STUDY ON UTILIZATION OF GOSSYPOL BY FUNGI ISOLATED FROM COTTONSEED AND MYCOTOXIN CONSTITUENTS OF COTTONSEED AND COTTONSEED BY - PRODUCTS.

BY

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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 at undergraduate level or as post graduate candidate, by any student, 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All information derived from published and unpublished works of others have been well acknowledge in this thesis.

Tijjani A.A.

25th February 2004

Date.

CERTIFICATION

This project entitled "**Study on utilization of gossypol by fungi isolated from cottonseed and mycotoxin constituents of cottonseed and cottonseed by products**" was carried out by **TIJJANI AZEEZ AMOSA** 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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DEDICATION

To my beloved late father, ***PA TIJJANI AJANI OLATUNDE "Baba Alaga" (1909–1999)***

And

In loving memory of my Cousin Master ***Yusuff Adesola Dauda***.

May their souls rest in perfect peace.

ABSTRACT

Some varieties of cottonseeds cultivated in Nigeria and cottonseed by products such as cottonseed cake, cottonkernel, cottonoil (crude) and refined cotton oil (edible) have been analyzed for their gossypol, protein and lipid content. Studies were also carried out to determine the mycotoxin and fungal contamination of cottonseed. The gossypol content of the samples ranged from 0.0256%. to 0.3802% to Aflatoxin, zearalenone and ochratoxin A were screened for in the cottonseed and cottonseed by products. The three mycotoxins were not detected in any of the samples. Studies were also carried out to determine the fungal contamination of cottonseeds, and the potential utilization of gossypol as sole source of carbon and energy by the isolates fungi. Of twenty – five fungal isolates obtained from cottonseed, only 2 (8%) of *Penicillium spp*. did not utilize gossypol as sole source of carbon and energy at varying concentration of 0.1%; 0.2% and 0.5%. *Mucor spp* and *Rhizopus spp* were the most efficient utilizers of gossypol. This was followed by *Aspergillus flavus*, *Aspergillus spp* and *Penicillium spp*. The results obtained provide a clear indication of the promising role fungal could play in utilizing and transforming gossypol into non-toxic compounds to improve the nutritive quality of cottonseed by products.

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CHAPTER ONE

1.0 INTRODUCTON

Gossypol is a yellow polyphenolic pigment found in the cotton plant (*Gossypium spp)*. These and other related pigments are found in raw and processed cottonseed, but can also be found in much lower levels in other parts of the cotton plant such as the leaves and the hulls. The amount of gossypol found in the seed is dependent on variety grown, climatic conditions, growing location, agronomic practices, and other factors. Gossypol is one of many intrinsic substances, which are thought to be responsible for the insect resistance of the cotton plant. It exists in two forms, free and bound, with the free form being toxic, and the bound form is thought to be non-toxic because it is bound to proteins (Cheeke *et al* ;1985).

Bernardi *et al.,*1980 reported that the heat associated with the reaction between gossypol and protein during processing of the seed reduces both the protein availability and the toxicity of gossypol.

The importance of fungi as poisonous agents has been appreciated for many years, but it is only recently that the scope and magnitude of the losses that they cause have become apparent. Partly this has come about because of the greater accuracy of definition of other diseases with which mycotoxiccoses have been confused. The mycotoxins are those mould metabolites which cause illness or death in man, or his domesticated animals following consumption of contaminated food. The illness itself is referred to as mycotoxicosis (Gbodi, 2000). Poisonous symbiotic and parasitic fungi

cause loss in yield , quality, nutritional value and viability of foods and feed stuffs especially cereals crop. Fungi and the toxins (mycotoxins) they produce, constitute health hazards to animals and man. Many diseases in animals are now known to be of fungal origin. Epidemic and endemic diseases of unknown aetiology are now associated with fungi Smith and Moss, 1985. The obvious reduced crop and livestock production and adverse effects on health in human, caused by mycotoxins have severe consequences on national and world economy.

Gossypol is biologically active compound whose mechanism of action in animals is not yet fully understood and because gossypol in the food chain may ultimately lead to its consumption by humans. Ruminants are able to detoxify large amounts of free gossypol by binding it to proteins in the rumen. Monogastrics on the other hand are unable to detoxify much of free gossypol, and are thus, more susceptible to its toxicity (Zucker and Zucker; 1947).

The toxic properties of gossypol have been known for some time in spite of an early report that it had none. Zucker and Zucker (1947) published papers stating that gossypol was an appetite depressants, and that purified gossypol had no toxic properties. The widespread publicity at that time relative to the possible use of gossypol in the treatment of obesity in man prompted Eagle (1960) to carry out a series of physiogical and toxicological studies of cottonseed pigment glands and pure gossypol. The investigators conclusions were as follows:

i. Untreated cottonseed pigment glands vary widely in their acute oral toxicity in the rat, and this toxicity is not proportional to their analyzed gossypol content.

ii. Pure gossypol is toxic to the rat, but to a lesser extent than any untreated cottonseed pigment gland tested.

iii. Pure gossypol fed at various levels in the diets of experimental animals caused body weight depression proportional to the amount fed.

iv. Cottonseed pigment glands were slightly less toxic when administered in water, on the other hand, gossypol was slightly more toxic when administered in oil than in water. Etc. The biological effects of gossypol are associated primarily with cardiac damage and dysfunction. However other effects produced by gossypol may include infertility in human reproduction, hypoprothrombinemia and anemia (cheeke *et al.,* 1985, Lin *et al.,* 1985).

Aflatoxins are toxic, carcinogenic chemicals produced by the fungal species, *Aspergillus flavus* and A. Parasiticus (Goldblatt; 1970). These fungi can infect crops before and after harvest to produce aflatoxins, thereby contaminating foods and feeds that threatens both human and animal health (Goldblatt,1970 Jelinek *et al.*, 1989). The first report of the toxicity of aflatoxins appeared nearly thirty years ago in Great Britain (Blount, 1961) when the cause of the extensively publicized turkey "X" disease was traced to contaminated peanut meal from Brazil. The disease caused the deaths of over 120,000 turkeys and other poutry and led to a huge research effort to investigate aflatoxin contamination of food and feeds in the USA and other countries. Aflatoxin was first detected in cottonseed and cottonseed meal about 25 years ago. Since then, Agricultural Research service scientists at the southern Regional Research center have investigated methods to control aflatoxin contamination of food and feed commodities (Goldblatt, 1970).

Initial research by scientists at the center centered New Orleans on the development of analytical techniques to quantify aflatoxins and on the development of methods to detoxify contaminated products (Goldblatt, 1965, pons and Goldblatt, 1970 park *et al.,* 1988).

Moulds grow on any stored feeds, the highest incidence being on feeds with high moisture content. Thus, a common fungal contamination is on mouldy corn or maize, which is a high moisture grain and difficult to harvest or store at the correct stage of maturity and moisture content. A degree of spoilage must be expected in corn grain with a moisture in excess of 20%.

The frequent occurrence of fungal growth on stored feeds ensures that mycotoxicoses occur more commonly in housed animals and in those confined to zero grazing in dry lots. Ingestion of food or feed highly contaminated with aflatoxin can lead to acute toxicity including hepatoxicity, teratogenicity, Immunotoxicity and even dealth (Kai *et al.*, 1996). Aflatoxin B1 (AFBI), the most abundant of this toxic chemical, is extremely mutagenic and is one of the most potent carcinogens ever tested in rat (Dyerackoye, 1990).

Many of the moulds on feeds do not produce toxic metabolites, but some do, although only at certain times. Many syndromes generally considered to be caused by fungal toxins. e.g. the hemorrhagic diathesis which occurs on feeding moldy lespedeza hay, corn grain, hay, chaff and bedding, have no recognized specific mycotoxic origin. On the other hand, there are some well-identified organ specificities. These include aflatoxin affecting the liver, ochratoxin and citrinin causing renal disease, ergotamine

and zearalenone affecting uterus, and tremogens affecting the nervous system. (Gbodi and Nwude 1988).

The type and abundance of mycoflora and mycotoxins to be found in an environment depends on the prevailing climatic conditions, (Agboola 1992). Optimal fungal growths and mycotoxins biosynthesis occur at temperature above 25°c and relative humility of between 65 – 85% in tropical and substropical area (Smith and Moss 1985).

Of all the agricultural crops, cereals is considered to be the ideal substrate for the growth of fungi providing the necessary carbonydrate for energy, protein to meet amino acid requirement and fats for additional energy (F.A.O. 1983). The susceptibility of grains to fungal growth and mycotoxin production have been demonstrated around the world (Jelinek *et al.*, 1989; Miller and Trenholm, 1994). In Nigeria, various fungi species and mycotoxins have been shown to occur naturally in grains [Okoye 1992]. Cottonseed constitutes a large potential source of high protein. With the state of economy in Nigeria today, there is rising cost of cereals, often employed in livestock feed, due to their high demand as staple human foods or also as a result of shortage due to insects or micro-organism damage on the field farm these automatically leads to deficit of available protein. There is need for alternative source of materials which otherwise are wastes or hazardous to man but can be utilized for our own benefit. Among such products are gossypol pigments and fungal contaminating cottonseed.

Cottonseed is an important by product of the ginned seed cotton. The seed produced in the Northern States of Nigeria contain 20% oil, 10 linters,

40% hulls and 30% Kernel (Anon, 1963). Cottonseed oil is one of the most important constituents of the seed, and it is of great economic value. The removal of the oil by crushing mills, leaves cottonseed meal or cake which has many uses. The cottonseed produced from ginned cotton is sold to oil millers for crushing, and distributed free to farmers for planting. In some parts of Nigeria like Sokoto State, the cottons seed is used in place of beans for making fried bean cake "akara" and in some parts of Plateau and Kwara States, cottonseed is used for special spices for soup in preference to the more popular condiment made from locust beans.

This study is important because cottonseed products have found wide use in Nigerian for livestock feed and for human food, and because little has been done on the economic ways of isolation and utilization of gossypol and fungal from cottonseed and cottonseed by products. Nigeria being a tropical country has all environmental for growth of aflatoxin-producing fungi and development of gossypol and gossypol like pigment in cottonseed plant it was thought necessary to investigate these in Northern part a major cotton producing area of Nigeria.

1.1 **Research objectives**

(i) To isolate and identify storage and advanced decay fungi infecting cottonseed and cottonseed by-products.

(ii.) To screen fungi isolates for potentials to grow on and utilize gossypol from cottonseed as sole source of carbon and energy.

(iii) To extract and identify mycotoxin that might be present in cottonseed by-products.

(iv) To analyze and determine free gossypol level in cottonseed and cottonseed by-products.

CHAPTER TWO

2. LITERATURE REVIEW

Cottonseed is obtained from cotton plant (*Gossypium spp linn*). Cotton belongs to the family Malvaceae and order malvales. Cotton by products are produced from the seeds of the many Gossypium species. They are G. *hirsutum, G. arboreum, G. herbaceum and G. barbadense* (Doller, 1968, Martinez, 1979).

The world production of cottonseed oil has been estimated at about 24.45 million tonnes, about 37.5 percent of which is produced in the USA. Other important cotton producing countries include USSR, China, India, Mexico, Brazil, Pakistan, Turkey, Sudan and Nigeria (Washington DC, 1985). Cotton is mostly cultivated in the northern part of Nigeria and sparsely extended to neighboring countries like Benin Republic (Institute of Agricultural Research Report, 1988).

Cottonseed is one of the most widely used economical source of protein in the animal feed industry (Waldrop 1986). Cottonseed is a valuable source of edible oil, cake, meal, flours, linters and hulls, all of which have several industrial and other applications.

2.1 Cultivation of Cotton

Cottonseed is cultivated across temperate, sub-tropical and tropical climatic zones, with crop performance strongly influenced by sowing and harvesting time (Mulkey, *et. al.,* 1987). Early sowing gives a higher yield while late sowing results in less vegetative growth and development of towel

bolls. The sowing time of cotton is largely determined by two factors, viz, soil temperature and seasonal conditions. Early sowing of cotton have several advantages besides giving greater yields of cotton. Optimum planting time of cotton varies with locality but is generally found to be between May and July in Nigeria.

2.2 Harvesting and Yield

The cotton-picking season in the northern region of Nigeria lasts from the middle of October to the end of December. Picking is invariably by manual labour throughout the country. The yield is low in the first picking, highest in the second picking, and lowest in the third (last picking). The entire crop is gathered in 3 or 4 pickings, the interval between pickings varying from 15 to 20 days (I. A. R. Report, 1988).

2.3 Processing Of Cottonseed

When cotton arrives at the cotton gin, it is sucked into the building through pipes placed in the trailers or trucks. In many plants it first enters driers that reduce the moisture content for easier processing. The cotton travels next to equipment that removes burrs, sticks, dirt, leaf trash, and other foreign matter. It then moves to the gin stand, where lint is separated from the seeds. After separation from the seeds, the lint is packed tightly into bales. For the processing of cotton fiber to make yarn, cloth, and cordage, see *Spinning;* Textiles (NCCA, 2000).

2.4 Marketing and Ginning

In advanced countries like the USA, the farmers do the ginning and dispose of lint and seed separately, thus-eliminating one of the major

middlemen between the producers and the consumers (Brown, 1986). In India, the Kapas as it is picked from the fields is readily sellable in the village market and through other middlemen. In other markets the Kapas are taken by commission agents to factory premises, where samples are ginned, and from the ginning percentages and other factors, the price is mutually settled. The cultivator get a much better price in the wholesale markets than in the villages also in India the products is sold by the farmers as Kapas through agents of private merchants (India central cotton committee, 1978). In Nigeria a number of intermediary agencies deliver the cotton to the ginning factory and sell the lint to the consuming mills. The average grower does not consider it worth while to carry the small quantity of cotton produced to the market but prefers to sell it at the farm house on the spot.

In Nigeria, most ginning and refinery factories are located in northern part of the country like Zaria, Kano, Gusau, Kontagora Funtua and Malunfashi. There are none or only few regulated markets in the country but there is a good network of co-operative cotton sale societies, which handles about 60 percent of the cotton produced .

These societies like National Cotton Association of Nigeria (NACOTAN), gin the seed cotton and sell the lint to merchants and agents of different mills. This body encourages government to give some incentives to the farmers and cottonseed crushers and to educate farmers on the best varieties of the cottonseed to plant.

2.5 Mycoflora and Mycotoxin Contaminating Cottonseed

The knowledge of the mycoflora and mycotoxin infecting cottonseed has been reported to be incomplete and imprecise (Uraquchi and Yamazaki, 1978). The mycoflora of cottonseed and cottonseed by- products may be classified as storage fungi and field fungi including pathogenic fungi and saprophytic fungi such as Aspergillus spp, Penicillium spp, Mucor spp, Rhizopus spp, Phoma Sorghina, Trichoderma spp, Curvularia spp, Nigrospora spp, etc. The microflora on and within foodstuffs is known to affect the quality, storage behaviour and processing of cottonseed and cottonseed by–products. Aflatoxin was first detected in cottonseed and cottonseed by-products about 29 years ago. However, the first report of the toxicity of aflatoxins appeared nearly 30 years ago in Great Britain when the cause of the extensively publicized turkey "X" disease was traced to contaminated peanut meal from Brazil. The disease caused the death of over 120, 000 turkeys and other poultry and led to a huge research effort to investigate aflatoxin contamination of foods and feeds in the USA and other countries (Blount, 1961).

Elegbede (1978), Gbodi (1986) and Okoye (1992) reported the dominant fungal genera in Nigerian food including cotton-by products as *Aspergillus, Penicillium, Fusarium, Trichoderma, Phoma Alternaria, Cheatomium, Curvutaria and Helminthoseporum.*_Abalaka and Elegbede (1982) investigated the distribution of aflatoxins and total microbial counts in cake of an edible oil extracting plant. They found aflatoxins B1, B2, G1 and G2 in groundnut kernels, cottonseed peelers, Crude groundnut oil and crude cottonseed oil. These investigators found that crude groundnut oil had a much higher aflatoxin B1 content than crude cottonseed oil (98.0 versus 65

ug/kg), but a higher B1 level was recorded for refined cottonseed oil (24 ugkg) than for refined groundnut oil (90ug/kg).

Gbodi, (1986) investigated the mycoflora and mycotoxins in acha (*Digitaria exilis Stapf*), maize (*Zea mays*) and cottonseed (Gossypium spp. Linn) in Plateau State, Nigeria. Ninety six fungi were isolated from acha and the mycotoxins found in acha were aflatoxins B1, B2, G1 and G2, *Zearalenone* and *Vomitoxin*. The investigator isolated 124 fungi, aflatoxin B1, B2, G1 and G2, *Zearalenone* and *ochratoxin* A from maize. Only aflatoxin could be detected in mouldy cottonseed.

2.6 Factors Influencing Mould Development and Mycotoxin Production in Cottonseed and Cottonseed By – Products

Fungal growth in cottonseed causes considerable losses to the cotton and cottonseed oil industries. These deteriorative changes in cottonseed are of considerable economic importance to oil millers. Consideration of the mycotoxin problem invariably leads to examination of the factors that can modify mould development and mycotoxin production. Many researchers (Altshul *et al.*, 1966; Malowan, 1969; Ashworth *et. al.,* 1971; Smith and moss, 1985; Diener *et. al.,* 1987; Cole *et. al.,* 1989; Goto and Manabe, 1989; Gbodi; 2000) reviewed and reported most of these factor to include Moisture Content, Temperature of the crop, deterioration of cotton seed strain of fungus and nature of substrates. The investigators concluded that the contamination may result from field damage or storage damage.

2.6.1Moisture Content

Altschul *et. al.,* (1966) have shown that seeds with less than 15 percent moisture content deteriorate as a result of enzyme activity within the seed. When seeds have more than 15 percent moisture, the initial deterioration also appears to be caused by these enzymes but as deterioration progresses and the seeds become less viable, they appear to become susceptible to micro-organism and ultimately the activity of the latter can become the pre dominant factor in the deteriorative process (Smith *et. al.,* 1985). Several investigators (Diener *et. al.,* 1987, Cole *et. al.,* 1989) have reported that increase in the moisture content of cottonseed in storage is accompanied by an increased rate of lipolysis. It is of interest to note that the lipolysis rate constant increases steadily with increase in moisture content of the seed (Malowan, 1969) and that seeds which exhibit a high respiratory intensity also show a high intensity of lipolysis. A linear relationship between moisture content and lipolysis similar to that observed between moisture content and respiration has been established (Altshul *et. al.,* 1966).

2.6.2 Temperature of the Crop

A series of experiments to determine the combine effect of temperature of storage and moisture on lipolysis was reported (Goto and manable, 1989; (cole *et. al.,* 1989). Lipolysis was revealed to be faster at higher temperature and moisture levels. It was established that lipolysis can be minimized by storing the seeds at very low temperature.

Simpson *et al.,* (1973) made a study of the effect of varying conditions of temperature and moisture content on the viability of two

upland varieties of cottonseed. The researchers found that conditions of low moisture and low temperature suppress free fatty Acid development in cottonseed also prevent the loss of viability in seed on storage.

2.6.3 Strain of Fungus

An important factor in control of fungal development and toxin production is the genetic capacity of the mould to produce mycotoxin. Fungi may attack food crops without necessarily producing any mycotoxin. This is because not all strains of fungi can produce toxins for instance only about 58% of all strains of *Aspergillus Flavus* produce aflatoxin (FAO 1977); Scott, 1978) in his review of mycotoxin in feed ingredients reported the occurrence of only aflatoxins in cottonseed. According to Gbodi 1989, the reason might be that although cottonseed could be infected with ochatoxin- and zearalenone- producing fungi, it may not be a good substrate for production of these mycotoxins.

2.6.4 Deterioration of Cottonseed

Gbodi, (2000) reported that mature, whole, unbroken kernels are less susceptible to mould attack than immature broken and defective kernel (Lillehoj and Zubee, 1975). Crop attacked by termites are also prone to fungal attack than undamaged crop. Thus, if because of climate change there is an early cessation of rainfall with resultant immature kernels higher fungal incidence in this crop may be observed. Presences of some chemicals in or on the subtrate may affect the growth of fungi or synthesis of mycotoxins. Dichlorvos for example has been cited (Gbodi, 2000) to inhibit aflatoxin biosynthesis by *Aspergillus flavus* (Hsieh, 1973; Yao and Hsieh 1974).

Feed damage is the deterioration that occurs while the seeds are hull in the cotton bolls in the field while storage damage is the deterioration which occurs after the cotton has been gained and the seeds stored at the gin or the oil mill. The two type of damage are recognized both by the appearance of the seeds and by the colour of the oil produced (Malowan, 1969) concluded from his observations that the atmospheric humidity under which cottonseed mature is of prime importance in determining the extent to which field damage may occur. Seeds which matured under conditions of low humidity suffered little field damage even though the moisture content was subsequently reduce by drying. The observation point to the importance of the location of the growing plant as a contributory factor to field damage. However, thus difference in the environment temperature accounts for the difference in the colours observed in cross-section of field damage and storage damage (Malowan, 1969; Diener *et. al.*, 1987; yayock *et. al.,* 1988; Goto and Manabe; 1989; Cole *et. al.,*1989).

2.7 Nature of Gossypol

Cottonseed is a product of the cotton plant consisting of two principal parts; namely, the hull or the spermoderm from which staple cotton and short fibres arise, and the kernel or embryo from which oil and the cake are obtained. In addition to these two principal elements of the seed, there is a third structure, a membrane which completely envelops the embryo. This membrane may be described as a residual tissue of the endosperm. When cotton is cut into sections and viewed under a microscope, innumerable dark specks are noticed on the cut embryo surface. These specks are pigment glands. They contain-pigment materials which impart a characteristic

yellow-red colour to cottonseed meal and oil. Gossypol is the most prominent component of these pigments (Dollear, 1968, Martinez, 1979). The outer structure of the gossypol pigment gland is rigid wall and not membranous. The walls of the gland are resistant to pressure and are reported to be made up of five to eight closely-fitting thick, curved plates, which are thicker at the centers than at the edges and are irregular in outline (Boatner, 1975). A relatively thick colourless transparent layer is known to envelop the gland.

Micro chemical evidence conclusively shows that the plates are composed of cellulose impregnated with pectin, hemicellulose and one or more un-identified uronic acid derivatives. The thick transparent encrustations of the glands consist of cutin. The pigment glands are strongly coloured visually, by appearing perfectly clear under a microscope in strong light showing there by that contents are homogenous and are in a state of single-phase solution, liquid or solid. When the gland is broken, it contents are not discharge except when the gland is soaked in liquids in which the pigments are soluble (Shirley, 1979). Boatner (1975) reported that, the behavior of the pigment glands is different in different liquids. Immiscible liquid, of low molecular weight that has no effect on the glands even after prolonged contact is called inert liquid (Boatner, 1975; Lyman; 1979; Martin, 1980).

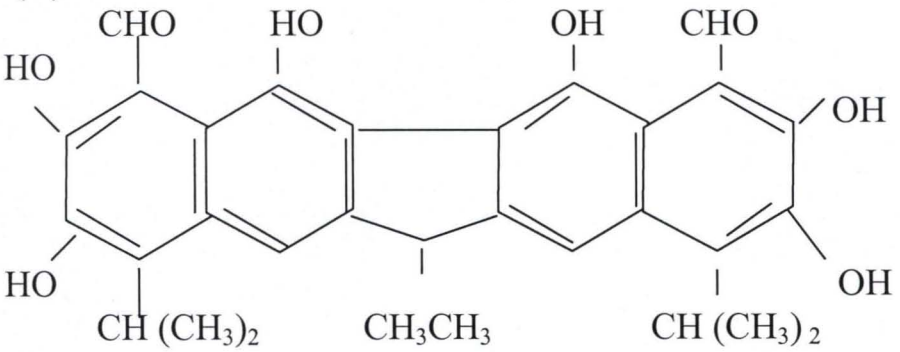
2.8 CHEMISTRY OF GOSSYPOL

Improved methods for the recovery of gossypol from cottonseed, based on the formation of three derivatives, acetic acid, aniline and water-soluble salt of gossypol has been cited by many investigators (Markman *et.*

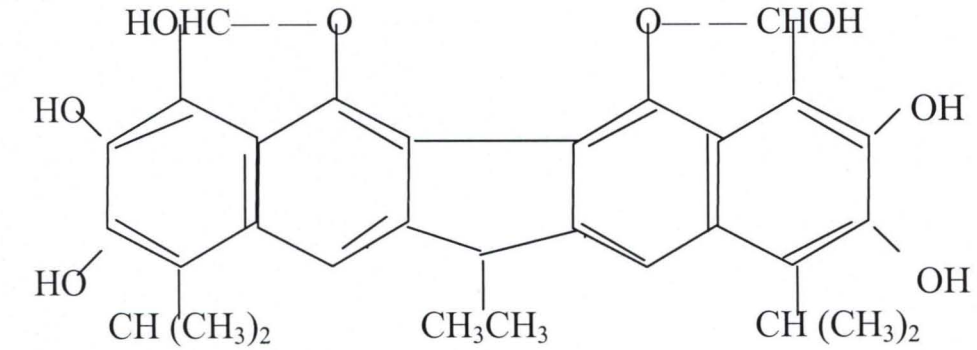
soluble salt of gossypol has been cited by many investigators (Markman *et. al.,* 1968, Pons 1977, ACS, 1978; Admasu and Chandravanshi, 1984,

Akanya *et. al.,*1989). Gossypol as reported by Admasu and chandravanshi (1984) is soluble in methanol, ethanol, isopropanol, n-butanol, acetone, ether and slightly soluble in glycerol, cyclohexane and high-boiling petroleum ether (bp 60-110 °c) and insoluble in low-boiling petroleum ether and water. Gossypol has also been described to exist in three tautomeric forms as presented in Figure 1.

(A)



(B)



(C)

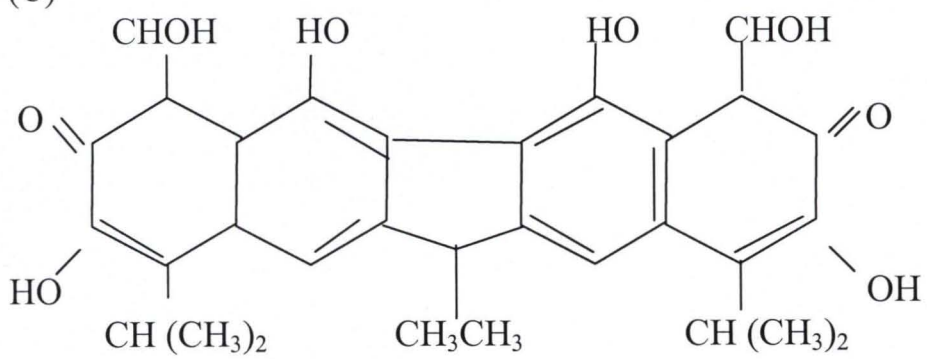


Figure 1: Structure and tautomerism of gossypol.

(A) hydroxy aldehyde; (B) lactol; (C) cyclic carboyl.

Clark (1927) proposed the molecular formular ($C_{30}H_{30}0_8$) for gossypol, which is in good agreement with the result of several workers (Bailey; 1948; Leiner; 1969; Martinez, 1979; Lyman; 1979). However, Tonin (1961) and Goldblatt (1969) could not get consistent analyses with their preparations of gossypol, which agreed with this formula.

Boatner (1975) was of the view that the method of purification of gossypol used by Clark (1927) and most subsequent investigators (Markman *et. al;*.,1968; Leiner, 1969, Pons, 1977;Admasu and Chandravanshi, 1984) through crystallization from hot solvents may be responsible for altering the heat-sensitive gossypol molecule.

In studies of certain Nigeria cottonseed varieties in (1962); presence of gossypol has been reported by the Tropical Institute (TPI) London in fairly high proportion. Gossypol is a polyhydroxy compound systematically known as 1,1,6,6,7,7,-hexahydroxy 5,5,-diisopropyl -3,3-dimethyl-(2,2-binaphtalene)-8,8-dicarboxy aldehyde (Bailey, 1948, leiner, 1969). However, in determination of gossypol, Boatner (1975) reported that maximum absorptivities occur at 520μM and 380μM and a minimum absorption occurs at 430μM. This seems to justify the conclusion of Hall *et. al.,* (1966) that the ratios of the absorption at 520μM and 430μM or at 520μM and 380μM are sufficiently specific for gossypol to distinguish it from other gossypol-like pigments, especially apo-gossypol and

gossypol preparation. Therefore, each method has its own advantages as well as limitations. A different procedure has been evolved for the determination of gossypol in mixed feed. The gossypol is complexed with 3-amino-1-propanol in dimethylformamide during extraction and the complex reacted with aniline in isopropanol-hexane solvent. The specific complexing reagent was necessary to recover gossypol from mixed feeds (AOCS; 1978). Comparison of the gossypol content determined by the aniline method and the recently developed chromatographic method has indicated that gossypol exists in cottonseed meal in forms other than free and bound (Martin; 1980).

In the analysis of cottonseed meal by Martin *et. al.,* (1969), the aniline method consistently showed 0.1- 0.2 percent more gossypol in the meal than the chromatographic method. In meals in which the aniline method showed 0.1 percent or less free gossypol, the chromatographic method would detect no free gossypol. This suggests the possibility that soluble gossypol derivatives occur in the meal. Lyman (1979) established that, the gland-free cottonseed meals from which glands had been separated by floatation contain primarily the soluble gossypol derivatives. Hexane extracted flakes yielded gland free meals containing not more than 0.02 percent of true free gossypol and as much as 0.11 percent of 'soluble bound gossypol' components. It was reported (Lyman, 1979, Martins, 1980) that the pigment glands were found on analysis to be essentially free from 'soluble bound gossypol'.

A new strain of gland-free cottonseed being developed in the USA (Berardi and Goldblatt, 1980) showed a scarcely detectable amount of 0.002 percent of free gossypol. The phospholipid gossypol concentration was

0.0002 percent and the soluble gossypol content was 0.001 percent. In the study of some varieties of Nigerian cottonseed Akanya *et. al.,* (1989) reported a free gossypol content of about 0.5 percent which was lower than the reported values for Nigerian varieties of cottonseed by (TPI, 1962). Akanya *et. al.,* (1989) established that hexane seems to be the most suitable solvent for extracting a gossypol free oil from varieties analyzed while acetone would be the most suitable for extracting a gossypol.

2.9 Toxicity of Gossypol

Morgan (1989) reported gossypol exists in two forms, free and bound, with the free form being toxic. The bound form is thought to be non-toxic because it is bound to proteins. The heat associated with the reaction between gossypol and protein during processing of the seed reduces both the protein availability and the toxicity of gossypol. Morgan (1989) described gossypol as a biologically active compound whose mechanism of action in animals is not yet fully understood. Ruminants are able to detoxify large amounts of free gossypol by binding it to proteins in the rumen. Monogastrics on the other hand, are unable to detoxify as much free gossypol and are thus more susceptible to its toxicity. Morgan (1989) established that most reports of toxicity indicate that it takes at least several weeks to develop after the onset of feeding.

Berardi and Goldblatt (1980) reported that the presence of gossypol in cottonseed presents two hurdles in the utilization of cottonseed meal for nutrition. The 'free gossypol' content causes adverse physiological effects in

animals and the binding of gossypol with proteins reduce the availability of the biochemically essential amino acid lysine.

Although it has been established that gossypol is a major factor in egg yolk discolouration, the work of Kemmerer *et. al*; (1963) showed that yolk discolouration was greatly enhanced by the presence of malvaceous oil components. This effect was attributed to the presence of malvalic acid. Their finding also suggested that the discolouring tendency of cottonseed meal in egg yolk might also be due to the presence of cyclopropeniod fatty acids. Hale and layman (1980) reported that monohydrate ferrous sulphate at 500ppm level supplemented the protective action of animal proteins against gossypol toxicity.

Gossypol has proven to be toxic to man, poultry, swine, dogs, rabbits, rodents and to a lesser extent ruminants (Cheeke *et. al.,* 1985; Patton *et. al.,* 1985; Eisele, 1986, Hudson *et. al.,* 1988). The biological effects of gossypol are associated primarily with cardiac damage and dysfunction. However, other effects produced by gossypol may include hypoprothrombinemia , anemia and infertility in males, and (Cheeke *et. al.,* 1985).

Gossypol is a non-hormonal fertility-inhibiting molecule which occurs naturally in unrefined cottonseed oil. An investigation of Jiangxi Province in China, conducted in 1929 (but not published until 1957}, showed a correlation between low fertility rates for couples and use of crude cottonseed oil for cooking. Continued research showed that cottonseed oil was specifically affecting only male fertility. Eventually researchers isolated the compound causing the contraceptive effect, gossypol, from the oil.

In the 1970's, the Chinese Government began large-scale research on the possible use of gossypol as a contraceptive. These studies involved over 10,000 subjects, and continued for over a decade. The researchers found that gossypol provided reliable contraception, could be taken orally as a tablet, and did not upset men's balance of hormones. At the conclusion of the trials, gossypol seemed a promising male contraceptive, but it also had serious flaws.

Some men in the Chinese studies remained azospermic after prolonged use of gossypol. The longer the men had taken the drug, and the higher their overall dosage, the more likely the men were to lose part or all of their fertility. Various studies reported between 5 and 25 percent of the men remained infertile up to a year after stopping treatment.

The Chinese studies also reported an abnormally high rate of hypokalemia among subjects, varying between 1 and 10 percent. Hypokalemia is characterized by low blood potassium levels. Symptoms include fatigue, muscle weakness, and at its most extreme, paralysis. Hypokalemia is usually the result of kidney malfunction, although exactly how gossypol affects renal function is unkown. The Chinese researchers found that most subjects recovered after stopping gossypol treatment and taking potassium supplements. A later study showed that taking potassium supplements during gossypol treatment did not prevent hypokalemia in primates.

Gu et al; (2000) reported on side effects of gossypol during a low-dose study. Subjects took 10 or 12.5 milligrams of gossypol for 16 to 18 months. The men took the pill daily for the first 2 months until they became

azoospermic. They then switched to a maintenance dose, taking the pill every other day for the duration of the study. After stopping treatment, all men returned to pre-treatment sperm counts. None of the subjects suffered from hypokalemia or muscle fatigue during this study. Lohiya et al; (1990) Reported a test of gossypol taken in combination with a potassium supplement for 120 days. The researchers observed "severe oligospermia" and "extensive renal potassium loss." Both sperm count and blood potassium levels returned to pretreatment levels within 105 days of the cessation of treatment. Meng et al; (1988) investigated the reversibility of gossypol's contraceptive effect. Sixty-one percent of the subjects regained normal fertility with "" median recovery time of 1.1 years." Twenty – two percent remained azoospermic after 1.9 years. "The failure of recovery was strongly associated with longer treatment, greater total dose of gossypol, smaller testicular volume, elevated FSH (follicle stimulating hormone) concentrations and, to a lesser extent, with greater body weight."

Sertoli cells play a crucial role in the development and maintenance of spermatogenesis. Monsees *et al;* (1998) investigated influence of gossypol on the secretory function of cultured sertoli cells, the researchers observed decrease of both lactate and inhibin accumulation apparently resulted from sertoli cell degeneration and death, because viability and mitochondrial function were also reduced. The results suggested that mitochondria of sertoli cells are a possible target for gossypol-induced infertility. In the search of endogenous effector molecules, which interfere with the functions of GAA. Javed and Khan (1999) have studied the in vitro effect of various amino acids on the inhibition of the purified LDH-X by GAA. Histidine, cysteine and glycine were shown to block the effect of GAA. The effects of these amino acids were concentration dependent. Histidine and glycine

protection was found to be complex type in which both the KM and Vmax were decreased compared to control. Arginine, glutamic acid, phenyalanine and valine were found to be ineffective against the inhibitory action f GAA.

To determine the effects of gossypol acetate on guinea pig epidiymal and vas deferens sperm maturity and in vivo susceptibility to in vitro capacitation and the acrosome reaction, Shi and Friend (1985) examined spermatozoa removed from 37 animals fed gossypol acetate (10 – 15 mg/kg/day) for 5 to 9 weeks, and 15 vegetable oil-fed, age-paired control animals. In gossypol-treated, reproductively immature guinea pigs, the number of spermatozoa in the epididymis was markedly reduced (P less than 0.01) compared to controls, whereas the presence of spermatids and spermatocytes increased in the epididymis with the duration of gossypol administration. In sexually mature guinea pigs (given 15 mg/kg/day for 5 weeks), the epididymal sperm survival and forward motility were decreased significantly (P less than 0.025 and P less than 0.01, respectively), although the density of mature spermatozoa was the same as in control animals. The percentage of induced acrosome reactions (26.4+/- 12%) was almost three-fold lower than that of control animals (72.8+/- 4.6%). Also, in 31.5+/-3.8% of spermatozoa from gossypol-treated animals, as compared to only 2.4+/-0.7% of controls, the cytoplasmic droplet failed to migrate to its proper position in the mid-piece and was retained in the neck region. With a few exceptions, spermatozoa from both experimental and control groups had comparable patterns of freeze – fractured membrane differentiations. Susceptibility to the induced acrosome reactions and the position of the retained cytoplasmic droplet reversed within 3 weeks after the end of

gossypol feeding. This study helps to established the suitability of the guinea pig for studies on gossypol-induced infertility (Shi and Friend, 1985)

Researchers in Brazil have undertaken trials of low dose gossypol. These researchers had promising results, but also had concerns about reversibility. In the mid 1990s, the Brazilian pharmaceutical company Hebron announced plans to market a low dose gossypol pill called Nofertil, but the pill never came to market. Its release to the public has been indefinitely postponed due to unacceptably high rates of infertility. Despite this controversy, the Brazilian public's interest in the drug is high. According to a Brazilian women's magazine, over 10,000 men have volunteered for clinical studies of Nofertil.

In 1998, the World Health Organization's Research Group on Methods for the Regulation of Male Fertility reviewed the research on gossypol and concluded that contraceptive research should be abandoned. In addition to the side effects mentioned above, the WHO researchers have major concerns about gossypol's toxicity. The toxic dose in primates is less than 10 times the contraceptive dose.

South to South, an alliance of 16 developing countries researching reproductive health, is looking into gossypol as an alternative to vasectomy. Phase II clinical trials of this method of sterilization are taking place in Austria, Brazil, Chile, China, the Dominican Republic and Nigeria with over 300 men.

Zucker and Zucker (1947) reported that gossypol was an appetite depressant, and that purified gossypol had no toxic properties. The

widespread publicity relative to the possible use of gossypol in the treatment of the obesity in man prompted Eagle (1960) to carry out a series of physiological and toxicological studies of cottonseed pigment glands and pure gossypol. The investigator concluded was as follows:

i. Untreated cottonseed pigment glands vary widely in their acute oral toxicity in the rat, and this toxicity is not proportional to their analyzed gossypol content.

ii. Pure gossypol is toxic to the rat, but to a lesser extent than any untreated cottonseed pigment gland tested.

iii. Repeated doses of gossypol at levels of 10-200mg/kg per day are fatal to the dog.

iv. The acetone and water-soluble fraction of a sample of cottonseed pigment glands was the most toxic material ever isolated from cottonseed.

v. The toxic factors of cottonseed pigment glands that were not extracted by petroleum naphthas or tetrachloroethylene, were partially extracted by ethanol, and completely extracted by diethyl ether and acetone.

vi. Gossypol combination products were considerably less toxic than cottonseed pigment glands and, in four out of five cases, were much less toxic than gossypol.

v. The sensitivity of various animal species to cottonseed pigment glands decreased in the order of guinea pig, rabbit, mouse, rat.

vi. Long-term storage of cottonseed pigment glands for even as long as 9.5 years had no effect on their acute oral toxicity or their analyzed gossypol content.

vii. Cottonseed pigment glands were slightly less toxic when administered in water, on the other hand, gossypol was slightly more toxic when administered in oil than in water.

viii. Pure gossypol fed at various levels in the diets of experimental animals caused body weight depression proportional to the amount fed.

ix. Cottonseed pigment glands are well detoxified when administered in 2 per cent ferrous sulphate solution, and fourteen other reagents cause detoxification of cottonseed pigment glands to varying degree.

x. Cottonseed pigment glands fed to experimental animals depressed body weight considerably more than could be explained on the basis of the gossypol content.

Five conferences on cottonseed processing were held in the Southern Regional Research Laboratory of the US department of Agriculture between 1950 and 1959 with emphasis on the nutritional role of cottonseed meal for animals. The scope of the next conference in 1960 was enlarged to consider the use of cottonseed protein for animals and man and the seventh conference in 1964 was devoted solely to cottonseed protein concentrates in human nutrition. In all these conferences, attention was focused on the role of gossypol. They agreed that the free gossypol content should not exceed 0.045 percent for human consumption.

2.10 Gossypol as Economic Chemical

The importance of gossypol as an economic chemical has been proven in many fields, pharmaceutical, medicinal, insecticide, oil processors, animal feed and protein. It has been reported that gossypol has no significant

physiological effect on the growth of the cottonseed plant but it offers its protection against insects and bacterial attack (Solomon, 1987).

Interactive effects of gossypol and chloroquine as determined by activities of some enzymes and cholestrol level were investigated in rats. Administration of either gossypol or chloroquine decrease the level of cholestrol. A greater decrease was recorded when both were given together. For interactive effects of gossypol and chloroquine it was suggested that gossypol can be employed as a male contraceptive among malaria - infected-populations (Achudume *et al.,* 1998).

The report of Waites *et. al.,* (1998) also suggested a relationship between environmental conditions and spermatogenesis. It has been shown that vitamin E supplementation did not affect gossypol concentrations in plasma and tissue. The highest gossypol concentrations were found in the liver followed by the heart and testis (Velasquez *et. al.,* 1999). In vitro lipid peroxidation of tissue indicated that gossypol acts as an antioxidant in the lipid peroxidation systems. The investigation concluded that cottonseed meal in the diets of bulls did not affect growth or vitamin E status (Velasquez *et. al.*, 1999).

National Institute of Health (NIH), Department of Health and Human services, NDHHS (1996) in their clinical trials in the U.S.A, demonstrated the use of GAA in breast and prostrate cancer. GAA was confirmed by the Institute to exhibit low toxicity relative to other chemotherapeutic agents and does not appear to cause cardiac failure or neurotoxicity. The investigators established the milder side effects of the use of GAA to include mild fatigue, muscle tremor, dry mouth, dry skin, and occasional nausea. This Health

Department concluded that patients treated with GAA, therefore, may be able to continue normal activities (NIH, 1996). Vlietinck *et. al.*,(1998) reported that many compounds of plant origin including gossypol have been identified to inhibit different stage in the replication cycle of human immuno deficiency virus (HIV). Gossypol play an important role in overcoming the problem of rancidity in nutrition industries because gossypol has been shown to have antioxidant properties.

2.11 Utilization of Gossypol and Cottonseed Protein Flours

Cottonseed flour is being investigated as a food source in various parts of the world where cottonseed is available. Cottonseed is the most widely distributed oilseed in tropical and sub-tropical areas all over the world. It was believed in 1969 that one-quarter of the flour potentially available from world cottonseed production could alleviate the edible protein shortages of hungry nations (Bressani, 1969). Cottonseed protein could plays an important role in over-coming the deficit of available protein in the world today, it could be used in three ways; as an additive in bakery products, as a filler in meat-like products and as a protein supplement to diets of cereal grains or of vegetable protein mixtures (Bressani, 1969).

Cottonseed protein as an additive in bakery products reduces dough stickiness, increases the water-binding capacity, increases shelf life, improves machine properties and reduces fat adsorption. Its use as filler in the manufacture of ground meat-type products such as sausages, meat loaves and hamburger patties, gives products of good nutritive value which supplement cereal diets. As a protein supplement, cottonseed flour could

well find its most important outlet. It could, for example, be used to supplement the diet when the cereal is itself of poor nutritional quality.
The traces of gossypol which remain bound in some form in the oil continue to exert a protective action. Gossypol a complex chemical compound occurring in crude cottonseed oil has been shown to have strong anti-oxidant properties and also possess anti-polymerizing properties in view of its polyphenolic nature (WHO/FAO/UNICEF, 1980).

2.12 Acute Aflatoxicosis

Aflatoxin was detected in the tissues of 22 out of 23 children who died of encephalopathy and fatty degeneration of the viscera or Reyes Syndrome (Enomoto, 1978). The levels of aflatoxin was found to be 93ug/kg, 123ug/kg, 127ug/kg in liver, stool; stomach of these children respectively. Between October 1974 and January, 1975 large number of villages in north west India suffered outbreaks of acute aflatoxicosis with severe liver disease and the deaths of 106 people (Enomoto, 1978). Analysis of the suspected samples showed that the maize was heavily contaminated by *Aspergillus* flavus and the affected people could have consumed between 2 and 6mg aflatoxin daily over a period of one month. On 4th November 1968, a three-year old boy was admitted to hospital in Northeast Thailand suffering from fever, vomiting, coma and convulsions (Smith and Moss, 1985). He died six hours later, and autopsy revealed fatty degeneration of the liver, kidney and heart, oedama in the brain with degenerated neurons. The death was investigated and there seemed to be no doubt that he had consumed a large amount of aflatoxin in heavily contaminated boiled rice. In 1970, the Philippines had its own case of presumably acute aflatoxicosis involving 34 children who consumed fried patties prepared from mouldy

flour (Bungay, 1994). All the children developed vomiting and abdominal pain and were hospitalized. Two hour after admission the little girl among them died. No autopsy was performed on the girl. Consequently only a presumptuous diagnosis of acute aflatoxicosis was offered.

2.12.1 Critical Factor in Aflatoxin Control

Many of the biochemical factors controlling aflatoxin formation by the fungus, *A. Parasticus* has been elucidated by food and feed safety researchers (Bhatnagar *et. al.*, 1989). Of all substances known today, both natural and artificially synthesized, aflatoxin displays the highest oral carcinogenicity in a wide range of animals. Several of the chemical intermediates in the aflatoxin pathway have been identified by scientist (Benelt and lee, 1977). Identification of Aflatoxin pathway intermediate allow these compounds to be used as enzyme substrates to detect aflatoxins pathway enzymes. Several critical enzymes including a methlytransferase (Bhatnagar *et. al.*, 1990), catalyzing key steps in the aflatoxin pathway, have been identified, purified and characterized.

An antiserum probe was made against the methytransferase protein to study the molecular regulation of this catalyst (Cleveland and Bhatnagar, 1989); and to identify the gene coding for this aflatoxin pathway enzyme. Cloning of aflatoxin pathway genes will provide molecular probes for future applications in biotechnology. Recent effort in several laboratories have focused on the investigation of the molecular trigger controlling the onset of aflatoxin biosynthesis and precision in activation of a specific steps/ genes in aflatoxin biosynthesis to produce aflatoxin non- producing fungi.

Cotty, (1989) reported genetic approach to control aflatoxin production in cotton. Researchers have identified varieties of corn (Widstorm, 1987; Scott and Zummo, 1988) and cotton (Cotty, 1989b) that demonstrated measurable levels of resistance, which in theory could provide germplasm for enhancement of host plant resistance to useful levels against aflatoxin producing fungi. Efforts to bread for resistance against these toxigenic fungi have been hampered by high variability from sample within a given genotype (Scott and Zummo 1988); and the resistance that was identified is apparently mutagenic and complex. Identification of biochemical "Marker" for resistance against *A. Flavus* would greatly facilitate selection of resistant progeny derived by classical plant breeding or through new genetic engineering technology. It is possible that chemical barriers in crops, the impede fungal development and aflatoxin formation may account for the partial level of resistance identified in corn (Widstorm, 1987; scott and Zummo, 1988).

Neucere and Clevelandna (1989) reported an attempt to characterize fungal or hydrolytic enzymes (chitinases and glucanases) detected in corn kernels that have anti fungal activity in other crop systems. Certain other proteins (presently unidentified) from these corn kernel have demonstrated fungal growth inhibitor properties. Volatile and non-volatile compounds have been identified in cotton leaves (Zerinqeu and McCormick, 1989) that inhibited both fungal growth and aflatoxin production.

Gbodi *et. al.,* (1995) reported the identification of other carcinogenic mycotoxins like sterigmatocystin in feedstuff could be responsible for the increased incidence of high cancer rate in Nigeria. This is because of the

difficulty involved in effective and economic control of aflatoxin contamination of food and feed by traditional agricultural methods.

Gbodi *et. al.*, (1995) also reported recent efforts in several laboratories have focused on developing an in depth understanding of the molecular biology of aflatoxin biosynthesis, how this information can be used to eliminate the toxin from food - chain, understand the regulation and evolution of the aflatoxin pathways and understand the biological significance of aflatoxin to the producing fungus (Kai *et al.,* 1996).

Agboola (1992) discussed the factors affecting fungal infection and mycotoxin contamination of agricultural crop after harvest. The investigator suggested post harvest technologies that can reduce mycotoxin contamination of food crops with special reference to grains, oilseeds, root and tubers. To avoid mycotoxin contamination, it was advised that crops be harvested promptly at maturity and dried quickly after harvest. Threshing should be carried out with great care to ensure that mechanical damage is not inflicted on the crop. Crops should be stored in clean, dry containers (Agboola, 1992).

Similarly Gbodi (2000) enumerates some general principle of storage, which minimize infection of crops with mycotoxin which are presented as follows:

i. Start with high quality, mature crop which is free from mechanical, insect termite or mould damage.

ii. Check the moisture content of the crop before accepting it for storage. Dry the crop if need, to at least the save moisture constant before storage. Also re wetting of dried produce should be avoided at all cost.

iii. Clean thoroughly and disinfect the storage container and the store before placing produce in them.

iv. Do not store warm produce. Warm grains should not be sealed up until it is cool.

v. Store in a clean, cool dry store.

vi. Store at the lowest temperature possible and ensure that the temperature of storage is as uniform as possible.

vii. Whenever possible, use controlled atmosphere or inert atmosphere for storage of grains.

viii. Control insects, rodents and birds during storage using factory and economical pest control. Pestcides should be used with care when needed. Use of chemical fungicides should be avoided if possible.

ix. Inspect the store and produce regularly to monitor moisture, temperature, insect infestation and rodent infestation. Take appropriate remedial action.

x. Adopt scrupulous hygiene around the store in the store and in the produce.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection and Processing of Samples:

One and half kilograms of visibly mouldy cotton seed were collected from two different areas, Sunseed (Nig) Ltd, Zaria in Kaduna State and Kontagora in Niger State. All the samples were collected from store places. The samples were collected in sterile polythene bags.

Similarly, cottonseed cake, crude cotton oil and refined cotton oil were collected from Sunseed Nigeria Limited, Zaria. Refined cotton oil (edible) was also bought at Sabongari Market, Zaria, Nigeria. All samples were transported to the Research Laboratory of Department of Biological Sciences, Federal University of Technology Minna, Nigeria for investigation.

Collected Cotton seed samples were each divided into two: one half was ground and stored at 20°C until analysed for mycotoxins and determination of free gossypol contents, and the other half was used immediately for fungal isolation.

3.2 Isolation of Fungi:

The cottonseed samples used for fungal isolation were delinted mechanically using scarpel blade. They were surface sterilized using 5.25%

sodium hypochlorite solution (Clorox, the Clorox Company, Oakland) and then washed with 10 successive 100ml of sterile distilled water. Ten seeds were placed into each of 10 petri-dishes containing Potato Dextrose Agar (PDA) plates with added 100mg/litre of chloramphenicol.

The plates were incubated at 28 ± 2°C and examined daily for 7-10 days. Fungi that developed on the plates were picked and sub-cultured onto fresh PDA plates to obtain pure cultures. The pure cultures were transferred to agar slants for further characterization and identification.

3.2.1 **Characterization and Identification of Microbial Isolates.**

The characterization of the isolates were based on macroscopic and microscopic appearances which comprised pigmentation, colour of aerial and substrate hyphae, type of hyphae, shape and kind of asexual spore, presence of special structures such as foot cell, sporangiophore or conidiophore and the isolates were determined according to the schemes of Alexopoulos (1962), and Domsch and Gramms (1970).

3.3 **Screening of Fungi for Ability to Utilize Gossypol.**

Fresh agar medium was prepared with the following concentrations: 0.1%, 0.2% and 0.5% gossypol acetate standard (Makor Chemical Limited, Jerusalem, Israel) was added to each demarcated plates to serve as the sole source of carbon and energy.

Discrete fungal colonies were randomly picked and sub-cultured on to fresh gossypol – agar plates. The plates were incubated at room temperature (28 ±2°C) and the growth was observed daily for 7-10 days. At the end of

incubation period, growth of inocula was determined visually by the turbidity of the culture as compared with the un-inoculated control. The growth characteristics of fungi were rated as high (+++), moderate (++), minimal (+) and no growth (-).

3.4 **Isolation and purification of gossypol:**

The rapid extraction of gossypol from cottonseed and cottonseed by-products method was adopted (Smith, 1985) Twenty grams of finely ground flaked meal of the cottonseed sample was weighed into a 500ml capacity Erlenmeyer flask and thoroughly mixed with absolute ethanol (98% ethanol). The suspension was allowed to stand for fifteen (15) minutes with little agitation and occasional stirring to yield an extract containing gossypol.

The extract was filtered to free it from residual meal under reduced pressure on Buchner funnel fitted with Whatman No 44 filter paper. Ten millilitre of ether (40 - 60°C) was added to the ethanolic extract and water was added drop by drop in sufficient quantities in order to cause the gossypol to precipitate from aqueous layer and dissolve in the ether layer. Gossypol acetic acid was thus obtained by the addition of 10ml of (80%) glacial acetic acid to the ether extract. The combined ether-acetic acid solution was evaporated from the mixture by heating at 30°C - 60°C for 30 minutes using magnetic stirrer hot plate (Stuant Scientific, U.K). The crude gossypol acetic acid was again hydrolyzed by dissolving in 10ml of ether and heating the combined ether solution was added with small amount of sodium dithionite, until all the ether had evaporated, and gossypol acetate obtained.

3.5 Analysis of Free Gossypol Contents of Cottonseed Samples

Oil was extracted from cottonseeds using the standard procedure of Soxhlex (Electrothemal, Britain). Petroleum ether (b.p 40-60 °C) was used.

3.5.1 Preparation of the Standard Curve

Standard curve was prepared using gossypol acetate standard (Makor Chemical Limited, Jerusalem, Israel). Exactly 2.232mg of gossypol acetate was weighed and transferred quantitatively to a 20ml volumetric flask using 8ml of acetone. To this were added glacial acetic acid (0.1ml) and distilled water (6ml). This was made up to 20ml with the acetone in a volumetric flask. These standard solution (stock) contained 0.112mg of gossypol per ml and it is stable at room temperature (28 $\pm$ 2°C) for one hour.

Exactly 1ml, 2ml, 4ml, 8ml, portions of stock solution were each pipetted into four different 20ml volumetric flasks, the flasks were made up to volume with acetone and then kept in the dark for colour development. After about fifteen (15) minutes, the absorbance of each solution was measured against a reagent blank at 440nm in a UV Spectrophotometer (Camspec, Model 201)

3.5.2 Determination of Free Gossypol Content of Cottonseed

One gram of finely ground dried cottonseeds was accurately weighed and transferred into 250ml conical flask. About 50ml of (70%) aqueous

acetone was added, a polythene screw cap was fitted on the flask and clamped on to A Wrist Action Shaker (Heidolph Unimax 2010) and shaken for one hour. The mixture was filtered under reduced pressure on Buchner funnel fitted with Whatman No 44 filter paper, the first 5ml of the filtrate was discarded and remaining filtrate was collected (AOCS, 1969). Exactly 10ml of the duplicate aliquot of the filtrate was pipetted into each of two separate 25ml conical flasks, each of the filtrate was labeled samples (A) and (B).

To sample (A), two drops of 0.10ml of 10% aqueous thiourea (E. Merk, Darmstadt) and one drop 1.2 MHCl (East Anglia Chemical GPR) was added to 25ml with equeous isopropyl alcohol (M & B Ltd, Dagemham). To sample (B) two drops of (10%) aqueous thiourea, one drop of 1.2 MHCl and 2mls of aniline were added. A blank was prepared equal of that aliquot solution from (70%) aqueous acetone (Charwell Health Essex, England) used as a blank using solution (A) as a reference. The sample aliquot designated as (B) and the reagent blank was heated in a boiling water bath (Nickel Electro Limited Westons Marc Avon, 29988) at 100°C for thirty (30) minutes after which the solution was removed from the bath and 10ml of 80% aqueous propan-2-ol was added to each solution to effect homogeneity and allowed to cool to room temperature and diluted to volume with 80% propan-2-ol. The absorbance maxima of designate solution B was measured at 440nm after standardizing (Camspec Model 201) UV Spectrophotometer equipped with clean cell of 1cm light path against the blank. Triplicate analysis was carried out for each of the sample analysed.

3.5.3 Determination of the Percentage Free Gossypol Content of Cottonseed

This was determined in cottonseed kernel, cottonseed cake, crude cotton seed oil and refined cotton oil (edible) from the absorbance value using formula below:

Calculation of free (toxic) gossypol) content of the cottonseed and cottonseed by-products samples (AOCS, 1969)

$$\text{Free gossypol \%} = \frac{5G}{WV}$$

where G = mg gossypol in the sample
W = Sample weight, in grams
V= Volume of sample aliquot used

Similarly: Quantity of gossypol in g/ml = $\frac{\text{weight of sample (gm)}}{\text{Volume of solvent (ml)}}$

3.6 Determination of Free gossypol Content of Cottonseed and Cottonseed By-Products Samples

The method of Mathur *et al.*, as modified by Akanya, (1976) was used. About 25mg of the oil extract were accurately weighed and 100mg of anhydrous sodium sulphate were added. 5ml of petroleum ether (40 – 60°C) were then added to the mixture and after mixing thoroughly, it was centrifuged for ten (10) minutes at speed five in an angle – head rotor MSF bench centrifuge. The supernatant liquid was discarded. The extraction was repeated twice and each time discarding the supernatant liquid. Diethyl ether 10ml was then added to the residue with shaking. After centrifuging again for another ten (10) minutes, the supernatant liquid was removed and the extraction was repeated twice. The combined ether extract was

evaporated to dryness in a fume cupboard. The resultant residue was dissolved in ethanol and then quantitatively transferred into a $25cm^3$ volumentric flask. This was then made up to volume with the same solvent. 2ml of this extract and 1ml of the ferric chloride bipyridyl reagent (Analar BDH Chemical Limited Poole, England) were pipetted into another $25cm^3$ volumentric flask and then made up to volume with absolute ethanol. After fifteen minutes in the dark, the absorbance was measured at 510nm against a reagent blank. Triplicate analysis was carried out for each sample. The quantity of free toxic gossypol was determined from a standard curve.

3.7 **Extraction, Identification and Quantitation of Mycotoxin.**

The procedure outlined in the AOAC (1980), as modified by Ehrlich and Lee (1984); and Gbodi, (1986) was used. Fifty grams of ground cottonseed sample was weighed into 500ml capacity conical flask and 25ml methylene chloride were added. The flask was shaken for thirty (30) minutes and the contents filtered under reduced pressure on Buchner Funnel fitted with 18.5cm circle rapid filter paper. At least, 200ml of the filtrate was collected. Aliquots (50ml) were placed in separate 100ml conical flasks fitted with glass stoppers for aflatoxin, and zearalenone assay.

On one of the above 50ml aliquot, aflatoxin assay was made following the procedure outlined in the AOAC method (1980). Chromatographic column was set up with glass wool and a beaker. To this was added methylene chloride (150ml). The methylene was drained half way into a beaker and 1 cup of unhydrous sodium sulphate was added and sides of the column was washed down with methylene chloride (Solvay Chemical, England). 130ml of hexane was added followed by 130ml diethyl ether. The

elution solvent were collected into a new beaker, it was heated gently over a water bath to evaporate. The plates were developed in ether-methanol-water (96:3:1). Ochratoxin assay was by the modified method of Patterson (1978). Two dimensional thin – layer chromatography using 2 solvent system were recommended. The plate was first developed in benzene: hexane (3:1), it was dried in air and developed secondly in benzene: acetic acid (95+5) after which the plate was sprayed with alcoholic aluminium chloride 20%. One molar phosphoric acid was substituted for 4M and methylene chloride was substituted for chloroform. The intensities of the fluorescence of the standards and samples were visually compared using UV light at 365nm.

For zearalenone assay, the modified method of AOAC (1980) was essentially used, except for the use of phosphoric acid and methylene chloride in place of chloroform as extracting solvent, as described by Ware and Thorpe (1978).

3.7.1 **Identification of Mycotoxins**

Aflatoxin was confirmed by spraying the thin layer chromatographic plate with aqueous sulphuric acid (50/50 $^{v}/_{v}$), dried and viewed under uv light at 365nm when the spots of ochratoxin A fluorescence changed from blue green to bright blue. Zearalenone was also confirmed by spraying the plate with alcoholic Aluminium Chloride and viewed under uv light at 365nm when the fluorescence intensity increased.

3.8 Determination of the Lipid Content of Cottonseed

One gram of the finely ground cottonseed sample was first dried to constant weight in a vacuum oven (55°C, 0.1mmHg) in order to determine the percentage moisture content.

150ml of solvent was added to the flask and the Soxhlex extractor unit was assembled. An extractor thimble, previously dried in an oven (60 – 80°C, 4 hours) was weighed, and 10g of cottonseed sample were accurately weighed into the thimble. Using forceps, the thimble was then placed into the extractor unit and cold water was circulated through the condenser while heating on a mantle. The heating temperature was adjusted until the solvent was refluxing at a steady rate. Extraction was carried out for six (6) hours after when heating was stopped, and the apparatus was allowed to cool. The thimble and content were removed from the unit and sample were dried to constant weight in an oven (60 – 80°C). it was allowed to cool in a desicator before being re-weighed. The flask containing the extracted oil was removed from the Soxhlex unit and the solvent was evaporated in a rotary evaporator. The flask and content were then dried to constant weight in an oven fitted with a convector fan at 60 – 80°C. The exractable lipid content of the sample was determined.

3.9 Determination of Protein Content of Cottonseed

The protein content was determined using the micro-Kjeldahl Method (Pearson, 1972). This is an estimation of the crude protein or the nitrogenous matter of the sample. One gram of the six-hour lipid extract above were accurately weighed into a 30ml Kjeldahl tube. To this was added potassium sulphate (1g), mercuric oxide (40mg) and copper sulphate

the tubes were heated on an electric heating mantle. After refluxing for about 4 –5 hours, the colourless digestion mixture was diluted with distilled water (5ml) to dissolve any solids.

The mixture was then transferred quantitatively to a Markham distillation apparatus. The digestion flask was then thoroughly rinsed with distilled water (about 10ml) and these washings transferred to the still. 0.1m hydrochloric acid (100ml) was placed in a receiving flask and (50%) NaOH solution (20ml) was added to the mixture in the still. With a current of steam, the ammonia distilled over until about 50ml of the distillate had been collected. Two drops of bromothymol blue indicator were added and the excess acid was titrated with 0.1M NaOH. Total nitrogen was determined by the Kjeldahl method and the result multiplied by 6.25 to give crude protein.

3.10 **Determination of Ash Content of Cottonseed.**

The total ash content was determined by heating ground sample using muffle furnance at 570°C for six (6) hours and the difference between initial and final weights taken and ash content calculated on percentage basis as described by (AO AC, 1980)

3.11 **Determination of Nitrogen Free Extract + Fibre.**

The method adopted for crude fibre determination was the procedure outlined in the Association of Official Analytical Chemists (1980).

Two gramms of the dried fat - free sample residue was transferred into 600ml beaker and 200ml of hot sulphuric acid was added. The mixture was boiled gently for 30 minutes and filtered while hot with Whatman No 44

boiled gently for 30 minutes and filtered while hot with Whatman No 44 filter paper. The residue was again transferred back to the beaker. 200ml of hot sodium hydroxide solution was added. After boiling for 30 minutes, the mixture was filtered through a porous crucible and was washed with boiling water. The residue obtained was again washed with 1% hydrochloric acid and then with boiling water. The residue was dried over night at 100°C after washing again with ethanol.

The contents. After washing, were ashed at 500°C for 3 hours using muffle furnance, after cooling, the weight of fibre was calculated on percentage basis.

Nitrogen Free Extract was calculated when the sum of % protein, % lipid, % crude fibre and % ash were subtracted from 100. The remainder accounts for the Nitrogen Free Extract (NFE).

Thus % NFE = 100 – (% protein + % Lipid + % Crude fibre + % ash).

CHAPTER FOUR

4.0 RESULTS

4.1 Fungi Isolated

A relatively limited number of fungal species have been isolated. The results obtained indicated that, the predominant fungi species in cottonseed and cottonseed by-products are *Aspergillus spp* (24%), especially *Aspergillus flavus (22%)*, this is followed by *Penicillium spp* (16%) and *Mucor spp* (16%). *Rhizopus spp* (12%) has the least frequency occurrence (Table 1).

Table 1. Frequency of isolation of fungi from cotton seed.

Fungi Isolates	**Frequency of Occurrence**
Aspergillus flavus	8 (32)
Aspergillus spp	6 (24)
Penicillium spp	4 (16)
Mucor spp	4 (16)
Rhizopus spp	3 (12)
TOTAL	25

Numbers in parenthesis represent percent frequency of occurrence of isolates.

4.2 Determination of Utilization of Gossypol by Isolated Fungi from Cottonseed

The result (Table 2) shows that *Mucor spp* are the most efficient utilisers of gossypol as sole sources of carbon and energy followed by *Rhizopus spp* at all the concentrations investigated *Aspergillus flavus,*

Aspergillus spp and *Penicillium spp* were unable to utilize gossypol at higher concentration (0.5%)

Table 2. Ability of fungi to utilize gossypol as sources of carbon and energy.

ISOLATED FUNGI TESTED	Concentration of prepared Gossypol from Cottonseed		
	(0.1%)	(0.2%)	(0.5%)
Rhizopus Sp AZF – 01	+++	++	+
Aspergillus Sp AZF – 02	++	+	-
Mucor Sp AZF – 03	+++	++	+
Rhizopus Sp AZF – 04	+++	+	+
Mucor Sp AZF – 05	++	+	+
Aspergillus Flavus AZF – 06	++	+	-
Mucor Sp AZF – 07	+++	+++	++
Rhizopus Sp AZF – 08	+	+	+
Aspergillus Sp AZF – 09	++	+	-
Aspergillus Flavus AZF – 10	+	+	-
Aspergillus Sp AZF – 11	++	++	-
Mucor Sp AZF – 12	++	++	++
Penicillium Sp AZF – 13	-	-	-
Aspergillus Flavus AZF – 14	++	+	-
Penicillium Sp AZF – 15	++	++	-
Penicillium Sp AZF – 16	-	-	-
Penicillium Sp AZF – 17	+++	++	-
Aspergillus Flavus AZF – 18	+	+	-
Mucor Sp AZF – 19	++	+	-
Aspergillus Flavus AZF – 20	+	+	-
Mucor Sp AZF – 21	++	++	-
Aspergillus Flavus AZF – 22	+	+	-
Aspergillus Flavus AZF – 23	++	+	-
Mucor Sp AZF – 24	++	++	-
Aspergillus Flavus AZF – 25	+	+	-

+++ = Maximum growth

++ = Moderate growth

+ = Minimal growth

- = No growth

AZF = Coded plates

4.3 Determination of the Free Gossypol Level of Cottonseed and Cottonseed By – Products.

The wholeseed was found to have the highest amount of (0.3802%) and (0.3811%) in the two different methods employed while the least amount of (0.0252%) and (0.0256%) of free gossypol was recorded for the edible oil in method of Mathur *et al.,* and AOCS official methods respectively (Table 3).

Table 3. Free Gossypol Content of Cottonseed and Cottonseed by-products

Sample Type	**Source**	**Value (% ± SD) free gossypol contents**	
		Method 1*	Method 2**
Wholeseed	Sunseed Nig. Ltd, Zaria	0.3802 ± 0.0129	0.3810 ± 0.0015
Wholeseed	Gineary, Kontagora	0.3814 ± 0.0081	0.3811 ± 0.0023
Kernel	Sunseed Nig. Ltd, Zaria	0.3604 ± 0.0279	0.3595 ± 0.0129
Cottonseed Cake	Sunseed Nig. Ltd, Zaria	0.1749 ± 0.0162	0.1708 ± 0.0005
Cotton Oil (Crude)	Sunseed Nig. Ltd, Zaria	0.2201 ± 0.0281	0.2204 ± 0144
Cotton Oil (Edible)	Sabongari Market, Zaria	0.0252 ± 0.0072	0.0256 ± 0.0067

* Method of Mathur *et al.,*

** AOCS method

4.4 Screening of Mycotoxin In Cottonseed

Three mycotoxins, aflatoxin, ochratoxin A; and zearalenone were screened for in each sample type. No mycotoxin was found in any of the samples.

4.5 Proximate Analysis of Cottonseed

Table 4. Proximate Analysis of Cottonseed

Parameter	**Value (%) (a)**	**Value (%) (b)**
Lipid	22.0	23.0
Moisture Contents	7.2	7.6
Total Ash	1.9	2.0
Protein	20.0	22.0
Nitrogen Free Extract + Fibre	56.1	53.0

(a) Cottonseed collected from Sunseed (Nig) Ltd. Zaria – Kaduna State.

(b) Cottonseed collected from Kotangora, Niger State.

CHAPTER FIVE

DISCUSSION

Little information is available on the mycoflora and mycotoxins infecting and contaminating cottonseed and cottonseed by-products. Uraguchi and Yamazaki (1978) reported the incomplete and imprecise knowledge of the mycoflora of cottonseed. The investigators gave a list of fungi isolated from field and stored seeds mainly from the U.S.A.

In a study of mycoflora and mycotoxins found in stored mouldy cottonseed in Plateau State, Nigeria, Gbodi, *et al.*, (1989), isolated many fungal species including *Aspergillus flavus,* the main fungus predominantly identified in the present study. De'mendonca and Alves (1973) in a study of fungi and bacteria infecting cottonseed in Mozambique identified some field and store fungi infecting cottonseed and cottonseed by products. Some of their findings included *Aspergillus spp, Aspergillus flavus and Rhizopus spp,* which were also isolated in the present study.

Aspergillus flavus strains produce many types of mycotoxin including aflatoxins, oxalic acid and tremorgens (Hesseltine, 1974). Aflatoxins are usually mixtures of four closely related compounds of known structure which have been termed aflatoxins B1, B2, G1 and G2. The B1 form is the one usually found in greatest amounts and it also appears to be the most toxic.

The results as presented in Table 1. is largely in agreement with the findings of Gbodi (1986) who gave the fungi presented in food and feed stuffs *as Aspergillus niger, Aspergillus quadrilineatus. Aspergillus nidulan.*

Aspergillus flavus. Gbodi, (1986) also reported that only aflatoxin was found in cottonseed samples. *Aspergillus spp and Aspergillus flavus* were also detected in the present study.

Judging from the isolates (fungi) growth when gossypol was the sole source of carbon, *Mucor spp* and *Rhizopus spp* (Table 2.) exhibited high ability in utilizing gossypol pigment effectively in different concentration levels. Eighteen (72%) of isolates from cottonseed did not produce turbidity at concentration of (0.5%) of gossypol pigment. This suggest that they could not utilize gossypol pigment at this concentration as sole source of carbon and energy.

Aspergillus spp, Aspergillus flavus and Penicillium spp could not utilize gossypol as sole source of carbon effectively at high concentration. The results of the present study revealed that the ability to breakdown gossypol pigments by fungi is not restricted to a few species, it as wide spread among the fungi infecting cottonseed. Of the twenty-five (25) fungi screened, only 2 (8%) fungi isolates could not use gossypol as sole source of carbon. And these were made up of two Penicillium isolates (Table 2).

Judging from the morphological characteristics, colour and growth pattern of the isolates (appendix 1) one could speculate that some isolates are mycotoxigenic in character.

World wide interest in protein malnutrition has stimulated a lot of research into finding alternative sources of protein. The fact that some fungi isolates were able to utilize gossypol effectively for their growth showed that they have enzymes that can break down gossypol. These fungal isolates can

be mass cultivated and enzymes for gossypol breakdown can be identified and extracted. The extracted enzymes may then be used for the treatment of cottonseed cake for the removal of gossypol. The degossypolised cake can be fed to human and monogastric animals without harmful effects. This would also serve as cheap source of good protein.

Knowledge of the amino acid content of cottonseed meal or of any material containing proteins will serve as a guide to its nutritive value. According to Hale and Lyman [1980] amino acids composition of cottonseed meal include the ten nutritionally essential amino acids [Arginine, Histidine, Isoleucine, Leucine, Lysine, methionine, Phenylaleanine. Threonine, Tryphtophan, Tyrosine, Valine].

Lyman [1979] revealed that the inactiviation of gossypol during processing of cottonseed meal is accomplished through the formation of an insoluble, inert gossypol-protein complex, which results not only in rendering the gossypol harmless but in a loss in nutritional value of the protein. Supplementation of cottonseed proteins with lysine was reported by Phelps [1982] to give excellent results in the feeding of rats, chicks and swine, again indicating the key role of this amino acid in the nutritive value of cottonseed cake and meal. Phelps [1982] reported that cottonseed meal is a little higher in methionine than soybean but somewhat lower in lysine.

Based on feeding studies Bressani [1980] recommended an all – vegetable protein mixtures for the supplementary feeding of young children. One of these mixtures contained 9 % of cottonseed flour. This formular was found to have a good protein quality in tests with chicks, rats and children. Another mixture containing 38 percent cottonseed flour was subjected to extensive biological testing. In chicks the mixture produced good growth and feed efficiencies, and no toxic effects were detected. The cottonseed

flours employed in the mixtures were specially prepared for human consumption and contained over 50 percent protein of a relatively high biological value. They had approximately one percent total and 0.04 to 0.06 percent free gossypol. On the basis of extensive and favourable biological and clinical findings. It was concluded that the mixture is of good quality, free of toxic materials and the products were recommended for the supplementary and mixed feeding of young children and as low-cost protein-rich foods of good quality for persons of all ages.

In a review of mycotoxin in feed ingredients Scott (1978) reported the occurrence of only aflatoxins in cottonseed. Similarly Gbodi (1986) in his study of mycotoxins in Plateau State reported the presence of only aflatoxin in mouldy cottonseed. Although, fungi isolated from present study are known to be potential mycotoxins producers. Except aflatoxin G1 and G2 that can not tested, aflatoxin, zearalenone and ochratoxin A, were not detected in any sample the possible reason why aflatoxins was detected in investigation of Scott, 1978, and Gbodi, 1986, but not detected in the present findings may be due to differences in the effects of environmental and physical status of the samples analysed.

According to Joffe; (1965) aflatoxin is produced only when certain environmental conditions such as temperature, humidity, level of oxygen, physiological properties, chemical and biochemical characteristics of the agricultural products which serve as the substrate for aflatoxin production are satisfied. For example, it is noteworthy that, of more than hundred *Aspergillus flavus* isolates obtained from a toxic Uganda groundnut meal, only 10 percent produced the flourescent aflatoxins. Another possible factor might be due to the different reagents used which effectively destroy aflatoxin during processing especially Hexane and acetone which effectively

destroy aflatoxen during processing. Park *et al;* (1988) in their studies of chemical inactivation of aflatoxins in groundnut and cotton seed meals reported that ammonia, methylamine, sodium hydroxide and formaldehyde reduced aflatoxin levels and would be of practical utility for large – scale treatments, they also presented data on the effects of various reaction parameters including time, temprature and moisture content on the efficiency of these reagents. Goldblatt 1970 reported that ammoniation with ammonia, either anhydrous or as a hydroxide under pressure, reduces aflatoxin in both contaminated cottonseed meals and groundnut meals by up to 99%.

Gbodi (1989) speculated that the reason why only aflatoxin was detected in cottonseed might be that although cottonseed could be infected with ochratoxin and zearalenone producing fungi, it may not be a good substrate for production of these mycotoxins.

The Tropical Institute (TPI), London, in its studies of certain Nigerian cottonseed variety in 1962 reported the presence of high gossypol [0.65%] for the Allen-type varieties. Akanya *et al;* (1989) in their studies of different cottonseed of samaru varieties an offshoot of the Allen-type reported much lower value of free gossypol content between the range of 0.35% - 0.393%. Similar value of 0.392% of free gossypol content (Table 3.) was obtained in the present study for samaru cottonseed variety. Free gossypol contents of *Moco* variety seed, an exotic variety from Brazil (*G. hirsatum var. marie galante)* being introduced into India varied between value of 0.952%. to 1.120% (Martinez, 1979).

The Regional Research Laboratory Hyderabad reported the free gossypol content of the kernels which lie between the range of 0.61-0.88%.

These values are high compare with the value of 0.3608% obtained from samaru type used in the present study.

Several studies have been made on the free gossypol contents of Indian cottonseeds and their kernels by Thirumala Rao *et al;* 1960, Lyman, 1979; Martinez 1979, Hale and Lyman, 1980. The free gossypol contents of Indian cottonseed kernels of *G. hirsutum, G. herbaceum and G. arboreum* (the latter two species categorized as desi) and *G. bardadense* varieties varied between 0.80% and 1.68%. The tropical climate of India might be responsible for presence of high gossypol.

Also, free gossypol contents of cottonseed cakes produced in India in a pilot plant were found to be around 0.10 percent (Thirumala Rao *et al*; (1960). In this study, a value of 0.1749% was obtained for cottonseed cake. The difference may be due to the variety of cottonseed used in producing the cake. However some physical and physiological factors are known that can determine the level of gossypol in cottonseeds. Such factors include, soil type, availability of water, temperature and humidity among others. Also the difference in extraction procedures of the oil mills may have left a meal with high concentration of the gossypol.

In the USA. Hale and Lyman (1980) reported high free gossypol contents of screw pressed (expeller) oils (crude) to vary from 0.25% - 0.47%. High free gossypol contents of typical Indian cotton oils (crude) was also reported by Lyman (1979) to lie between the range of 0.254% to 0.353%. In this study cotton oil (crude) showed free gossypol content of 0.2204% (Table 3).

The free gossypol contents of typical Indian cottonseed oils (edible) *Lakshmi and Buri* had values of 0.088% and 0.099% respectively (Lyman

for refined oil (edible) of 0.025% is the only value below the 0.045% free gossypol, the WHO/UNICEF/FAO (1980) recommended value for human consumption.

The two different methods employed for the gossypol determination in this study viz AOCS. official tentative method and Mathur *et al*; method gave very similar values. It is of interest to note that each method has its own advantages and disadvantages. AOCS (1969) is least laborious if pure gossypol is required from cottonseed and sufficient facilities are available and time is not important. Mathur *et al*; is very useful and most advantageous because it is a less laborious and faster method. The two methods require expensive solvents and chemicals for their determinations. Also solvents require for both methods are toxic and highly combustible and should be handled with care.

The source of the cottonseed used in the oil extraction mill is the Nigerian Cotton Board, which markets seeds obtained from farmers directly to the mills. These seeds have been provided earlier to the farmers by the seed multiplication centres, which provides specific varieties to specific zones of the cottonseed growing region (IAR Report, 1988). Investigation revealed that most varieties of cottonseed found in northern Nigeria like Zaria, Kano, Funtua and Gombe Oil Mill were improved varieties of Samaru type - an off shoot of the Allen type varieties. Cottonseeds collected at Kontagora gineary are traced to these varieties.

Akanya *et al*; (1989) reported that protein content for all the varieties of cottonseed lie between the range 33.00 – 35.40%. These values are much higher compared with the values of 20.0% and 22.0% recorded in the present findings (Table 4a – 4b). The reason may be due to the difference in

present findings (Table 4a – 4b). The reason may be due to the difference in the climatic conditions prevailing during the period of the growth of cottonseed. Considering the Indian seeds specieswise, Murti *et al;* (1961) reported that sea island cottonseed has the highest protein content of 20.9% which is about the same as that of cottonseed reported in the USA (20.8%). The protein content of the seed of the American types grown in India with value of 19.9% is slightly lower than that of the USA seed (20.8%).

Akanya *et al;* (1989) reported lipid content of samaru types varieties of cottonseed to range between 23.10% - 38.43%. In the present study, lower lipid contents were obtained from seeds collected from different locations (22.0% and 23.0%). This variation may be attributed to the difference in climatic factors under which they were cultivated.

In this study moisture content of the seed samples showed values of 7.20% and 7.6% for seeds collected from Sunseed (Nig.) Ltd. Zaria and Kontagora respectively. Murti *et al;* (1961) reported the moisture content of desi type seeds obtained from India and America to be 8.0 and 7.9% respectively. These values are higher than that obtained in the present studies. Afzal (1967) published the analysis of 88 samples of cottonseed of both desi and American type obtained from 10 provinces of Indian. He classified cottonseed into three categories: Low fuzz (desi), low fuzz (American type) and high fuzz American type, moisture percentages quoted for these categories were 6.8%, 6.7% and 6.3% respectively. These values are lower than that obtained in the present studies.

In this study, the value obtained for total ash (1.9%) for seeds collected from Sunseed (Nig.) Ltd. Zaria is similar to that obtained (2.0%) for seeds collected from Kontagora. The present study showed that the cottonseed

collected from Sunseed (Nig.) Ltd. Zaria and Kontagora contained 56.10% and 53.0% of Nitrogen Free Extract + Fibre.

Cottonseed constitutes a large potential source of high protein. The present study revealed an interesting relationship between protein quality and gossypol. These findings also reveal that many isolates utilized gossypol as sole source of carbon and energy. The results obtained provide a clear indication of the promising role fungal could play in utilizing and transforming gossypol into non-toxic compounds to improve the nutritive quality of cottonseed by products, the nutritional potentials and economic value of cottonseed and its by products could be utilized and exploited to increase protein supply in Nigeria. These could play significant role in overcoming the deficit of available protein in the world. Thus, economic ways of isolation and utilization of gossypol and fungal from cottonseed and cottonseed by-products.

SUMMARY AND CONCLUSION.

SUMMARY

The present findings revealed that the predominant fungi species in cottonseed and cottonseed by – products in Nigeria are *Aspergillus spp* especially *Aspergillus flavus*, the sub-dominant fungi species are Penicillium *spp* and *Mucor spp. Rhizopus spp* has the least frequency level. Judging from their growth, when gossypol was sole sources of carbon, *Mucor spp* and *Rhizopus spp* exhibited high ability in utilizing gossypol pigment effectively in different concentration levels. No Aflatoxins, Ochratoxin A, Zearalenone were detected in all the samples analyzed.

Determination of free gossypol shows that the cottonseed have the highest amount of free gossypol 0.3802% and 0.3810% in the two different methods of AOCS (1969) and Mathur *et al;* Of all the samples obtained for free gossypol that of refined oil was the only one (0.025%) was below the maximum recommended value of 0.045% for human consumption (WHO/UNICEF/FAO 1980).

Mucor spp and *Rhizopus spp* were found to be effective users of gossypol for their carbon sources. These two spp can be mass cultivated and gossypol catabolizing enzymes could be extracted from them to improve the nutritive quality of cottonseed and by-products.

CONCLUSION

From this study the following conclusion can be made:-

1. The main fungi found in cottonseed and cottonseed by – products are *Aspergillus flavus*, *Penicillium spp; Mucor spp, Rhizopus spp and* Aspergillus spp.

2. Many isolates utilized gossypol as sole sources of carbon, but *Mucor spp and Rhizopus spp* were the most efficient utilizers of gossypol.

3. Aflatoxin, Zearalenone and Ochratoxin A, were not detected in the samples.

4. Of all the samples analyzed for free gossypol content of cottonseed and cottonseed by – product, the value reported for refined oil (edible) of 0.025% is the only value below the maximum recommended value of 0.045% for human consumption (WHO / UNICEF / FAO 1980).

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APPENDIX

Appendix 1A: Morphological Characteristics and Identification of Fungi Isolates

Isolate No	Colour of Aerial Hyphae	Colour of Substrate Hyphae	Shape and Kind of Asexual Spore	Presence of Special Structure	Appearance of Sporangiophore Conidiophore	Characteristics of Spore Head	Probable Organism
AZF - 01	Black	Black	Black oval tiny Sporangiospore	Rhizoid stolon and columella	Long sporangiophores arising at the node where the rhizoid form	Large and black sporangia with (cup shaped) apophyses carrying columella	*Rhizopus sp*
AZF – 02	Green	Brown	Oval greenish conidia	Foot cell present	Long, erect non - septate	Multi nucleate globose vesicle with radiating steringmata	*Aspergillus sp*
AZF – 03	White and cottony	Dark gray	Round black sporangiophore	Round columella present	Unbranched sporangiophore	Round black sporangia containing sporangiophore	*Mucor sp*
AZF – 04	White and closely woven	Light brown	Black oval sporangiophore	Much branched rhizoids and stolon	Long, erect sporangiophore	Sporangiophore terminating at black sporangia	*Rhizopus sp*
AZF – 05	White brown	Brown	Round brownish spore-like	Round columella present	Unbranched sporangiophore	Round black sporangia containing sporangiophore	*Mucor sp*

Appendix 1B: Morphological Characteristics and Identification of Fungi Isolates

Isolate No	Colour of Aerial Hyphae	Colour of Substrate Hyphae	Shape and Kind of Asexual Spore	Presence of Special Structure	Appearance of Sporangiophore Conidiophore	Characteristics of Spore Head	Probable Organism
AZF – 06	Green	Brown	Globose greenish conidia	Foot cell present	Long, erect sporangiophore	Multi–nucleate globose vesicle	*Aspergillus Flavus*
AZF – 07	White and cottony	Dark gray	Round black sporangiophore	Round columella present	Unbranched sporangiophore	Round black sporangia containing sporangiophore	*Mucor sp*
AZF – 08	Black	Black	Black oal tiny sporangiophore	Rhizoid stolon and columella	Long sporangiophores arising at the node where the rhizoids form	Large and black sporangia with (cup shaped) apophyses carrying columella	*Rhizopus sp*
AZF – 09	Bluish green	Dark brown	Globose greenish conidia	Foot cell present	Long, erect, non - sepatate conidiophore	Multi nucleated vesicle with radiating sterigmata	*Aspergillus sp*
AZF – 10	Green	Brown	Globose greenish mature conidia	Foot cell present	Long, erect sporangiophore	Multi–nucleate globose shaped	*Aspergillus Flavus*
AZF – 11	Yellow	Brown	Oval greenish conidia	Foot cell present	Long, erect, non – sepatate conidiophore	Swollen vesicle bearing sterigmata	*Aspergillus sp*
AZF – 12	White and cottony	Dark gray	Round black sporangiophore	Round columella present	Unbranched sporangiophore	Round black sporangia containing sporangiophore	*Mucor sp*

Appendix 1C: Morphological Characteristics and Identification of Fungi Isolates

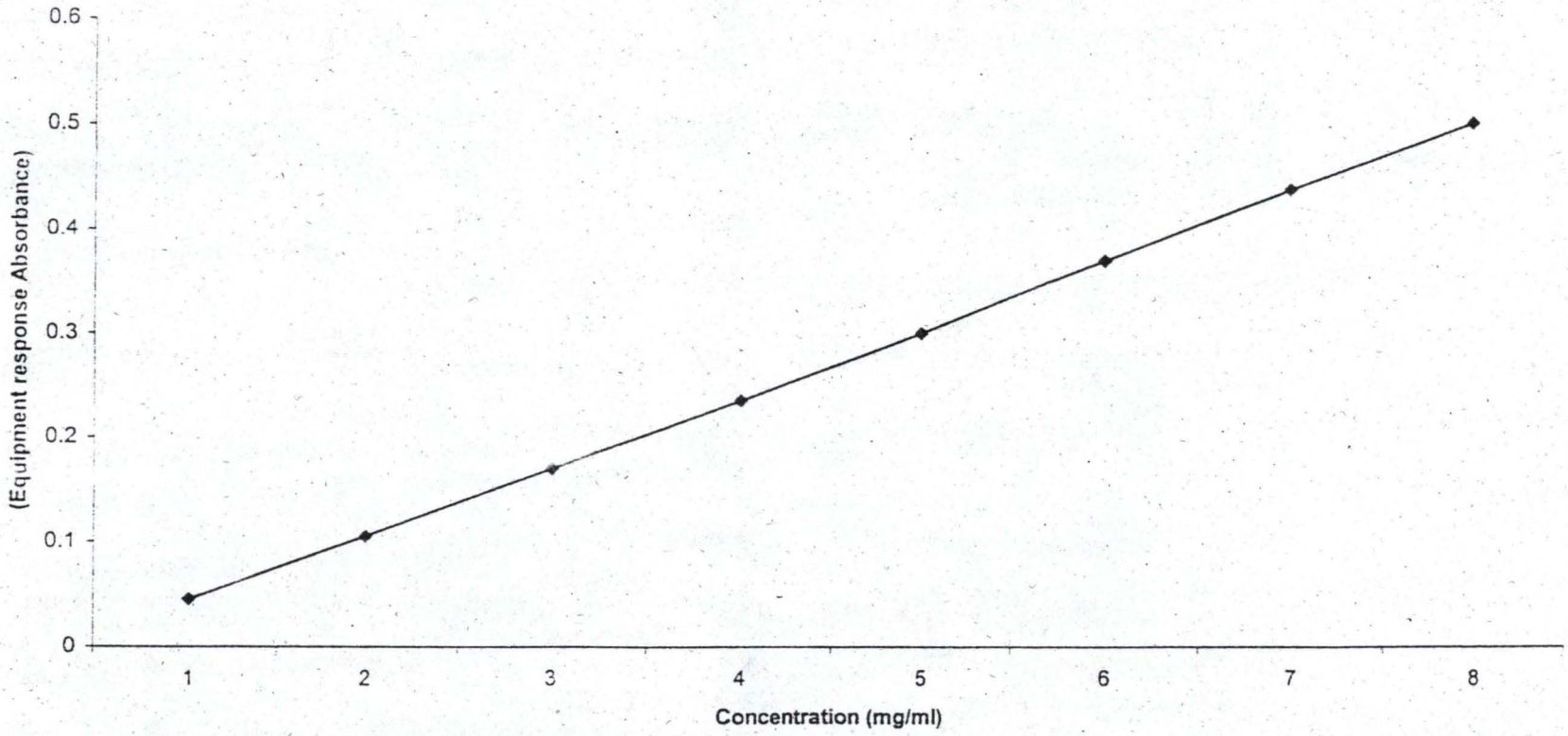
Isolate No	Colour of Aerial Hyphae	Colour of Substrate Hyphae	Shape and Kind of Asexual Spore	Presence of Special Structure	Appearance of Sporangiophore Conidiophore	Characteristics of Spore Head	Probable Organism
AZF – 13	Green yellow	Dark brown grew	Globose greenish conidia	Foot cell present	Long, erect conidiophore	Assymetrical	*Penicillium sp*
AZF – 14	Green	Brown	Globose greenish mature conidia	Foot cell present	Mature conidiophore	Multi–nucleate globose vesicle	*Aspergillus Flavus*
AZF – 15	Light green	Brown	Globose greenish conidia	Foot cell present	Simple, long, erect conidiophore	Broom-like group of sterigmata	*Penicillium sp*
AZF – 16	Green	Brown	Oval conidia	Foot cell present	Well branched and septate conidiophore	Primary and secondary sterigmata bearing short chain of conidia	*Penicillium sp*
AZF – 17	Light green	Brown	Globose greenish conidia	Foot cell present	Long, erect conidiophore	Assymetrical	*Penicillium sp*
AZF – 18	Green	Brown	Globose greenish mature conidia	Foot cell present	Long, erect conidiophore	Multi–nucleate globose vesicle	*Aspergillus Flavus*
AZF – 19	Green	Brown	Oval greenish conidia	Foot cell present	Long, erect and non – septate conidiophore	Multi–nucleate globose vesicle	*Aspergillus sp*
AZF – 20	Yellow	Brown	Oval greenish conidia	Foot cell present	Long, erect conidiophore	Multi–nucleate globose vesicle	*Aspergillus Flavus*
AZF – 21	Green	Brown	Oval greenish conidia	Foot cell present	Long, erect and non – septate conidiophore	Multi–nucleate vesicle	*Aspergillus sp*

Appendix 1D: Morphological Characteristics and Identification of Fungi Isolates

Isolate No	Colour of Aerial Hyphae	Colour of Substrate Hyphae	Shape and Kind of Asexual Spore	Presence of Special Structure	Appearance of Sporangiophore Conidiophore	Characteristics of Spore Head	Probable Organism
AZF – 22	Yellow	Brown	Globose greenish mature conidia	Foot cell present	Long, erect conidiophore	Multi–nucleate globose vesicle	*Aspergillus Flavus*
AZF – 23	Green	Brown	Globose greenish conidia	Foot cell present	Long, erect conidiophore	Multi–nucleate shaped vesicle	*Aspergillus Flavus*
AZF – 24	Bluish green	Brown	Oval greenish conidia	Foot cell present	Long, erect and non – septate conidiophore	Swell into multi–nucleate vesicle	*Aspergillus sp*
AZF – 25	Green yellow	Brown	Globose greenish mature conidia	Foot cell present	Long, erect and non – septate conidiophore	Multi–nucleate globose vesicle	*Aspergillus Flavus*

Appendix 2

CALEBERATION CURVE OF STANDARD PRIMARY GOSSYPOL ACETIC ACID



NOTE: The above Linear Graph represents a Caliberation for Standard Primary Gossypol at 440 nM Wavelength (using Visible Spectrophotometer M201 Camspec Model with 325-900 nM Wavelength range.

APPENDIX III
DETERMINATION OF LIPID CONTENT OF COTTONSEED

First, the moisture content was determined as;

$$\% \text{ moisture (w/w)} = \frac{\text{Loss in weight} \times 100}{\text{Net weight of sample}}$$

And then

$$\% \text{ lipid (w/w)} = \frac{\text{Weight of lipid extracted} \times 100}{\text{Weight of dried cottonseed sample.}}$$

Where the weight of lipid extracted is given by:
The loss in weight of the thimble and content after extraction and is equal to the weight of the lipid in the flask after removal of the solvent.

APPENDIX IV
DETERMNATION OF CRUDE FIBRE

Crude fibre is determined as that fraction remaining after digestion with standard solutions of sulphuric acid and sodium hydroxide under controlled conditions.

CALCULATION

Crude fibre (% of fat free DM)

$$= \frac{\text{(Weight Ccrucible + dried residue) – (Weight crucible + ashed residue) x 100}}{\text{(Weight of sample)}}$$