several mycotoxins have been shown to cause cancers in a variety of animal species. The aflatoxins were the first mycotoxins to be studies for their carcinogenic effect and are now recognized as hepatocarcinogens.

Mutagenecity: Mycotoxins have been shown to be mutagenic. Aflatoxin B, is the most potent mutagen of the aflatoxins and a strong parallel exists between the ability of the aflatoxins to be mutagenic and carcinogenic.

Teratogenecity: Mycotoxins which are potent inhibitors of protein synthesis in eukaryotes must be expected to impair differentiation in sensitive primordial. Prenatal effects have been documented with experimental animals and this suggests that many mycotoxins are embryotoxic and teratogenic.

Oestrogenism: Zearalenone for example has been shown to lead to enlargement of vulva, uterus and nipples and prolapsing of rectum when some animal species are exposed to them.

Hepatotoxicity: The hepatic tissues of the liver absorb toxic substance from the blood stream and thus remove them from circulation, detoxify them and then excrete them. Many mycotoxins however induce both non-specific liver injury, moderate to extensive necrosis and heamorrhage.

Nephrotoxicity: The kidney is the major organ involved in the excretion or elimination of waste products and foreign substances that are not utilized within the body. Several mycotoxins have been shown to induce variable levels of renal damage, both in field intoxications and experimentally dosed animals.

Neurotoxicity: Several mycotoxins characterized as tremorgen has been isolated from fungi.

Dermal toxicosis: Some mycotoxins when applied to the skin of animals, can cause local irritation, inflammation, desquamation, subepidermal heamorrhaging and general necrosis.

2.4.2.3 Human Mycotoxicoses

The poisonous nature of some toadstools has been appreciated for many centuries. Many of the toxins of these fungi (some of which are non poisonous and are eaten) are well characterized. Such poisonings following the deliberate or unwitting consumption of these larger fungi are referred to as mycetism (Smith and Moss, 1985). However, the study of mycotoxins and their health effects on humans is still in its infancy.

Acute Mycotoxicoses can cause serious and sometimes fatal diseases in humans (Peraica *et al*., 1999). Some specific mycotoxins and their effects are discussed below

Aflatoxins:- Aflatoxins are produced primarily by some strains of *Aspergillus flavus* and by most, if not all, strains of *Aspergillus parasiticus*, plus related

species, *Aspergillus nomious* and *Aspergillus niger* (Cornell, 2002). They are four major aflatoxins: B1,B2,G1and G2. M1 and M2 are oxidative metabolic products of B1and B2 in human (Peraica, 1999; Cornell, 2002). Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds (Peraica, 1999). The main target organ of their carcinogenecity is the liver. Evidence of acute aflatoxicosis has been reported from many parts of the world. The syndrome is characterized by vomiting abdominal pain, pulmonary edema, convulsions, coma, and death (Cornell, 2000). Aflatoxins have also been implicated in sub acute and chronic effects in human, like primary liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis (Smith and Moss, 1985). Moreover, aflatoxins have been suggested as the aetiological factor in encephalopathy and fatty degeneration of viscera, similar to Reye Syndrome which is common in countries with hot and humid climate (Peraica 1999, Lawley 2000b).

<u>Ochratoxins</u>:-Ochratoxins are produced by *Aspergillus ochraceus* mainly in tropical regions and by *Penicillium verrcosum* in temperate regions. Ochratoxin A and B and different in the presence of chlorine atom in the structure of ochratoxin A. In any case, ochratoxin B is loss toxin than A. Other related molecules are ochratoxin C, ochratoxin and ochratoxin B (Lawley, 2000c). IARC classified ochratoxin A as a compound possible carcinogenic to humans (Peraica, 1999). It has also been suggested that ochratoxin A may be the causative agent of both endemic nephropathy and urothelial tumours (Peraica, 1999).

<u>Trichothecenes</u>: A large group of chemicals characterized by a double bond between C9 and C10 and an epoxy ring at the C12, C13 position in the chemical structure. These biologically active mycotoxins are wrongly referred to

as *Fusarium* toxins as several other fungal genera including *Trichoderma, stachybotrys, Myrothecium Verticuimonosporium, Cephalosporium and Trichothecium* can also produce them (Lawley 2000d). Over 148 trichothecenes have been isolated (Peraica, 1999). They are often classified as Group A (e.g. T-2 toxin, HT-2toxin, neosalinial, and diacetoscirpenol) and Group B (e.g. deoxynivalenol, also known as vomitoxin, nivalenol and fusarenon x) depending on whether they have a side chain on C7. Another group known as thermacrocyclic trichothecenes includes satratoxins, and verrucarins (Lawley, 2000d). The first recognized trichothecene mycotoxicosis was alimentary toxic aleukia in the USSR in 1932 (Nirenberg and O'Donnell, 1990; Peraica *et al* 1999), which begins with burning sensations of the mouth, throat, oesophagus and stomach, continues with vomiting, diarrhea and gastric cramps, and finally progresses to severe leukopenia and death.

Trichothecenes are considered primarily to be blister agents, which at lower concentration can produce considerable incapacitation and death within minutes to hours (Wannemacher and Wiener, 2000). Acute oral, parenteral, dermal or aerosol exposure to trichothecenes produce gastric and intestinal lesions. Heamatopoietic and immunosuppressive effects are radiomimetic, central nervous system toxicity causes anorexia, lassitude and nausea; suppression of reproductive organ function, and acute vascular effects leading to hypotension and shock (Wannemacher and Wiener 2000; Peraica, 1999) chronic exposure to sub acute doses are responsible for alimentary toxic aleukia. Because of their anti-personnel properties, ease of large scale production and apparent proven delivery by various aerial dispersal systems, trichothecenes particularly T-2 toxins have excellent potential for weaponization. Evidence also support the contention that trichothecenes were used as biological warfare agents in South

East Asia, and Afghanistan by the former soviet Union and its surrogates (Wannemacher and Wiener, 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 COLLECTION OF SAMPLES

Two sealed packs each of thirty different commercial brands of cigarettes and four packs of the thirty second brand, were acquired from Kasuwan Gwari market in Minna, Niger State of Nigeria. The cigarette brands collected were:

Brand Name	Label	Origin
St. Moritz	BF1	Foreign
Dunhill	AF2	"
Regal	AF3	"
Dorchester	AF4	"
Dorchester Menthol	BF4	"
London	AF5	"
London Menthol	BF5	"
Rothmans	AF6	"
Rothmans consulate	BF6	"
Marlboro	AF7	"
Benson and Hedges	AF8	"
Benson and Hedges	AL8	Local
Benson and Hedges Menthol	BL8	Local
Super Kings	AF9	Foreign
Business Club	BF10	"
Standard	AF11	"
Harbour	AF12	"
Bond	AF13	"
Gold Leaf	AL14	Local
Aspen	AF15	Foreign
Aspen Menthol	BF15	"

Keranis Millennium	AF16	"
Forum cool Menthol	ABF17	"
High Society	AL18	Local
Excel	AL19	"
Yes	AF20	Foreign
Target	AL21	Local
Ashton	AF22	"
Ace	AL23	Local
SM	ABL24	"
Mars	AL25	"
Three Rings	AL26	"
Golden Eagle	AF27	Foreign

3.2 ISOLATION OF FUNGI FROM SAMPLES

3.2.1 Preparation of Potatoes Dextrose Agar

3.2.1.1 Preparation of PDA Plates

Five hundred grams of thinly sliced peeled white potatoes was heated with distilled water at about 60°C for 1 hour and then filtered through muslin cloth. Ohaus Brain weigh B3ooD was used to weigh 0.5g of chloramphenicol, 20g of glucose and 15g of agar agar were added to the potato broth and was made up to 1000ml with distilled water. The mixture was shaken to homogeneity and then autoclaved to sterilization in a Prestige medical clinical autoclave. Cooled PDA broth (20ml) was pipetted into sterile Petri dishes using sterile pipette in a previously fumigated inoculating hood. It was then allowed to cool and gel.

3.2.1.2 Preparation of PDA Slants

Preparation of PDA slants, was done as described in section 3.2.1.1 except was that kimax glass tubes were used instead of petri dishes

3.2.2 Inoculation of Samples

Three cigarette sticks of each sample were emptied into a sterile porcelain dish in a previously fumigated inoculation hood. A forcep was used to mix the tobacco leaves together and then inoculated to an appropriately labeled PDA plates. Triplicate plates were inoculated for each sample and later transferred to another previously fumigated inoculation hood and then left at room temperature. Three plates of uninnoculated PDA plates were kept as control. The plates were then observed after 48 hours for fungal growth.

3.2.3 Isolation of Pure Cultures

Some plates had multiple fungal colonies growing on them. Such plates colonies needed to be separated unto different plates. A sterile inoculating loop was used to touch the PDA close to the colony to be separated to make it sticky. The sticky end was used to touch the colony to be separated such that some spores or mycelia stick on to the loop. This was then carefully transferred to an appropriately labeled freshly prepared PDA plate. This was repeated until all the different colonies have been transferred into separate plates. The newly inoculated plates were then transferred to another previously fumigated inoculation hood together with three uninnoculated plates which served as control. The plates were then observed for fungal growth after 24 hours. The plates that still had mixed colonies growing on it were further separated until pure culture

were obtained. By the same method, the pure culture obtained were transferred into PDA slants for storage and further investigation.

3.2.4 Identification of Pure Isolates

Identification of the pure isolates were carried out in the microbiology laboratory of the Biological Sciences Department of Federal University of Technology, Minna. Niger State of Nigeria. The results were further compared to those obtainable from the Adelaide University, Australia, Mycology online resources.

3.2.5 Culturing of Isolate on Rice Medium

Distilled water (100ml) was added to 250g of rice in 1000ml conical flask and then allowed to stand for 24 hours at room temperature for moisture equilibration. It was then autoclaved to sterility using a Prestige medical clinical autoclave . The pure isolates were inoculated onto appropriately labeled sterile rice medium in a previously fumigated inoculation hood. The inoculated rice medium were then maintained in a Grieve laboratory oven LW 201C at about 35°C for 21 days.

3.3 EXTRACTION OF FUNGAL CULTURED RICE MEDIUM

After 21 days of maintaining the fungal culture on rice medium at 35°C, 750ml of methylene chloride was added to the conical flask containing the rice culture and allowed to stand for 1 hour. The culture was then pulverized using a Christison commercial blender. The pulverized rice culture was then poured into an Erlenmeyer flask fitted to another Erlenmeyer flask and then Speedevac 2 suction pump through a Buchner funnel fitted with Whatman paper No 4. The filtrate collected was then taken to the Soxhlet apparatus for evaporation of the methylene chloride.

After evaporation, the residue was mixed in with petroleum ether in ratio 1:15ml and then put into the refrigerator. After 24 hours, the mixture was filtered through Whatman no? filter paper. The petroleum ether passed through as filtrate and was discarded. The filter paper containing the precipitate was taken into a Grieve laboratory oven LW 201C and maintained at 40°C to dryness.

3.4 TOXICITY SCREENING OF CRUDE EXTRACT

Lethality test of the crude extracts were carried out on healthy male mice with average body weight of 300g. the principle employed was to administer the test limit for acute oral toxicity test generally considered to be 5.0g/kg body weight, (Wallace, 1982), a dose level at which if no mortality is observed, a higher level would not be necessary and the test substance can be said not to be acutely toxic. Due to the ease of intra peritoneal administration, a pilot study was carried out to compare the result between mortality observed from oral administration and those observed from intraperitoneal administration. More so that some researches have shown similarity in mortality between oral and intraperitoneal administration of trichothecene to mice rats and guinea pig (refer to Appendix A)

3.4.1.1 Pilot Toxicity Test by Oral Route

Two and a half milligram of crude extract was suspended in 10ml of "Mazola" corn oil manufactured by Unilever for Best Foods UK Ltd. The animals to be used (weighing 300g on the average) were appropriately labeled by marking them using a pigment ink marker. The animals were fasted for about three hours prior to dosing. 3ml of test corn oil extract containing 0.75mg of crude extract was administered to the animals each by gavage. Four hours later another 3ml of test corn oil extract was

administered to the animals by gavage. This was repeated for thirty (30) different test substance. Three animals were also administered with corn oil without the test substance to serve as control. Three animals which served as control, were also administered 6ml of corn oil without any of the extracts by gavage. This was administered twice over four hours.

3.4.1.2 Toxicity Tests by Intraperitoneal Route (IP)

Two and a half milligram of crude extract was suspended in 10ml of "Mazola" corn oil manufactured by Unilever for Best Foods UK Ltd. The animals to be used (weighing 300g on the average) were appropriately labeled by marking them using a pigment ink marker. Corn oil (3ml) containing 0.75mg of crude extract was administered to three different test animals by IP injection using 5ml syringe and needle. Four hours later, 3ml of test substance was administered to the test animals again. This was repeated for thirty (30) different test substances. Three animals which served as control, were also administered 6ml of corn oil without any of the extracts IP. This was administered twice over four hours interval.

3.4.2.3 Screening of Extracts for Lethal effects

Two and a half milligram of crude extract was suspended in 10ml of "Mazola" corn oil manufactured by Unilever for Best Foods UK Ltd. The animals to be used (weighing 300g on the average) were appropriately labeled by marking them using a pigment ink marker. Intraperitoneal route of administration was employed. 3ml of corn oil extract administered IP twice at four hours interval. Three test animals were administered with same volume of corn oil alone as control. The animals were then observed for 14 days for clinical symptoms and mortality.

3.5 SCREENING FOR AFLATOXINS IN CIGARETTE TOBACCO

3.5.1 Preparation of Samples

Ohaus Brain weigh B3ooD was used to weigh 50g tobacco of each cigarette sample into a 500ml conical flask. Exactly 25ml of 0.1M H_3PO_4 was added to the conical flask, to which 250ml of methylene chloride was also added. This was shaken for thirty minutes on Gallenkamp flask shaker and filtered through cotton wool. Approximately 50ml of the filtrate was collected to a 100ml conical flask.

3.5.2 Purification and Defatting

A column was set up with a small plug of glass wool tamped at the tapered end of the column. About 150ml of methylene chloride was poured into the column and then emptied halfway. One scoop of anhydrous sodium sulphate was added to the column and methylene chloride was used to wash the sides. Chromatographic silica gel type G-60 was mixed with methylene chloride in a beaker and poured into the column and allowed to settle. Three additional scoops of anhydrous sodium sulphate was added to the column and methylene chloride was used to wash the sides; the methylene chloride was drained off to the top of the column. 50ml of the filtrate sample was added to the column and drained off down to top of column into a beaker. About 130ml of hexane was added to the column and drained to the top of the column into the beaker. 130ml of petroleum ether was added to the column and drained of to the top of the column into the beaker. The beaker was changed for a new beaker. 130ml of ether:methanol:water (96:3:1) was added to column and eluted at maximum flow and collected off column into a new beaker. A few boiling chips were added to the eluate and then evaporated nearly to dryness on steam bath. This was then put into vials and stored in the refrigerator.

3.5.3 Screening for Aflatoxins

The vials containing the extract residue was uncapped and 200µL of benzene:acetonitrile (98:2) was added to it and then recapped. The content was shaken vigorously. As rapidly as possible, Micropipette was used to spot 2, 5, and 10µL of the extract residue on imaginary line 4cm from bottom edge of TLC plate. On the same plate, 2, 5, and 10µL of aflatoxin standard was spotted using Human micropipette. The plate was then developed in a chromatographic tank using diethyl ether:methanol:water (96+3+1) as the developing solvent. The plate was then removed from the tank after the solvent front has reached 10cm and solvent evaporated at room temperature to dryness. The plate was then illuminated from above on a long wave UV lamp in a darkened room for characteristic blue or green fluorescence, which confirms the presence of aflatoxins.

CHAPTER FOUR

4.0 RESULTS

4.1 ISOLATION OF FUNGI

Thirty two (32) different brands of cigarette were analyzed. One of them, Benson and Hedges was of two types; one locally produced and the other imported. The two were analyzed separately, thus making a total of 33 different samples that were analyzed. Fungi were isolated from all of them except Benson and Hedges (locally produced), Benson and Hedges menthol and Golden Eagle. Some of the fungal isolates are known toxigenic moulds. The tests were carried out under good laboratory practices because there was no growth on the control media. (Table 4.1)

London menthol had the lowest incidence of fungal contamination with only one fungal colony growing on only one of the three plates. The fungus isolated from this sample, *T. tonsurans* also was very rare as it did not grow on any other sample tested. Two other samples, Dunhill and London also had low incidence of fungal contamination. Only two isolates were obtained from them. The highest incidence of fungal contamination, was found in Ashton and Mars (seven isolates each). However, a distinction was found in Marlboro, which had a slightly lower incidence of six isolates, that were all of different species. (Table 4.2)

There were a total of seventeen (17) different species of isolates obtained with a highest incidence of seventeen each occurring for *Syncephalastrum spp* and *Aspergillus flavus*. The lowest incidence of one isolate each occurred for *Trichophyton tonsurans* and *Trichophyton verrcosum (Table 4.3)*. Two of the isolates, *Microsporum spp* and *Rhodotorula rubra* were found growing together alone on a plate in two different samples , St. Moritz and Marlboro. The total number of isolates obtained from all the samples was one hundred

and fifteen(115). London had another distinction although there were two isolates obtained from the culture, they were of the same type. (Fig 4.2)

Table 4.1: Type and occurrence of Fungi isolates on cigarette tobacco culture

Samples	Plate I			Plate II			Plate III		
	Isolate I	Isolate II	Isolate III	Isolate I	Isolate II	Isolate III	Isolate I	Isolate II	Isolate
St. Moritz	*Microsporum Spp*	*Rhodotorula rubra*	-	*A. fumigatus*	-	-	-	-	-
Dunhill	*Syncephalastrum spp*	-	-	*Penicillium Spp*	-	-	-	-	-
Regal	-	-	-	-	-	-	*A. fumigatus*	*Cladosporium*	-
Dorchester	*Cladosporum spp*	-	-	*A. fumigatus*	*Syncephalastrum spp*	-	-	-	-
Dorchester*	*Paecilomyces spp*	*Penicillium spp*	-	*Exophiala spp*	-	-	*Syncephalastrum spp*	-	-
London	*Cladosporum spp*	-	-	*Cladosporium spp*	-	-	-	-	-
London*	-	-	-	*T. tonsurans*	-	-	-	-	-
Rothmans	*Microsporum Spp*	-	-	*Syncephalastrum spp*	-	-	*A. fumigatus*	*Syncephalastrum spp*	-
Consulate	*Syncephalastrum spp*	-	-	*Sependonium spp*	-	-	-	-	-
Marlboro	*Fusarium spp*	*A. niger*	-	*A. flavus*	*Exophiala spp*	-	*Microsporum spp*	*Rhodotorula rubra*	-
B&H (import)	*Penicillium spp*	-	-	*Syncephalastrum spp*	*A. flavus*	-	*A. niger*	-	-
B&H (local)	-	-	-	-	-	-	-	-	-
B&H*	-	-	-	-	-	-	-	-	-
SuperKing	*T. schoenleinii*	-	-	*A. flavus*	*Syncephalastrum spp*	-	-	-	-
Biz club	*T. verrucosum*	-	-	-	-	-	*Syncephalastrum spp*	*Sependoniun spp*	-
Standard	*Fusarium spp*	*A. niger*	*Syncephalastrum spp*	*Fusarium spp*	-	-	*Fusarium spp*	-	-
Harbour	-	-	-	*Syncephalastrum*	-	-	*A. niger*	*Penicillium spp*	-

* Menthol

Table 4.2: Number and Type of Isolates isolated from samples

Samples	Total No. Of Isolates	No. of Types of Isolates
St. Moritz	3	3
Dunhill	2	2
Regal	2	2
Dorchester	3	3
Dorchester Menthol	4	4
London	2	1
London Menthol	1	1
Rothmans	4	3
Consulate	2	2
Marlboro	6	6
B&H (import)	4	4
B&H (local)	0	0
B&H Menthol	0	0
SuperKing	3	3
Business club	3	3
Standard	5	3
Harbour	3	3
Bond	4	3
Gold Leaf	4	3
Aspen	4	3
Aspen menthol	4	4
Keranis millennium	5	5
Forum Cool Menthol	3	3
High Society	4	2
Excel	5	3
Yes	4	3
Target Super	4	3
Ashton	7	5
Ace	3	3
SM	4	3
Mars	7	5
Three Rings	6	4
Golden Eagle	0	0

Table 4.1: Continues

Samples	Plate I Isolate I	Isolate II	Isolate III	Plate II Isolate I	Isolate II	Isolate III	Plate III Isolate I	Isolate II	Isolate
Bond	*Fusarium spp*	*Rhizopus spp*	-	*Syncephalastrum spp*	-	-	*Fusarium spp*	-	-
Gold Leaf	*A. flavus*	*A. niger*	-	*A. flavus*	-	-	*Rhodotorula rubra*	-	-
Aspen	*A. niger*	-	-	*A. niger*	*A. fumigatus*	-	*Sependonium spp*	-	-
Aspen*	*A. flavus*	*Penicillium spp*	-	*Cladosporum spp*	-	-	*A. niger*	-	-
Keranis	*A. fumigatus*	*Fusarium spp*	*T. Schoenleinii*	*Exophiala spp*	-	-	*Mucor spp*	-	-
Forum	*Sependonunium spp*	-	-	*Penicillium spp*	*Fusarium spp*	-	-	-	-
High Society	*A. flavus*	-	-	*Penicillium spp*	*Fusarium spp*	-	-	-	-
Excel	*A. fumigatus*	-	-	*A. fumigatus*	*Fusarium spp*	*Paecilomyces spp*	*A. flavus*	*Fusarium spp*	-
Yes	*A. flavus*	*T. schoenleinii*	-	-	-	-	*A. flavus*	*Fusarium spp*	-
Ashton	*A. flavus*	*Syncephalastrum spp*	*Rhizopus spp*	*A. flavus*	*Fusarium spp*	*Syncephalastrum spp*	*Penicillium spp*	-	-
Ace	*Paecilomyces spp*	-	-	*Cladosporum spp*	-	-	*A. fumigatus*	-	-
SM	*Syncephalastrum spp*	*Fusarium spp*	-	*Mucor spp*	-	-	*Mucor spp*	-	-
Mars	*T. schoenleinii*	*A. niger*	-	*Cladosporum spp*	*Penicillium spp*	*Syncephalastrum spp*	*Cladosporum spp*	*Penicillium spp*	-
Three Rings	*A. flavus*	*Cladosporum spp*	*Syncephalastrum spp*	*A. flavus*	*Cladosporum spp*-		*A. flavus*	-	-
Golden Eagle	-	-	-	-	-	-	-	-	-
Target Super	*T. schoenlenii*	-	-	*Paecilomyces spp*	*A. flavus*	-	*T. schoenlenii*	-	-

* Menthol

Table 4.3 Incidences of Fungi Isolated from 33 Samples of Cigarette

Fungi	Incidence
Aspergillus flavus	17
Aspergillus fumigatus	11
Aspergillus niger	9
Cladosporium spp	10
Exophiala spp	3
Fusarium spp	12
Microsporum spp	3
Mucor spp	3
Paecilomyces spp	4
Penicillium spp	9
Rhizopus spp	2
Rhodotorula rubra	3
Sependonium spp	4
Syncephalastrum spp	17
Trichophyton schoenleinii	6
Trichophyton tonsurans	1
Trichophyton verrucosum	1

4.2 ELABORATION OF SECONDARY METABOLITES

Fifty nine of the isolates representing 51% of the total number of isolates elaborated secondary metabolites on culturing on rice media. Nine out of the total 17 species of fungi were found to be toxigenic. All the isolates of *A. flavus*, *Rhizopus spp* and *Fusarium spp* elaborated secondary metabolites, of the *Cladosporium spp* however, it was only one out of the ten isolates that elaborated secondary metabolites. Table 4.4

With the exception of *A. flavus*, *A. fumigatus* and *Fusarium spp*, all the isolates that grew as single colonies on the initial plate were found not to elaborate secondary metabolites. Also, it was only the isolates of *Penicillium spp*, *A. niger* and *Paecilomyces* that was in a mixed culture before they were separated into pure cultures that elaborated secondary metabolites on rice media.

Table 4.4: Incidence of Toxigenic Fungi from 33 sample of cigarette

Fungi	Incidence
Aspergillus flavus	17/17*
Aspergillus fumigatus	10/11*
Aspergillus niger	6/9*
Cladosporium spp	1/10*
Fusarium spp	12/12*
Penicillium spp	6/9*
Paecilomyces spp	3/4*
Rhizopus spp	2/2*
Trichophyton tonsurans	2/6*

*total number of isolates from the 33 cigarette samples

4.3 LETHALITY OF CRUDE EXTRACTS OF FUNGI ISOLATES

The extracts from all the isolates of *Fusarium spp* showed 100% mortality. This was followed by extracts from *A. flavus*, 74.5% and *Penicillium spp*, 72.2%. There was only one isolate of *Cladosporum spp* that was toxigenic but the secondary metabolite produced was not acutely toxic or lethal as it could not induce mortality on any of the test animals. At a dose of Secondary metabolites elaborated by the isolates of *A. niger A. fumigatus, Rhizopus spp* and *T. tonsurans* also induced low mortality in the test animal. (Table 4.5, 4.14)

It was observed that all the isolates of *A. fumigatus* that grew as mixed colonies with either *Fusarium spp* or *A. flavus* on the initial culture exhibited 100% lethality.

The pilot test showed that the results obtained from oral toxicity testing and IP test compared favourably. There were only three instances out of ten in which IP administration induced slightly higher mortality than those administered PO. However, the difference was not of statistical significance (Appendix B, C and D)

Table 4.5: Comparison of IP and PO Screening of Extracts

		Mortality	
Isolate	No of Test Animals	Oral Route	Intraperitoneal Route
i	3*	3	3
ii	3*	2	3
iii	3*	3	3
iv	3*	0	0
v	3*	0	0
vi	3*	1	2
vii	3*	3	3
viii	3*	3	3
ix	3*	1	2
x	3*	2	2
Control	3*	0	0

* per test route

4.6: Lethality of extracts of isolates of *A. flavus* on mice

Isolate	No of Test Animals	Mortality
i	3	3
ii	3	3
iii	3	3
iv	3	2
v	3	1
vi	3	1
vii	3	1
viii	3	1
ix	3	2
x	3	2
xi	3	2
xii	3	2
xiii	3	3
xiv	3	3
xv	3	3
xvi	3	3
xvii	3	3

Table 4.7: Lethality of extracts of isolates of *A. fumigatus* on mice

Isolate	No of Test Animals	Mortality
i	3	3
ii	3	3
iii	3	3
iv	3	0
v	3	1
vi	3	0
vii	3	0
viii	3	0
ix	3	0
x	3	1

Table 4.8: Lethality of extracts of isolates of *A. niger* on mice

Isolate	No of Test Animals	Mortality
i	3	1
ii	3	2
iii	3	0
iv	3	1
v	3	0
vi	3	1

Table 4.9: Lethality of extracts of isolates of *Cladosporum spp* on mice

Isolate	No of Test Animals	Mortality
i	3	0

Table 4.10: Lethality of extracts of isolates of *Fusarium spp* on mice

Isolate	No of Test Animals	Mortality
i	3	3
ii	3	3
iii	3	3
iv	3	3
v	3	3
vi	3	3
vii	3	3
viii	3	3
ix	3	3
x	3	3
Xi	3	3
xii	3	3

Table 4.11: Lethality of extracts of isolates of *Paecilomyces spp* on mice

Isolates	No of Test Animals	Mortality
i	3	1
ii	3	2
iii	3	2

Table 4.12: Lethality of extracts of isolates of *Penicillium spp* on mice

Isolates	No of Test Animals	Mortality
i	3	3
ii	3	2
iii	3	0
iv	3	3
v	3	3
vi	3	2

Table 4.13: Lethality of extracts of isolates of *Rhizopus spp* on mice

Isolates	No of Test Animals	Mortality
i	3	0
ii	3	1

Table 4.14: Lethality of extracts of isolates of *T. schoenlenii* on mice

Isolates	No of Test Animals	Mortality
i	3	1
ii	3	1

4.4 DIRECT SCREENING FOR AFLATOXINS

On direct screening for aflatoxin in the cigarette tobacco, six of the cigarettes samples; Marlboro, Excel, Yes, Ashton, Three Rings and Mars, were found to contain aflatoxin contamination. Of all of them that had aflatoxin contamination it is only Mars that did not yield colonies of *A. flavus* on PDA culture. The other cigarette samples like Benson & Hedges, SuperKing, Gold Leaf, Aspen menthol, and High Society which produced colonies of A. flavus did not have aflatoxin contamination (Table 4.15)

Table 4.15: Aflatoxin contamination of Cigarette Samples

Cigarettes that yielded *A. flavus* on culture and had aflatoxin contamination	Marlboro, Excel, Yes, Ashton, Three Rings
Cigarettes that did not yield *A. flavus* on culture but had aflatoxin contamination	Mars
Cigarettes that yielded *A. flavus* on culture but had no aflatoxin contamination	High Society, SuperKing, Gold Leaf, Aspen menthol,

CHAPTER FIVE

5.0 DISCUSSION

The result showed that cigarette tobacco contains fungi contaminants, that could endanger human lives. Known toxigenic fungi like *A. flavus, Fusarium, A. niger, Penicillium and A. fumigatus* (Mold-Help 2001) were isolated from 26 of the 32 commercial brands analyzed. Other fungi like *Paecilomyces spp, Rhizopus spp, Trichophyton schoenleinii, Mucor spp* and *Cladosporium spp* whose toxigenic status has not been clearly established were also isolated from 16 of the 32 commercial brands of cigarette analyzed. *Syncephalastrum spp, Sependonium spp, Exophiala spp, Trichophyton tonsurans, Trichophyton verricosum, Microsporum spp* and *Rhodotorula rubra* were also isolated from the tobacco culture. Aspergillus spp were the most common (37 isolates) and prevalent (found in 21 of the 32 commercial brands) isolates. Syncephalastrum, 17 isolates with a prevalence of 14 was a distant second. Of the Aspergillus spp, *A. flavus* was the most common isolate.

A team of Dutch researchers that analyzed 98 cigarettes from 14 commercial brands has also obtained a similar result as reported on line by New Scientist 2002. It was reported that cigarette tobacco is a source of fungal spore; as all the brands analyzed contained some fungal contamination. However, *A fumigatus* was the most common fungal isolate reported. Although the fungal isolates obtained could cause mycoses in humans like Aspergillosis, sp, penicillosis and peacilomycosis, the presence of the fungal spore in the cigarette cannot predispose the smoker to cases of mycoses as such spores would have been destroyed in the smoking process. The Dutch researchers also reported no trace of fungi in the fume of cigarette, containing fungal contaminants, smoked down by a smoking machine (New Scientist 2002). The real danger and threat to the health of smokers is posed by those fungal spores that are able to elaborate mycotoxins, and quite a number of

the isolates did. In fact, more than half of the fungal isolates elaborated mycotoxin on natural media. A lot of factors like temperature, water activity and nutrient of the media influences the elaboration of mycotoxins by fungi, and thus even some of the isolates that did not exhibit toxigenecity could have done so under a different condition. So the threat of mycotoxin induced ailments in smokers is real. This is also because of the fact that mycotoxins are heat stable (Smith and Moss, 1985) and could be inhaled by the smoker even after the cigarette tobacco and the fungal spores that produced them are combusted.

Fungi of the genus *Aspergillus* are very ubiquitous in nature (DoctorFungus 2003). The ones isolated are not mere laboratory contaminants however because non of the control produced any fungal colony. *Aspergillus* includes over 185 species (Asan and Ekmekci 1994), and about 20 of which have been described to be pathogenic to humans (BoDD 2002). Together with *Penicillium spp* and *Fusarium spp*, they are considered to be the most toxigenic mould (Smith and Moss 1985). This genus is also of particular importance because it contains many species that are capable of growth and metabolism at low water activities (as found in cigarette) and are thus associated will spoilage of food materials which are too dry to be attacked by other micro-organisms (Smith and Moss 1998). The genus is metabolically very versatile and includes several species that produces metabolites which may or may not be toxic to humans and animals (Mold-Help 2003). Some of the important toxigenic species of Aspergillus and their mycotoxins are: *A. flavus* – aflatoxins and aflatrem; *A. fumigatus* – viriditoxin, fumagilin, gliotoxin and verrucalogen; *A. ochraceus* – ochratoxin, penicillic acids. *A. versicolor* –sterigmatocystin and cyclopiazonic acid.

Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic, nephrotoxic, hepatotoxic and carcinogenic (Peraica *et al*., 2003) Some of the other mycotoxins are known to cause lung and liver tumour in laboratory animals. The

analyses showed that mycotoxins obtained from *A. flavus* was very lethal to mice and those from *A. fumigatus* and *A. niger* induced low lethality in test animals. This only shows that the toxins produced by *A. flavus* were more acutely toxic to mice. However the real danger lies in the chronic exposure of smokers to these mycotoxins. Moreso, aflatoxin has been reported to cause mutations in p53 tumour suppressor gene at codon249 in liver cells, the same site often mutated in lung cancer, which incidence is very high among smokers.

The result also showed that out of the seventeen cigarette samples from which *A. flavus* was isolated, only five of them contained aflatoxin contaminants. This means that it is only in these five samples that the fungus had elaborated mycotoxin. This number may be small compared to the total number of seventeen, but it proves that aflatoxin can be produced on cigarette tobacco, Thus, the presence of the fungus producing it should be alarming. It may just be that in the other twelve samples, the spore had not been on the cigarette long enough to be able to elaborate mycotoxins and if left on the shelf for a longer period may start elaborating the toxins. This is very likely since cigarettes do not have expiry dates. It was also discovered that one cigarette sample that did not yield *A. flavus* on culture had aflatoxin contamination. It may either be that the *A. flavus* spore that elaborated the mycotoxin on the tobacco leaf had been destroyed during the manufacturing process of the cigarette and the aflatoxin it produced was not destroyed, or it could be that the aflatoxin contamination had been produced by *A. niger* which was cultured from the sample. Aflatoxin can also be produced by *A. niger* (Cornell 2002). The fact that aflatoxin contaminant was found in cigarette tobacco should be a serious cause of concern bearing in mind the carcinogenicity of this mycotoxin.

Fusarium has over 142 species, varieties and forms (BoDD, 2002). The species of *Fusarium* cover a spectrum of activities from those which are fairly specific plant

pathogens to those which are saprophytic or even causing biodegradation of industrial products (Thomas, 1934). *Fusarium spp* produces a lot of mycotoxins (sequiterpene metabolites) called trichothecenes, as well as zearalenone, fusarin moniliformin, and butenolide (Smith and Moss, 1985). The analysis showed that the mycotoxins elaborated by the *Fusarium* were very highly toxic as they induced 100% mortality in the test animals. Of great concern however, is the implication of chronic exposure to low levels of concentration by smokers, as some of these mycotoxins have been associated with carcinogenesis, particularly oesophageal cancer (Doyle, 1999). The high lethality of these mycotoxins is corroborated by a report by EMAN (2000) which shows that trichothecenes from Fusarium are acutely toxic at low concentrations when given orally or by intraperitoneal injection to mice. The report said that dosed animals become listless or inactive and develop diarrhoea and rectal heamorrhaging. Also necrotic lesions may develop in the mouth parts, the mucosal epithelium of the stomach and small intestine erodes. These may be accompanied by heamorrhage, which may develop into gastroenteritis followed by death.

Mycotoxins elaborated by isolates of *Penicillium spp* also pose a potential health hazard to smokers at it showed high acute toxicity on mice, the potential of chronic toxic effects not withstanding. The more important toxigenic species of *Penicillium* and their mycotoxins are:*P. citrinum* - citrinin; *P. expansum* – patulin and citrinin; *P. islandicum* –luteoskyrin, islanditoxin and cyclochlorotine; *P. purpurogenum* – rubratoxin; *P. roquefortii* - roquefortine; and *P. viridcatum* – ochratoxins, citrinin, viridicatin, xanthomegnin and viomellein

Ochratoxin is nephrotoxic and hepatotoxin with evidence that it impairs the immune system. It is also a suspected carcinogen. Citrinin causes renal damage and patulin is believed to cause heamorrhaging in the brains and the lungs (Mold-Help, 2003). Other mycotoxins cause varying forms of adverse effects on humans. Thus,

the presence of this fungi in cigarette tobacco may pose potential health risk to smokers.

Another fungus that showed toxigenecity in this work is *Paecilomyces spp*. This genus contains several species, but the most common are *Paecilomyces lilacinus* and *Paecilomyces variotii* (DoctorFungus, 2003). They are not classified as important toxigenic moulds but some of them are capable of producing metabolites of varying toxicity including bysochlamic acid, byssotoxin A, viriotin and patulin (Smith and Moss, 1985). Some of them are nephrotoxic (Mold-Help, 2001). This may explain why the metabolites by the isolates in analyzes were moderately acutely toxic to mice. The chronic effects over a long term exposure to these toxins are not clearly understood, but the potential chronic health risk to smokers, should not be ignored.

Some species of *Mucor, Absidia* and *Rhizopus,* all of the Mucoraceae family, have been implicated in food spoilage as plant pathogen. Although there are occasional reports of ability to produce toxic metabolites, there is no strong evidence to implicate this group of fungi in mycotoxicosis (Smith and Moss, 1985). However, the two isolates of Rhizopus in the analyzes were found to be toxigenic while the three isolates of Mucor did not elaborate any metabolite. Nonetheless, the metabolites of Rhizopus were found to be of very low toxicity. Their implications in human health problem is not clearly understood but that they are produced at all, could be source of adverse health effects to smokers on chronic exposure to them.

Cladosporum spp and *Trichophyton schoenleinii* also had some isolates that elaborated metabolites. Again, the metabolites were found to be of very low acute toxicity. The effect of long term exposure to these toxins are not clearly understood and so it could be a potential health hazard to smokers.

Isolates of *Syncephalastrum spp*, which was the next most occurring species after *Aspergillus spp*; *Sependonium spp*, *Rhodotorula rubra*, *Trichophyton verrucosum*, *Trichophyton tonsurans*, *Microsporum spp* and *Exophiala spp* did not elaborate any metabolite on culture in natural media. These fungi are not known to produce mycotoxins. However, it could be that the species isolated do not produce any form of secondary metabolites or that the experimental conditions were not conducive for their production.

Another interesting feature of the results is the possible synergistic effect of the presence of multiple fungal spore in a cigarette tobacco on mycotoxin production. Apart from isolates of *A. flavus*, Fusarium spp and *A. fumigatus* which produced mycotoxin when isolated as single colonies of contaminant on a cigarette tobacco, all other fungal isolates which grew as single colonies on tobacco culture did not produce any form of secondary metabolite. Also interesting is the fact that some isolates like *Paecilomyces spp*, *Cladosporium spp* and *T. schoenleinii* which otherwise did not produce metabolite, elaborated mycotoxins when present as one of multiple colonies cultured from a tobacco culture. Fungus may be stimulated by the presence of other competing fungi to elicit the production of mycotoxins (Smith and Moss, 1985.

Although the intraperitoneal route was chosen for ease of administration of toxic metabolites to the test animals, exposure of smokers to these mycotoxins is by inhalational route. Mycotoxins are about 40 times more toxic when inhaled than when injected parenterally (Peraica *et al.,* 1999; Wannemacher and Wiener, 2003). This increases the risk posed by these mycotoxins to smokers. It also means that those toxins that did not show serious toxicity through the intraperitoneal route could actually be toxic through the inhalational route, which in any case is the route through which smokers are exposed to these mycotoxins.

This research has not sought to discover new adverse effects of cigarettes that smokers are now exposed to, but to elucidate the underlying cause for some of the health hazard that smokers have always been exposed to and have fallen victim of. There is a strong correlation between lung cancer death rates and smoking. Constituents of tar and the gas phase of cigarette smoke have always been implicated in cigarette induced carcinogenesis. The scientific thought has been along this line for too long and other possible predisposing factors to cancer in tobacco has been ignored. This research has established the presence of aflatoxin contamination and of fungal contaminants of cigarette tobacco, and that some of these fungi produces mycotoxins, which smokers may have always been exposed to. Mycotoxins may have been ignored and classified within the tar constituent being particulate itself. Since some of the mycotoxins, and especially aflatoxin, are known carcinogens, chronic exposure to them by smokers could be another predisposing factor to cancer in smokers.

CONCLUSION

The curing and aging process of tobacco in cigarette production involves microorganisms including Fungi. Fungi produces spores which may not be destroyed during the processing of the tobacco leaves into cigarettes. Cigarette tobacco contains fungal contaminants. Some fungal spores are capable of producing toxic metabolites known as mycotoxins. Cigarette tobacco is conducive for the production of aflatoxin. Some mycotoxins at low concentrations could be lethal when administered to mice and by projection could be acutely toxic to humans that are exposed to them. Some of the mycotoxins are also known carcinogens, therefore exposure to very low concentrations over a long period of time could initiate carcinogenesis in humans. There is a high correlation between incidence of cancers and smoking and the mycotoxins could be the carcinogen or co-carcinogen involved.

RECOMMENDATION

Cigarette is the only legal substance that produces adverse effects when used for its intended purpose. The evidence available suggests that the diseases associated with smoking relate to the intake of the smoke constituents in a dose-dependent way; the greater the exposure to smoke constituents, the greater the risk. Those who smoke many cigarettes are at a greater risk than those who smoke few and smokers of many years standing are more threatened than those with a shorter smoking history. A real world view suggests that in the foreseeable future, many millions of adults would continue to consume tobacco. A growing world population and rising incomes broadly suggests increased consumption; this may be off set by trends for smokers to consume less per day, to quit and to modify their consumption to accommodate changing approaches to smoking in public places.

In Nigeria, poverty and deprivation subsists, cases of HIV/AIDS that makes patients immunosuppressed and so incapable of fighting diseases is on the increase. With health facilities and personnel already overstretched and on adult population expected to rise to 120 million by 2025 (UN, 1998). It is all too evident that care and nursing facilities will be largely unavailable to manage the enormous health crisis Nigeria could be facing in the future. There is therefore a need for a great deal of discipline, commitment and courage on the part of the government and individuals to mitigate the effects of smoking. The government should enforce the ban on advertising tobacco. Also the decree banning smoking in public places should be revived and enforced. More concerted efforts should be put in place to educate the public on the dangers of smoking. Also, effective programmes to rehabilitate addicts and to encourage others to quite should be intensified.

This research has not answered some critical questions like the acute toxicity of mycotoxins by inhalational routes, the chronic effect of sustained exposure to low

levels of these mycotoxins and the carcinogenecity of the mycotoxins, and should only be regarded as a pilot study in this field to elicit and generate interest in this course of research. Therefore more efforts and funds should be channeled into elucidating some of this grey areas.

Seeing that cigarette tobacco could contain fungi contaminants, capable of producing toxic metabolites, especially aflatoxin, it is very necessary that the government puts tobacco production, consumption and importation under a regulatory body like NAFDAC, so that the levels of contamination of cigarette by toxigenic moulds and aflatoxins can be properly monitored and controlled.

Finally, equity amongst individual depends on who owns what, while equity amongst nations depends on who knows what. The progress of a nation depends mostly on the qualities of its research which are mostly promoted by any government that considers development a priority. The authorities of the Nigerian government and private corporation have neglected this vital project under the cover of economic crisis. Government and corporate bodies should therefore invest on quality researching that can better the well being of the citizenry, especial heath - wise.

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APPENDIX A

Comparative Toxicity of T-2 Toxin by Various Routes of Administration

	Mammals Tested						
	Mouse	**Rat**	**Guinea Pig**	**Rabbit**	**Cat**	**Pig**	**Monkey**
Route of Administration	**T-2 Toxin LD_{50} (mg/kg)**						
Intravenous	4.27	0.7	2.0			1.2	
Intraperitoneal	9.1	2.6	3.0				
Subcutaneous	2.1	2.0	12.0		<0.5		
Intramuscular		0.9	1.0	1.1			0.8
Intragastric	9.6	2.3	3.1				
Intranasal		0.6					
Intratracheal	0.16	0.1					
Inhalational	0.24	0.05	0.6				
Intracerebral		0.01					
Dermal in DMSO	6.6	4.3	2.2	10			>8.0
Dermal in Methanol		>380	>80				

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APPENDIX B

PO	IP
3	3
2	3
3	3
0	0
0	0
1	2
3	3
3	3
1	2
2	2

F-Test: Two-Sample for Variances

	PO	*IP*
Mean	1.8	2.1
Variance	1.5111111 1111111	1.4333333 3333333
Observations	10	10
df	9	9
F	1.0542635 6589147	
P(F<=f) one-tail	0.4692750 82724622	
F Critical one-tail	3.1788931 0457235	

APPENDIX C

PO	IP
3	3
2	3
3	3
0	0
0	0
1	2
3	3
3	3
1	2
2	2

t-Test: Paired Two-Sample for Means

	PO	IP
Mean	1.8	2.1
Variance	1.51	1.4
Observations	10	10
Pearson Correlation	0.92	
Pooled Variance	1.47	
Hypothesized Mean Difference	0	
df	9	
t	-2	
P(T<=t) one-tail	0.04	
t Critical one-tail	1.83	
P(T<=t) two-tail	0.08	
t Critical two-tail	2.26	

APPENDIX D

PO	IP
3	3
2	3
3	3
0	0
0	0
1	2
3	3
3	3
1	2
2	2

z-Test: Two-Sample for Means

	Variable 1	***Variable 2***
Mean	1.636363636 36364	1.909090909 09091
Known Variance	0	0
Observations	11	11
Hypothesized Mean Difference	0	
z	ERR	
P(Z<=z) one-tail	ERR	
z Critical one-tail	1.644853630 87206	
P(Z<=z) two-tail	ERR	
z Critical two-tail	1.959963992 57784	