STUDIES ON MYCOFLORA AND MYCOTOXINS IN ACHA, MAIZE AND COTTON SEED IN PLATEAU STATE, NIGERIA.

BY

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DECLARATION

I sincerely declare that this work was carried out by me in the Department of Physiology and Pharmacology Ahmadu Bello University, Toxicology Section of National Veterinary Research Institute, Vom and in Southern Regional Research Centre of United State Department of Agriculture, New Orleans U.S.A. No part of this thesis has ever been submitted to any Institution for award of diploma or degree.

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CERTIFICATION

This Thesis entitled: "Studies on mycoflora and mycotoxins in acha, maize and cottonseed in Plateau State, Nigeria" by T.A. Gbodi meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Toxicology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The production of toxic metabolites by moulds and their resulting mycotoxicoses in animals and man have been known for many years, but despite this knowledge little attention was shown by scientists to such diseases.

Little has been done on mycotoxicoses in animals in Nigeria and work done on mycotoxins so far is confined to aflatoxins.

Thus, study on moulds and mycotoxins in acha (Diqitaria exilis Stapf) maize (Zea mays) and cottonseed (Gossypium spp Linn) grown in Plateau State of Nigeria and toxicity of moulds infecting acha was carried out. Mouldy acha, maize and cotton seed samples were collected at three different seasons from some local government areas of Plateau State. These grains and seeds were screened for their mycoflora and for seven mycotoxins.

Ninety-six fungi were isolated from acha. These included Aspergillus ochraceus, A. sydowii, A. terreus, A. niger, A. nidulans, A. flavus, A. cremeus, Fusarium solani, F. moniliforme., F. oxysporum, F. roseurn, F. equiseti, F. semitectum. Phoma sorghina, et-cetra. Phoma sorghina was the commonest fungal

contaminant of acha during the cold dry harmattan season (November - February) and during the humid rainy season (June - October). Fusarium moniliforme and Aspergillus spp. were also commonly found. During the hot dry season (March - May), Aspergillus spp were the most prevalent fungi, followed by Fusarium spp. and Phoma sorghina in that order. Zearalenone was the most prevalent mycotoxin of mouldy acha during the harmattan, while aflatoxins were most prevalent in samples collected during the hot dry period. The mycotoxins found in acha were, aflatoxins B_1, B_{11}, G_1, G_2, zearalenone, and vomitoxin. Zearalenone was only detected in one of the samples collected during the rainy season.

One hundred and twenty-four fungi were isolated and identified from maize. Among these were: Aspergillus nidulans, A. f lavus, A. niger, A. f umigatus t Fusarium moniliforme, Neurospora spp Penicil1ium funiculosum, Trichoderma sppi et-cetra. Fusarium spp. were the comiiionest fungi found in maize during the harmattan and hot dry seasons, while Trichoderma spp. were common during the rainy season. The mycotoxins found in maize were aflatoxins B_1, B_2, G_1, G_2, zearalenone and ochratoxin A.

The highest level of aflatoxin was found in samples from Langtang (B. =960, B_ = 543 ug/kg) and the highest level of ochratoxin A(50 ug/kg) was found in sample from Jos area.

The fungi isolated from cottonseed were Aspergillus flavus, Cunninghamella spp.$_f$ Curvularia spp.$_t$ Nigrospora spp. Phoma sorghina and Trichoderma sp$_p$. Phoma sorghina was the only fungus isolated from mouldy cotton seed collected during the harmattan. Cunninghams!la spp. were isolated from all three samples collected during the hot dry season and from one of the six samples collected during the rainy season. Only aflatoxins could be detected in mouldy cotton seed with the highest level (271 ug/kg) occurring in samples from Keffi.

Ninety six fungal isolates from mouldy acha were cultured on rice and extracted with methylene chloride. The extracts were then put in corn oil and screened for toxicity in mice. Fifty of the 96 (52%) isolates were found to produce toxic metabolites. 20 (20.8%) were mildly toxic to mice; 13 (13.5%) moderately and 17 (17.7%) were very toxic. Aspergillus guadrilineatus, A. nidulans, Fusarium solani and Phoma sorghina produced the most toxic extracts.

Two extracts of the A. quadrilineatus; the petroleum ether soluble extract (PER) and the petroleum ether defatted crude extract (PEDCR) were tested for acute and chronic toxicity in mice, chicks and cattle. The per os_LD_{50} of PER was 1148mg/kg and that of PEDCR was 507mg/kg in mice, and 556mg/kg in chicks. The intraperitoneal LD_{50} of PEDCR in mice was 20.8mg/kg. The toxic signs of PER and PEDCR in mice and chicks included tachypnea, tachycardia, anorexia, somnolence, diarrhoea, coma and death. The main post mortem findings in acute toxicity were congestion of heart, liver, kidney and lungs and sloughing of the wall of stomach and haemorrhagic entiritis. The histopathologic findings in dead animals included oedema and mild degeneration of the myocardium, necrosis of kidney tubular epithelial cells, hepatocytes and bronchioles. The only clinical observation in calf acutely dosed with culture of A. quadrilineatus on maize were transient whole body tremors which occurred one hour after dosing, tachycardia and profuse salivation. No significant histopathologic changes were found in the organs of the sacrificed calf.

Rats fed on diets containing different percentages of A. quadrilineatus cultured maize gained less weight as compared with those on normal diets.

Other clinical signs in rats included soft faeces, lethargy, arched back, rough hair coat and death in the case of rats on 100% substituted diet.

No abnormal clinical signs were seen in cattle on substituted diets except that cattle on normal diet had superior weight gain as compared to those on substituted diets.

Post mortem findings in rats included hyperaemia of the mucosa of the stomach and intestine, sloughing of the mucosa of the stomach, petechial haemorrhages on the liver and gastro-enteritis. No significant post mortem findings were observed in cattle on substituted diet except that the mucosa of the stomach and duodenum of cattle on 100% substituted diet were sloughed off.

Histopathologic findings in rats included liver congestion, hepatocellular degeneration and necrosis, congestion of the venous sinuses of spleen characterised in some cases by mild to moderate lymphocytes depletion of the periarteriolar lymphatic sheath. Varying degrees of interstitial inflamation with peribronchiolitis and atelectasis and severe alveolar oedema were seen in lungs of some rats.

Submucosal oedema of the stomach and granular degeneration of the tubular epithelial cells of the kidney particularly of the medulla were seen. Severe meningeal congestion, haemorrhage and sub-meningeal degeneration were seen.

In cattle the histopathological findings included degeneration and necrosis of the liver. Multifocal areas of haemorrhage in the liver and extensive tubular necrosis and degeneration of glomerular tufts were seen.

Chronic feeding of A. quadrilineatus substituted diets to cattle had an increasing effects on PCV, neubrophils, serum cholesterol, triglyceride, SGOT, serum alkaline phosphatase and lactate dehydrogenase, and it had a decreasing effect on lymphocytes, monocytes, Hb, and albumin when compared to control animals values.

The mycotoxins produced by A. quadrilineatus were extractable from rice culture by chloroform. Column chromatographic separations in silica gel using different elution solvents and biological tests showed that the mycotoxins came off in the diethylether, chloroform and mostly in ethylacetate fractions.

Use of different available mycotoxin standards on silica gel G coated chromatoplates revealed that sterigmatocystin is one of the minor metabolites

produced by A. quadrilineatus. Two other more toxic mycotoxins were isolated and purified from the crude chloroform extract and their column, preparative thin layer chromatographic, infrared and UV-spectrophotometric characteristics were established.

The infrared spectra of the two purified mycotoxins suggested that the carbonyl group of their structures were similar to that of aflatoxin.

It was concluded that: in Plateau state, Nigeria both acha and maize can be infected naturally by many mycotoxin producing fungi, when they are poorly stored and in the field; that some mycotoxins like zearalenone, ochratoxin A and vomitoxin which were presumed to occur only in temperate cereals products can be produced on acha and maize in the Plateau State of Nigeria because of the temperate-like climate of the Plateau; that aflatoxins were the major mycotoxins contaminating mouldy acha, maize and cottonseed. A. quadrilineatus is the most toxic toxin-producing fungus isolated from acha. It can produce sterigmatocystin and seven other toxic metabolites which can induce both acute and chronic toxicity in mice, rats, chicks and cattle.

The mycotoxins produced by A. quadrilineatus can be extracted by chloroform and can be separated by column and preparative thin layer chromatography. Two of the mycotoxin produced by A. quadrilineatus have similar structures and probably contained 3 carbonyl bands (C=0). There is need for further work to establish the chemical structures of the two mycotoxins isolated. It was also concluded that there is great need for screening common feed stuffs for both fungal and mycotoxin infection and contamination. There is also need for Government Regulations on monitoring of feeding stuffs meant for animal and human consumption.

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GENERAL INTRODUCTION

Poisonous symbiotic and parasitic fungi occur as a major problem in almost all cereal grains. They cause loss in yield, quality, nutritional value and viability of these grains. Of utmost importance are the diseases and subsequent losses they cause in livestock. Epidemic and endemic diseases in the human and many diseases of unknown etiology are suspected to be of fungal origin.

The production of toxic metabolites by moulds and the resulting mycotoxicoses in animals and man have been known for many years, but inspite of this knowledge, little attention has been paid to such diseases. Thus, mycotoxicosis has been described as the neglected disease. According to Christensen (1980), many regions of Europe suffered severe and repeated epidemics of ergotism for over 300 years, between A.D. 900 and 1300. The cause and effect relationship between consumption of ergot and the development of convulsive or gangrenous ergotism was recognised in some places only by 1700. The apathy shown in mycotoxicosis altered drastically with the outbreaks of aflatoxicosis in the United Kingdom in 1960 when over 100,000 turkey poults were lost within three months (Blount, 1961).

Following the outbreak of aflatoxicosis problem in the United Kingdom, an investigation was carried out in Nigeria to assess the importance of aflatoxin in groundnut production in Nigeria (Darling, 1963). Low levels of aflatoxin in bagged groundnut at Kano and Portharcourt and on damaged groundnut kernels in the field were reported. Bassir, (1969); Eka (1977) and Nwokolo and Okonkwo (1978) isolated various amounts of aflatoxin B_1 from various feeds and foodstuffs offered for sale in Ibadan and Zaria markets and in savanna and forest regions of Nigeria.

Nigeria like many other African countries has a relatively high incidence of liver cancer. In the western part of the country the incidence is 5-9 per 100,000 males and liver cancer constitutes about 72% of all cancers (Edington and Mclean, 1965). The incidence of liver cancer in the U.K. is 3.0 and 2.7 per 100,000 males in the United States of America (Hutt, 1971). In Northern Nigeria liver cancer constitutes 19.2% of all cancers (Edington, 1977). The hospital records of University of Nigeria Teaching Hospital at Enugu showed that liver cancer constitutes about 10% of all cancers (Nwokolo and Okonkwo 1978), Keen and Martin (1971), Alpert et al., (1971) and Linsel and Peers(1977) have produced

evidence from different parts of the world suggesting that the level of aflatoxin contamination of local foods is directly related to the incidence of liver cancer in those communities. Of recent, Shreeve et al., (1979) has detected aflatoxin, ochratoxin and zearalenone in tissues and milk of dairy cows fed naturally contaminated feed. This shows that the possible exposure of human to the hazards of moulds and their toxins is not limited to consumption of grossly contaminated grains or other human foods. Thus, there is a need to investigate the degree and pssible effects of exposure of animals and man to these hazardous mould metabolites.

Although some fungi can infect more than one species of hosts, most have preferred hosts. Thus, the types of moulds and mycotoxins found in a particular area or country depends on the available hosts, environmental conditions, like temperature and humidity. For example mycotoxins, palmotoxin B_o and G_o were isolated from palm wine in Ibadan by Bassir and Adekunle (1968). Also in Western Nigeria a seasonal disease known as "Ijeshashakes", is said to be a fairly common diagnosis upon hospital admission. The cause is unknown although cynogenic glycosides or tremorgenic mycotoxin is suspected (Wright and Morley, 1958). Thus, there is need to investigate

different regions of Nigeria for moulds and mycotoxins.

Literature search has shown that little has been done on mycotoxicosis in animals in Nigeria and work done on mycotoxins so far is confined to aflatoxins.

This study on moulds and mycotoxins in acha (Digitaria exilis Stapf) maize (Zea mays) and cotton seed (Gossypium spp Linn) in Plateau State was carried out with the following objectives in view.

1. To isolate and identify the moulds infecting the seeds of the three crops.
2. To identify and quantitate the mycotoxins that might be present in the above crops.
3. To screen the isolated fungi from acha for mycotoxin production and its toxicity in mice, chicks and rats.
4. To establish acute and chronic toxicity of crude chloroform extracts of the most toxic cultured fungus from acha in mice, chicks and cattle.
5. To isolate and identify the mycotoxin produced by the selected toxic fungus.

CHAPTER 1

LITERATURE REVIEW

1.1 Types of Fungi

It is common knowledge that cereal grains, stored grains, processed foods and other feed stuffs of plant and animal origin harbour large numbers of fungi under certain natural conditions. These fungi include parasitic species that are host-specific, and saprophytic species which even tend to be selective in their choice of hosts and environment. Christensen, (1957) divided the fungi into three distinct groups; field fungi, storage fungi and advanced decay fungi.

The principal genera of field fungi are:- Alternaria, Fusarium, Helmithosporium and Cladosporium. All the field fungi require a high seed moisture content for growth. Their distribution depend on type of crop, region or geographic location and climatic conditions. The storage fungi consists of the genera Penicillium, Aspergillus and Sporendonema plus a few species of yeast. The major factors influencing the development of storage fungi on stored grain are temperature, duration of storage, degree of earlier

invasion of the grain by field fungi before arrival at given site, amount of foreign material and the activity of insects and mites. (Semeniuk and Alcock, 1954; Christensen and Kaufmann, 1965; Golumbic and Kulik, 1969; Christensen, 1972). The advanced decay fungi consists of the genera such as Fusarium and Chaetomium, which grow after considerable damage from other microorganisms has occurred (Christensen and Kaufmann, 1965).

1.2 Mycoflora and mycotoxins associated with maize

The major field fungi that have been associated with maize are species of Alternaria, Cladosporium, Nigrospora, Helminthosporium, Trichoderma and Fusarium. The genera most responsible for field and storage fungal infections are Aspergillus and Penicillium. The chief species of Penicillium producing mycotoxins associated with freshly harvested corn is P. oxalicum. P. cyclopium and P. breicompactum are the chief species isolated from stored maize kernels. Also frequently seen contaminating freshly harvested or stored maize and producing mycotoxins are Penicillium citrinum, P. auticae, P. palitans and P. puberulum. Fusarium moniliforme is one of the mycotoxin producing Fusaria species found in damaged corn seeds (Uraguchi and Yamazaki, 1978).

The mycotoxins that have been isolated from stored and field corn from different parts of the world are T-2 toxin, vomitoxin or deoxynivanenol, zearalenone, citrinin, ochratoxin A and aflatoxin (Scott, 1978).

1.3 Mycoflora and mycotoxins associated with cotton seed.

Before harvesting, cotton seeds are said to harbour mainly pathogenic Fusaria such as Fusarium moniliforme and F. roseum. Aspergillus flavus and A. niger are other mycotoxin producing moulds that are also associated with freshly harvested cotton seed. Such storage fungi as A. versicolor and A. candidus and Penicillia increase with duration of storage period (Uraguchi and Yamazaki, 1978).

The main mycotoxin that has been isolated from field and stored cotton seeds is aflatoxin (Scott, 1978).

1.4 Mycoflora and mycotoxins associated with Acha (Digitaria exilis).

There is no reported mycoflora work on both field and stored acha. Aflatoxin B has been isolated from a market sample in Nigeria (Okonkwo and Nwokolo 1978).

1.5 Moulds and Mycotoxins in Nigeria Feeds and Feeding Stuffs.

With the outbreak of Turkey "X" disease and the

enormous economic loss, in 1961 in the United Kingdom, the Federal Government of Nigeria initiated the formation of a committee to assess the importance of aflatoxin in relation to groundnut production in Nigeria and to co-ordinate research measures designed to reduce the incidence of fungal contamination and mycotoxin formation.

Darling, (1963) supplied the first report on aflatoxin problem in groundnut in Nigeria. He found great variation in aflatoxin levels from time of delivery to stacking. He also attributed the presence of aflatoxin to post harvest mould growth. Bags of groundnut that he examined had aflatoxin content of less that 0.5 ppm but a few had a range of 0.5-2.0 ppm. The aflatoxin contents of most accidentally wetted groundnut kernel and cake ranged between 0.1-0.5 ppm; some were in excess of 2.0 ppm.

Peers working at Vom in 1965 examined the aflatoxin of groundnuts stored at the Zaria mill before processing and at various stages along production line. Thirty-five per cent of the nut entering the mill had 0.10-0.20 ppm aflatoxin. When normal sorting and clearing were completed only 17% had the same level of aflatoxin; the rest had less than 0.1 ppm at all stages of processing.

If an additional manual sorting stage was inserted after blanching the proportion of nuts with 0.1-0.2 ppm aflatoxin fell to 8%.

Between 1961 and 1965, McDonald and Harkness (1965) conducted investigations at Mokwa and Kano into aflatoxin and factors contributing to its presence in Northern Nigeria groundnuts. These locations were representatives of the riverine and northern savanna zones respectively. They found Aspergillus flavus in the four varieties of groundnuts examined, and wide distribution of aflatoxin in both zones. They did not find any consistent pattern of fungal or toxin incidence in relation to soil types or climatic zone. Kernels from undamaged pod samples from both zones were almost entirely free from A. flavus, but pods from the same samples that had been broken during harvesting or deeply scarified by termite attack showed considerable contamination by A. flavus and other fungi. They also showed that under Mokwa conditions, aflatoxin was not present in the crop at lifting and that contamination of the kernel with A. flavus and aflatoxin did not occur until at least 6 days after lifting. Thereafter the contamination increased until the pods dried to a safe moisture content level of 8-10%.

Bassir and Adekunle (1968) isolated two metabolites of A. flavus from palm wine which they characterised and named palmotoxin Bo and Go. They showed later (Bassir and Adekunle, 1969) that the toxicity of palmotoxin Bo was comparable to that of aflatoxin B_1 in embryonated eggs.

Bassir (1969) isolated aflatoxin B_1 from various mouldy food materials offered for sale in Ibadan markets. Highest concentration was found in bitter leaf which yielded 94ug/kg. Mantle and Waight, (1968) reported the occurrence of a fungus Sphacelia sorghi on Sorghum vulgare and showed that sclerotia contain a polypeptide alkaloid, dihydroergosine.

Alozie et al. (1980) investigated aflatoxin contamination of eight Nigerian foodstuffs and eight beverages. All samples of beverages and five of the foodstuffs were contaminated with aflatoxin, "ewedu" (Corchorus olitorius) "dawadawa" (prepared from locust bean, Parkia Clappertoniana, Keay) and "Shoko yokoto" (Celosia spp.) were not contaminated. They also found that all the beverages used as culture media for A. flavus supported the growth of the fungus and aflatoxin elaboration. All foodstuffs also supported the luxuriant growth of A. flavus.

Eka (1977) examined samples of foods and feed-stuffs (Palm oil, groundnut oil, palm wine, bread, rice, oranges roasted meat, onion, plantain, tomatoes, melon, "fura," "nono", "danwake" "koko," "alele" and "kuli-kuli" from markets around Zaria for aflatoxin contamination and their ability to support the growth of aflatoxin producing strains of A. flavus. He reported that a large number of these foods and feeding stuffs were contaminated with and supported the growth of A. flavus; only a small proportion of the items were contaminated with aflatoxin. Okonkwo and Nwokolo (1978) in their survey of aflatoxin content of common foods in the savanna, and forest regions of Nigeria found that groundnut and dried fish were highly contaminated with aflatoxin (900 and 600 - 700 ppb respectively), whereas various cereals like millets, guinea corn and rice were moderately contaminated (150-300 ppb). They also found that low risk foods such as yam tubers became contaminated after storage, but less so, when wrapped in cellophane or newspapers. Immersion of foods in a 1% solution of milton reduced by almost 50% the level of aflatoxin. Exposure to direct sunlight caused a decline (13%-16%) of aflatoxin levels in foods.

Okonkwo and Obionu (1981) investigated the seasonal variations in aflatoxin levels of Nigerian market foods. They found wide variations between seasons, the level being higher during the rainy season than in the dry season. Cassava, a major staple food showed a significantly higher difference in aflatoxin level between the rainy and dry season. The aflatoxin levels of guinea corn and rice was negligible from December to April.

Salifu (1978) detected aflatoxin, patulin and zearalenone in excess of 20ug/kg in hard dough and ripe stage of four varieties of sorghum in the field in northern Nigeria. Dada (1979) did not find aflatoxin B_1, B_2 and zearalenone in all the sorghum varieties he studied, but a metabolite which showed black band and blue green flourescence was associated with Phoma sorghina culture. Elegbede (1978) worked on stored samples of local cultivars of sorghum obtained from farmers in Zaria environs and detected low levels of zearalenone and ochratoxin A. He did not detect aflatoxins.

Abalaka and Elegbede (1982) investigated the distribution of aflatoxins and total microbial counts in cake of an edible oil extracting plant. They found that aflatoxins B_1, B_2, G_1 and G_2 were present in groundnut kernels, groundnut pellets, cottonseed pellets, crude groundnut oil and crude cotton seed oil. Both the refined groundnut and refined cottonseed oil showed traces of four aflatoxins. They also found the groundnut kernels from government-controlled sources contained higher aflatoxin B_1 levels (600ug/kg) than kernels derived from miscellaneous sources (45ug/kg). They noted that crude groundnut oil had a much higher aflatoxin B_1 content than crude cottonseed oil (98.0 versus 65ug/kg) but a higher B_1 level was recorded for refined cottonseed oil (24ug/kg) than for refined groundnut oil (9.0ug/kg). These findings which have been confirmed by unpublished work at Vom should be of concern especially to the food regulatory agencies in the country. Analysis of feedstuffs at Vom over a period of 15 years showed aflatoxin contamination to the level of 100-166 ug/kg (Gbodi , 1980 unpublished data).

1.6 Mycotoxicoses in Domestic Animals

There is no doubt that the problem of mycotoxicoses has always been with man from time immemorial. For a long time scientists and farmers have suspected the harmful effects of mouldy feeds in man and animals, but earlier reports on the toxicity of fungi have been very confusing (Thomson and Sifton, 1922; Steyn, 1933). Such were the confusions that Eckles et al. (1924) whilst reviewing the work of other investigators on death of animals allegedly caused by ingestion of mouldy silage, stated that in no case was there evidence that fungi could be incriminated. They reported feeding mouldy silage to cattle, sheep and horse without ill effects, and feeding cattle and sheep with filtrates of Czapek's medium upon which had been cultured several fungi, including strains each of A. glaucus, A. niger, Penicillium expansum, Rhizopus nigricans, a species of Fusarium and other fungi and also with no apparent ill effects. From these studies, they concluded that moulds were not harmful to animal health.

Forgacs and Carll, (1962) pointed out that many scientists tend to approach the cause of animal diseases through the process of elimination.

If the causal agent is not found to be bacterial, viral or nutritional, it is concluded to be chemical in nature. Even though this is true, the possibility that the source of such toxic chemicals may be fungal in origin is usually ignored. It is comparatively of recent that the "Koch's postulate" has been successfully demonstrated with respect to mycotoxicoses. This is because of the difficulties involved in the isolation and characterization of fungal toxic metabolites coupled with the fact that some fungi do undergo mutation with repeated subculturing and most need the right conditions to elaborate toxins which may be difficult to reproduce experimentally.

The first paper in which reference was made to a toxic mould was published as early as 1891, when there was a report on the toxicity of bread prepared from grain infected with Gibberella zeae (Walt, 1965). Hoyman (1941) is said to have established that the feeding of cereals infected with this mould caused emesis and subsequent death in pigs. Alsberg and Black (1913) reported that from 1870 to 1900 many shipments of corn imported into Italy from the United States were suspected to have been made toxic by fungi

growing on them, but Christensen (1971) claimed that there was no solid experimental evidence to support this suspicion.

Many researchers still question the magnitude of diseases caused by mycotoxins, but the following are known diseases of animals caused by metabolites of fungi: Swine nephropathy in Denmark, Ireland and Sweden, and in poultry in Denmark caused by ochratoxin; trout hepatoma in the United States and Italy due to aflatoxin, facial eczema in sheep in New Zealand caused by spirodesmin, salivation factor in cattle eating fungus infected leguminous hay in which slaframine is produced; swine and poultry reproductive troubles caused by zearalenone, death of cattle from T-2 toxins; death of farm animals from Fusarium toxins (referred to as alimentary toxic aleukia in Russia); vomiting and rejection of corn infected with Fusarium by swine; acute poisoning and death of poultry fed aflatoxin (Turkey "X" disease); tremortin toxin causing death in swine, stachybotryotoxicosis in horses, sheep and goats, and penicillic acid poisoning of swine (Hesseltine, 1978).

There is no published report of mycotoxicosis of livestock in Nigeria, even though mycotoxins have been reported in Nigerian feedstuffs. This may be due to the problem of diagnosis as the biological effects of mycotoxins are very varied: many of the symptoms are vague and resemble vitamin deficiencies.

1.6.1 Mycotoxicoses in Cattle

1.6.1.1. Ergotism

One of the earlier recognised mycotoxicoses in cattle is ergotism. Atanosoff, (1920) reported that in 1608 after a period of heavy fogs there was a general outbreak of ergotism among cattle in Germany. Cases of ergotism were recognised and described in cattle in the USA between 1820 and 1850 (Christensen, 1980). Burfening (1973) reported that there were serious episodes of ergotism in cattle in Kansas, Missouri and Illinois, and that as late as 1970, ergotism was reported from Ireland in cows fed ergot infected silage, and in 1966 ergotism was reported in cattle in England.

1.6.1.2 Facial Eczema

Another mycotoxicosis long recognised in cattle is facial eczema. The first report in 1897 came from New Zealand (Cunningham et al., 1942). Clare (1955) claimed that the descriptions of this disease in sheep and cattle had been recorded as early as 1882. It was formerly thought that facial eczema was confined primarily to New Zealand (Clare, 1952, 1955), but Janes (1959) reported the disease and the causative fungus, Sporidesmium bakeri, to be present in Australia. Another disease strikingly similar to facial eczema was reported in cattle that grazed on mouldy Bermuda grass in the Southern United States (Kidder et al., 1951, 1961, Gibbons, 1953, 1959 and Whitehair et al., 1951). The fungus associated with the mouldy Bermuda grass toxicosis is a strain of Periconia minutissina (Kidder and Beardsley, 1961; Kidder et al, 1961).

Other natural outbreaks of Bermuda grass tremors of cattle in the U.S. were reported (Diener, et al., 1975). This afflicted 25000 cattle in over 500 herds in Louisiana in 1971 and resulted in the death of 600.

1.6.1.3 Stachybotrychosis

In 1931, massive intoxication of calves, horses swine and poultry developed in the Ukraine and similar fatal cases were recorded in Central Europe. The major symptoms were shock, stomatitis, dermal necrosis, haemorrhages, thrombocytopenia, leukopenia, nervous disorders and death from respiratory failure. The disease was found to be caused by ingestion of toxin produced by Stachybotrys alternans (Fortuskny et al., 1959; Yoshio, 1977). Sporadic outbreaks in cattle in recent years have also been reported from Czechoslovakia (Gdovin et al., 1966) Hungary (Danko, 1972) Romania (Mitroiu et al., 1973), Yugoslavia (Ozegovic et al., 1971) and Bulgaria (Kunev, 1973). Schofield (1924) isolated a fungus from a field outbreak of mycotocicosis and reproduced the toxicosis experimentally. He also investigated sweet clover poisoning in cattle; by culturing on sweet clover stalks a species of Aspergillus which he isolated from natural outbreak and reproduced the disease in rabbit.

1.6.1.4. Aflatoxicosis

In 1941, a unique disease of cattle was reported by Carll and Forgacs in New York State (Olafson, 1947).

This disease was observed in other states of the USA and was thought to be caused by chlorinated naphthalene but other workers reproduced the disease by feeding various mouldy feedstuffs and fodders in the absence of naphthalene compounds. Forgacs and Carll (1962) listed the symptoms. Sippel and his co-workers in 1953 reported natural outbreaks of poisoning from mouldy corn and peanuts among some cattle. They were able to reproduce the symptoms of toxicosis by feeding mouldy corn from enzootic areas. Albright *et al.*, (1964) reported a case of mouldy corn toxicosis in cattle, when out of a group of 29 heifers and steers (5-8 months old), 20 died or became moribund during a 73-day period following the feeding of a ration which contained toxin-producing *A. flavus*, *Penicillium cyclopium* and *P. palitans*. A similar disease was reported by Hsu *et al.*, (1972) to cause death in 20% of lactating Holstein cows over a 5 month-period. This Wisconsin dairy herd was fed for prolonged time on a diet containing 60% mouldy corn infested with *F. tricinctum*. They isolated T-2 toxin from the cultured mould, (Smalley *et al.*, 1970).

Aflatoxicosis, the disease that ravaged the turkey industry in UK in 1960 also caused some losses in cattle. Loosmore and Markson (1961) investigated an outbreak of a disease in calves which they subsequently established to be caused by feeding aflatoxin contaminated Brazilian groundnut meal. Clegg and Bryson (1962), Loosmore et al. (1964) also reported other similar outbreaks. Gopal et al. (1968) recorded an outbreak of aflatoxicosis in dairy cattle in India that resulted in the death of 58 crossbred cattle of which 25 were below one year of age. A similar toxicosis was reported in India by Ganti et al. (1965) and Purushothan and Mahendar (1976). Hurter (1966) in South Africa reported outbreak of aflatoxicosis which caused high mortality in pigs and cattle. A. flavus contaminated groundnuts and nubbings were incriminated. Lynch et al.(1970) linked the loss of six lactating dairy cows within a two month period on a farm in Maryland to toxicity caused by aflatoxins and other toxins produced by isolated A. flavus and A. clavatus from dairy ration. Adamesteanu et al.(1974) reported on a chronic proliferative,degenerative, hepatosis which developed during the foetal stage in 60 newborn and

two stilborn calves from a large herd in Romania. They considered the cause to be due to aflatoxins. A. flavus was isolated from maize silage and it produced aflatoxin B_1 and G_1 when cultured. Meronuck (1981) associated the incidences of decreased reproduction and reduced feed intake to toxicity caused by aflatoxins produced by A. flavus in corn fed to dairy animals. Smith et al. (1976) reported that over a 22 month period out of 278 submissions to North Carolina Diagnostic Laboratory for suspected aflatoxicosis, 94 cases were confirmed for aflatoxicosis. Kellerman et al. (1976) reported on a tremorgenic neurotoxicosis which decimated a herd of cattle in northern Transvaal. A. clavatus was isolated from the suspected sorghum beer residue and a pure culture of the fungus was used to reproduce the symptoms in yearling Friesland steer. Another outbreak in East Germany involved 346 heads of cattle fed malt germs contaminated principally by A. clavatus, 28% of the animals either had to be slaughtered or died (Schultz et al., 1965). Aspergillus clavatus contaminated malt sprouts had caused yet another outbreak of disease in cattle as reported by Abadjieff, (1966). Eight herds totalling 1050 cattle of which 346 developed symptoms, 28% died or had to be slaughtered, 62% recovered and 10% remained incoordinated and dribbled urine.

In 1964, Aust reported the death of 20 out of a pen of 29 heifers and steer due to haemorrhagic syndrome caused by contamination of corn by a species of Aspergillus. A similar mycotoxicosis was also reported by Dyson and Reed (1977) in the UK. This was caused by feeding mouldy barley to 80 Friesian cows.

1.6.1.5 Lupinosis

In 1974, Marasas described in detail mycotoxicosis called lupinosis which is caused by the toxin of Phomopsis leptostromiformis.

1.6.1.6. Slaframine Toxicosis

In 1959, O'Dell et al., and others reported a disease in cattle which occurred from eating clover hay, it caused slobbering and Smalley et al.(1962) demonstrated that the fungus responsible for the production of this toxic material was Rhizoctonia leguminicola which produced slaframine toxin.

1.6.1.7 Miscellaneous mycotoxicosis

Other mycotoxicoses reported are a syndrome in cattle which resembled migran and ryegrass staggers (Shreeve et al., 1978). A tremorgenic fungus was isolated from the pasture associated with the condition. Still and co-workers (1971) reported a case of bovine abortion when, during two month period, 13 out of 71 cows aborted. They isolated many fungi of which

A. ochraceus was experimentally shown to be responsible for the abortion. Dijkstra (1963) linked cases of abortion in cattle to A. fumigatus present in mouldy hay and ensilage. Seven thousand and six hundred aborted foetuses were involved. Mirocha et al.(1968) implicated zearalenone in a case of decreased fertility in dairy cattle. The cattle were fed F. graminearium contaminated hay. Iizuka and Iida (1962) reported from Japan mycotoxicosis in dairy cattle caused by A. oryzae contaminated malt sprout. Fisher et al., in 1967 reported two cases of reduced appetite and milk production in two dairy farms in Australia. The cattle were fed maize from which F. culmorum was isolated; its extract proved toxic in rabbits. Wilson et al. (1981) reported on a neurologic disease of fungal origin in three herds of cattle in Tennessee USA. The fungus incriminated were Claviceps paspali and P. verruculosum which were present in the hay fed to the animals.

Yamamoto (1954) isolated a toxic strain of Penicillium urticae from malt that caused a large number of deaths in dairy cows in Japan (Hori and Yamamoto, 1953). The same fungus was isolated from malt and shown to be responsible for intoxication

and death in cattle in Germany (Schultz, 1968; Schultz et al., 1969). Similar toxicosis was recorded in Russia by Konovalov et al., in 1977. Another case of haemorrhagic syndrome in cows in England was reported by Anderson (1978) where twenty cows died on nine farms.

1.6.2. Mycotoxicoses of sheep and goats

One of the earliest known mycotoxicoses of sheep is facial eczema. The disease is caused by the ingestion of spores of a fungus called Sporidesmium bakeri. A toxic principle known as sporedesmin which is elaborated in the spores is hepatotoxic and results in severe occlussive damage to the bileduct system followed by photosensitization. Giltruth first described the disease in New Zealand as early as 1908 (Hesseltine, 1978). Since then the disease has been reported in South Africa (Marasas et al.,1972) and Australia (Janes, 1959, Connole and Johnston ,1967). Mitton, et al. (1975) reported a case of abortions in a flock of sheep in France. The animals were fed maize ensilaged without preservative. High content of zearalenone was found in the maize and alcoholic extract of the maize tested on rats confirmed its oestrogenic potency.

Few reports of mycotoxicoses in goats have been documented. Minne et al. (1964) reported groundnut poisoning in pigs and goats, Aspergillus flavus and aflatoxins were isolated from the groundnut feed. Wanasinghe (1974) reported an outbreak of aflatoxicosis involving 194 goats in Sri Lanka. The majority of goats below one year of age died. Both A. flavus and aflatoxin were recovered from the defatted residue from grated coconut fed to the animals (Samarajewa et al., 1975).

1.6.3 Equine Mycotoxicoses

Before the outbreak of the "Turkey X" disease in turkey poults in Britain in 1960, research in the field of mycotoxicoses had been sporadic in Western countries. In the USSR a systematic study of mycotoxicoses was initiated as early as 1938 following the successful study of equine stachybotryotoxicosis. Thus, one of the earliest and most thoroughly studied mycotoxicosis is equine stachybotryotoxicosis (Forgacs and Carll, 1962). The disease is caused by ingestion of food upon which toxic strains of Stachybotrys atra have grown and formed toxin. The disease usually affects horses, but other animals and human beings are susceptible to the toxin.

The first case of this toxicosis in horses was reported in the USSR in 1931 (Forgacs and Carll, 1962). Massive intoxication of horses, swine, calves and poultry were reported in other countries like Hungary (Danko and Tanyi, 1968) and Romania (Draghici et al., 1971).

Mouldy corn poisoning or encephalomalacia is another fungal disease of horse. It has been recognised in the USA since 1950 as a highly fatal disease in horses. It was said to be responsible for the death of thousands of horses in Maryland around the turn of the century and in central Illinois and other Midwestern states in the 1930s' (Carlton, 1976; Wilson et al., 1973, Kellerman et al., 1972; Badiali et al., 1968). It has also been noted in China, Greece, South Africa, Germany and Egypt.

Mayer in 1953, writing of clinical aspect of alimentary toxic aleukia reported that in 1880, the war horses in Russia were poisoned by Fusarium sporotrichoides contaminated bread fed to the horses. Another mycotoxicosis of horses reported in Russia is "Dendrodochis toxicosis" caused by ingestion of Dendrochium toxicum infected feedstuff, roridins and verrucarine were the suspected toxic agents.

In 1930, Muller attributed the poisoning of horses to hay infected with Aspergillus fungus. A disease in horses fed bean-hulls was reported in Japan; Fusarium solani and its toxin neosolaniol and T-2 toxin were implicated (Ueno, 1977).

A possible case of equine aflatoxicosis was reported by Greene and Oehme (1976) in Manhanttan, Kansas, USA, Aspergillus flavus, Penicillium sp. and aflatoxin B_1 were isolated from the feed.

1.6.4 Mycotoxicoses in Swine

One of the earlier recognised mycotoxicoses in swine is what is called porcine nephropathy. It is a common mycotoxicosis in Scandinavian countries and it is responsible for a lot of economic losses in the pig. Several reports of this disease have been documented (Krogh et al., 1973). Krogh, (1979) reported that the disease has been in existence in Scandinavian countries for more than half of a century and found to be associated with food borne ochratoxin A in Sweden. More recently, Rutquist et al. (1978) reported the occurrence of nephropathy in fattening pigs in two farms in Sweden. These were caused by P. verruculosum which subsequently produced ochratoxin A in the pig feeds.

A similar case was also reported by Buckley in Ireland in 1971.

By far the most common mycotoxicosis of pigs reported from many parts of the world is the hyperoestrogenism caused by feeds contaminated with zearalenone producing fungi. One of the earliest reports of this disease was by McNutt et al.(1928). McErleen (1952) also reported this disease in swine in Ireland, and was associated with the feeding of Fusarium infected barley. The sows developed enlarged mammary glands and in more severe cases, prolapse of the vagina and rectum. Also in 1930, Munkdur and Cochran reported extensive poisoning of hogs in Iowa. The animals were fed barley contaminated by Gibberella saubinetti and the disease was produced in hogs by feeding experiment. Stob et al. (1962) observed vulvae hypertrophy in a number of swine herds that had been fed mouldy corn. A similar disease was observed in Minnesota where the symptoms were tumefaction of the vulva (Jubb and Kennedy, 1963) prolapsed vagina and hypertrophy of the mammae. In all these cases Fusarium contaminated feed was incriminated. Similar cases were reported in Yugoslavia, Romania, Hungary, Czechoslavakia, (Ozegovic, 1970) and in Italy

(Simonella, 1972). One serious outbreak of hyperoestrogenism was also reported from Romania by Popescu et al. (1977). It involved 1000 sows and 12800 piglets. Fusarium, Aspergillus and Penicillium infected feed was incriminated and T-2 toxin, zearalenone and ochratoxin were isolated from the feedstuffs. Other outbreaks were reported by Rosiles and Lopez (1977), Debreczeni and Rejto (1969) and Bristol and Djurichovic (1971) in Canada.

Another mycotoxin to which the swine is susceptible is aflatoxin. Most notable natural outbreaks of the disease was in 1960 during the extensive "Turkey" disease outbreak. Swine too were fed the contaminated Brazilian groundnut meals. Perhaps some of the earlier outbreaks of aflatoxicosis in swine included the report by Boiarkii (1950) in which he linked the outbreaks to Aspergillus and Penicillium fungi. In 1952 a disease called mouldy corn toxicosis occurred in Western Florida and Georgia in the USA. A total of 24 oubreaks affecting 23 pigs were reported, Aspergillus flavus and Penicillum rubrum were isolated from the mouldy corn. When these fungi were cultured on sterilized corn and fed to swine a similar toxicosis

was produced (Thasnakorn, 1970). Loosmore and Harding (1961) described an outbreak of poisoning in a large group of pigs following the feeding of groundnut meal. The meal was later shown to have been contaminated with aflatoxin. Blevins et al. (1969) reported an outbreak of a disorder characterised by bloody diarrhoea and abortion among pregnant sows and gilts consuming a ration in which aflatoxin B_1 was detected and from which Penicillium rubrum, Aspergillus sp and Rhizopus were isolated. Morbidity reached 33% and mortality 10%. Iwasaki in Japan (1976) reported an incidence of high liver cirrhosis among hogs brought by husbandry man. Mortality increased from 1-2% to 6-7% during the period of collected outbreak. Aflatoxin produced by A. flavus in feed stored for a long period was incriminated. Fong and Hutagalung (1977) in Malaysia reported an incidence of aflatoxicosis in swine caused by A. flavus and aflatoxin contaminated corn used in the feed. Connole and Hill (1970) reported aflatoxicosis in pregnant sows in Australia. Ketterer et al. (1982) reported five field cases of aflatoxicoses with abortion in pigs in Southern Queensland in Australia. Hurter (1966) reported incidence of aflatoxicosis in pigs in Northern Transvaal area of South Africa.

Among 278 submissions of farm animals made to the Northern Carolina Diagnostic laboratory during a 22 month period, 94 cases of aflatoxicosis were confirmed and 88% were in swine (Smith et al., 1976).

Stachybotryotoxicosis has also been reported in swine. Several outbreaks have been reported (Gajdusek, 1953; Formenchev, 1959, Kosheval, 1962) Bulgaria (Vachev et al., 1970) and Hungary (Szabo et al., 1970).

Ergotism has been reported in three pig breeding herds in Denmark (Barnikol et al., 1982). Examination of the wheat fed to the pigs revealed 1-2.9% ergot.

1.6.5. Canine Mycotoxicoses

One of the earliest recognised mycotoxicosis in dogs was that called "hepatitis X" which was observed by practising veterinarians in the USA long before early 1950's. But it was only during the early 1950's that a relationship was established between the syndrome in dogs and mouldy corn poisoning in cattle and swine. Newberne 1973 reported that sporadic episodes of the disease continued throughout the United States, which resulted in significant losses. Seibold and Bailey (1952) described a form of toxicosis in dogs designated "hepatitis X".

Following detailed experimental studies they concluded that "hepatitis X" in dogs and mouldy corn toxicosis in swine had the same aetiology. In 1958, a number of hunting dogs died from acute hepatitis after consuming canned food containing mouldy corn (Wogan, 1965). The condition was eventually related to aflatoxin. In 1959 Bailey and Groth reported a toxicosis in dogs that had consumed a diet containing corn obtained from a region where mouldy corn poisoning in swine was prevalent. The cause was linked with aflatoxin.

Schutz et al. (1965), in Berlin, reported a toxicosis involving 10 dogs in a kernel. The dogs developed rapid emaciation, progressive general weakness, apathy, dehydration, anorexia, vomiting and bloody diarrhoea. Four of them died, A. flavus in the mouldy oat flakes fed to the dogs was incriminated.

Ketterer et al.(1975) in Australia reported aflatoxin poisoning in dogs fed mouldy bread. A. flavus was isolated from the bread and aflatoxin B_1, 6.7 ppm aflatoxin B_2, 100ppm and aflatoxin G_1, 40 ppm were detected in samples of the vomitus.

Greene et al. (1977) in the U.S. reported an outbreak of chronic liver disease in a colony of dogs

and traced significant levels of aflatoxin B in the commercial dog food fed to the dogs.

Bost and Gastellu (1976) in France reported a case of food intoxication in 44 dogs that died; aflatoxin was incriminated.

An outbreak of hepatitis in man and dogs in Western India was reported by Krishnamachari *et al*. (1975). A large number of dogs developed ascites, icterus and died two to three weeks later. The disease was associated with the consumption of maize heavily contaminated with *A. flavus*.

Price (1978) reported an outbreak of canine aflatoxicosis in Kenya. Aflatoxin B_1 was detected in the liver and kidneys of many affected animals.

1.6.6 Avian Mycotoxicoses

Early controlled studies of feeding mouldy grain to chickens showed that chicken was apparently resistant to the effects of fungal metabolites (Creek, 1965). Forgacs (1966 a & b) reviewed early work on poultry mycotoxicoses. A typical case was the poultry haemorrhagic syndrome (Baker and Jacquette in 1953). However, the outbreak of the so called "Turkey X" disease due to aflatoxin and which caused death in more than 100,000 turkey poults in great Britain jolted the

Western world. Since then numerous reports have appeared on outbreaks of aflatoxicosis in different parts of the world. One of the earliest and most comprehensive reports was by Blount, (1960, 1961). Other reports from Britain were those of Chapman (1961), Gibson and Harris (1961), Carnaghan and Sargeant (1961), etc. Aflatoxicosis in poultry was also reported in Australia in 1965 by Gardiner and Oldroyd and by Hart 1965. Archibald et al. (1962) reported outbreaks of aflatoxicosis in Canadian broiler chickens. An outbreak was also reported in Kenya and Spain (Allcroft et al. (1961), Hungary (Derzsy et al., 1962), Senegal (Richiv et al., 1964) India (Gupta, 1965) and in Zimbabwe (Anon, 1961). The first report of aflatoxicosis in broiler chickens and laying hens in U.S.A. was by Smith and Hamilton in 1970.

In 1977 Mohiuddin et al., reported outbreak of aflatoxicosis in tufted pochard ducks in India. Smith et al. (1976) reported that out of 278 of farm animals submitted to North Carolina Diagnostic Laboratory during a 22 month period 94 were cases of aflatoxicosis, and 5% occurred in poultry

Other mycotoxicosis recognised in poultry is stachybotryotoxicosis. The first outbreaks were reported from Russia by Sarkisov (1954).

CHAPTER II

MYCOFLORA AND MYCOTOXINS FOUND IN ACHA (Digitaria exilis Stapf) IN THE PLATEAU STATE.

2.1 INTRODUCTION

Acha (Digitaria exilis Stapf) belongs to the grass family. It is indigenous to West Africa, especially the Plateau State of Nigeria where it is grown for its small yellow grains and straw. It thrives relatively well on the Plateau high lands where the poor sandy soil will not support the growth of other more popular cereals. Acha is also grown in Sierra Leone, in Guinea and in Togo (Oyenuga, 1968). Acha is the staple food of many Plateau people. The grain is used as porridge and for making a gruel-like drink, called 'kunnu' in Hausa. The straw is used as fodder for cattle.

Oyenuga (1968) provided information on the proximate analysis of acha grain and straw. Nwokolo and Okonkwo (1978), in their survey of the aflatoxin content of common foods in savanna and forest regions of Nigeria, found low levels of aflatoxin in the acha grain samples collected from a market in the Plateau. Apart from these two reports, little is known of the fungi that infect field and stored acha and the mycotoxins that these fungi can elaborate.

The purpose of this study was to investigate the mycoflora and some mycotoxins of field and stored mouldy acha grains in the Plateau State in view of the importance of the crop as a staple food in Plateau, Kaduna and Bauchi States.

2.2 MATERIALS AND METHODS

2.2.1 Sample Collection

Samplesof mouldy acha grains were collected during the dry harmattan period (November - February), the hot dry period (March - May) and the hot, wet and humid period (June - October) from Bassa, Jos, Barkin Ladi, Mangu, Bokkos and Pankshin Local Government Areas, the chief producing areas in Plateau State. The samples collected between November and May were mainly stored samples. The mouldy samples were stored mostly in local storage mud barns called 'rumbu' (in Hausa). The samples collected between June and October were taken from the field at harvest. About 2 kg of each sample were collected. One half was stored at $-20^{o}C$ for mycotoxin analysis and the other half was used for fungal isolation.

2.2.2 Isolation of fungi

About 10 g of the grain sample were washed with ten successive 100ml volumes of sterile distilled water and was surface-sterilized using 5.25% sodium hypochlorite solution (Clorox, The Clorox Co. Oakland, CA, USA). Ten grains were placed in each of ten petri dishes containing potato dextrose agar (PDA) and chloramphenicol (100 mg/litre). The dishes were incubated at 27°C and examined daily for 7-10 days. Fungi from plated contaminated acha were isolated in pure culture. Isolates were transferred to PDA slant media bottles and to fresh PDA in petri dishes for identification.

2.2.3 Identification of fungi

Identification of isolates was carried out partly by the Crop Protection Department of Ahmadu Bello University and partly by the USDA Southern Regional Research Center, New Orleans, U.S.A., and the Commonwealth Mycological Institute, Kew, Surrey, UK.

2.2.4 Analysis of mycotoxins

The samples were screened and analysed for aflatoxins B_1, B_2, G_1 and G_2, zearalenone and ochratoxin A. Only six randomly selected samples were assayed for vomitoxin. Aflatoxins and ochratoxin A

standards were obtained from Makor Chemicals Ltd, Jerusalem, Israel. Zearalenone and vomitoxin standards were provided by the USDA Southern Regional Research Center. A multimycotoxin method developed and used in the USDA Southern Regional Research Center (Ehrlich & Lee, 1984) was used. It utilized methylene chloride and phosphoric acid for the simultaneous extraction of aflatoxin, ochratoxin A and zearalenone. Vomitoxin, which is insoluble in methylene chloride, was extracted from the residue with methanol and water. A separate portion of the initial methylene chloride/phosphoric acid extract was subjected to a specific clean-up procedure for each mycotoxin. Each of the procedures was a modification of a published procedure for each toxin, as described below.

2.2.5 Extraction

After 50 g of a Hammer mill-pulverized sample was weighed into a 500 ml Erlenmeyer flask; 25 ml 1 M-phosphoric acid and 250 ml methylene chloride were added. The flask was shaken for 30 min. and the contents were then filtered under reduced pressure in a Buchner funnel fitted with a 18.5 cm circle rapid filter paper.

At least 200 ml of the filtrate was collected and, from this, 50ml aliquots were placed in separate

100-ml Erlenmeyer flasks with glass stoppers, for aflatoxin and zearalenone assays. The filter cake was reserved for vomitoxin assay.

2.2.6 Toxin assays

Aflatoxin was assayed in one of the 50-ml aliquots following the thin-layer chromatographic method out-lined by the Association of Official Analytical Chemists (1980). The chromatographic plates were developed in ether-methanol-water (96:3:1, by vol.) and the aflatoxins were quantitated densitometrically using a Schoeffel SD 3000 densitometer. Ochratoxin was assayed by a modification of the method of Paulsch et al. (1982), with 1 M-phosphoric acid substituted for 4 M-phosphoric acid, and methylene chloride substituted for chloroform. The intensities of the standards and samples were compared densitometrically. For the zearalenone assay, the procedure was that of Ware & Thorpe (1978) except for the use of methylene chloride/phosphoric acid in place of chloroform as the extracting solvent. Vomitoxin was extracted from the residue of the methylene chloride/phosphoric acid extraction according to the method of Vesonder et al. (1973) and was quantitated by the high-pressure liquid chromatographic method of Ehrlich & Lee (1984), using a Model ALC 502/401 liquid chromatograph (Waters' Associates, Inc. Milford, MA, USA). Using composite material the lowest detectable levels by these procedures were for aflatoxins: < 0.5 and for ochratoxin A = 10, zearalenone = 20 and vomitoxin = 10 ug/kg. The

recovery percentages were 76, 83, 77 and 57 for aflatoxins B_1, B_2, G_1 and G_2 respectively. The recovery percentages were 88, 81 and 84 for ochratoxin A, zearalenone and vomitoxin respectively.

2.2.7 Confirmatory Tests

To confirm the presence of aflatoxin, the thin-layer chromatographic plate was sprayed with aqueous sulphuric acid (50:50, v/v) dried and viewed at 365 nm, when the spots fluoresced yellow. Ochratoxin was confirmed by spraying the plate with alcoholic aluminium chloride (20 g/100 ml alcohol) and also by exposure of the plate to ammonia vapour and viewing at 365 nm. The spots of ochratoxin fluorescence changed from blue green to bright blue. Zearalenone was also confirmed by spraying the plate with alcoholic $AlCl_3$ and viewing at 365 nm, a procedure that increased the intensity of the fluorescence.

2.3 RESULTS

Table 2.1 shows the different species of fungi isolated from mouldy acha samples. In all, 123 isolates were cultured and identified. In nine acha samples collected during the cold dry harmattan period (November - February), Phoma sorghina was the most frequent contaminant (7/9) followed by Fusarium moniliforme (4/9) and then by Aspergillus spp.

(3/9). In seven mouldy samples collected during the hot dry period (March - May), Aspergillus spp. (7/7) were the commonest contaminants, followed by Fusarium spp. (4/7) and then by Phoma sorghina (3/7). The 12 mouldy acha samples collected during the humid and wet period of the year (June - October) were contaminated most frequently by Phoma sorghina (9/12), followed by Fusarium spp. (6/12), Aspergillus spp. (6/12) and Curvularia lunata (5/12).

The results of the mycotoxin analyses of nine samples collected during the cold dry harmattan period are shown in Table 2.2. Four of the nine samples were contaminated with zearalenone (200-309 ug/kg). Vomitoxin was not detected in any of three randomly selected samples, and neither aflatoxin nor ochratoxin A were isolated from any of the samples.

Eight of the samples collected during the wet season were assayed, and none was contaminated with either aflatoxins or ochratoxin A(Table 2.3), only one had zearalenone (18 ug/kg).

Of the seven mouldy samples collected during the hot dry period (March-May) four were contaminated with aflatoxins B_1 (2-20ug/kg) and two with B_2(8-12ug/kg) and three contained zearalenone (200-600ug/kg). Vomitoxin (5-58ug/kg) was found in all the three samples randomly selected for this assay.

2.4. DISCUSSION

Survey studies may be biased or unbiased. The study reported here was biased, since only mouldy samples of acha were collected for fungal isolation and mycotoxin assay. Scott (1978) underlined the importance of biased studies; whereas unbiased surveys give useful incidence data, biased analysis illustrates that contamination of a feedstuff by a particular fungus or mycotoxin is possible under natural condition. This, to our knowledge, is the first report on the mycoflora and mycotoxins of acha grown in Nigeria. In many parts of the country, only feedstuffs that are unfit for human consumption are fed to domestic animals. From our investigations during collection of the samples, it was apparent that mouldy grains were kept and fed to domestic animals but, in some cases, mouldy grains were also washed and used for human food. This explains the reluctance of many villagers to part with their mouldy samples. The feeding of mouldy samples may influence the performance of the local livestock. The detection of appreciable amounts of aflatoxin, zearalenone and vomitoxin should be a pointer to the deleterious role that mycotoxins could play in animal production in Nigeria.

It will be necessary to conduct more detailed, unbiased studies of this type to give a clearer picture of the incidence of fungal and mycotoxin contamination and the possible health hazards to the people who use acha as a staple food. However, the presence of many known mycotoxin-producing fungal species in both stored and freshly harvested mouldy acha should be of concern. In some of the samples, aflatoxin-producing and zearalenone-producing fungi occurred together. All the three samples contaminated with vomitoxin also contained zearalenone. Similar findings of multiple occurrence of toxins in a sample have been observed by other workers (Ehrlich & Lee, 1984). Thus, an animal may be exposed to more than one mycotoxin at a time. The secreening in this study was limited to seven mycotoxins, but the high incidence of Phoma sorghina in both stored and field acha suggests the likely presence of other metabolites of these species in acha. Phoma spp. are known to produce mycotoxins, including cytochalasin A (Uraguchi & Yamazaki, 1978) and tenuazonates (Steyn & Rabie, 1976). The latter metabolites have been implicated in a haematological disorder called 'Onyalia', a disease that occurs widely amongst Black African

population south of the Sahara (Rabie et al, 1975). The abundance of Aspergillus spp. in acha after relatively long storage tends to correlate with the incidence of aflatoxin in these stored samples. Although a high incidence of Fusarium spp. was found in the field samples, zearalenone was only found in one of the eight samples collected. This study indicates that Phoma and Aspergillus spp. are the chief fungal contaminants of stored acha, while Phoma and Fusarium spp. predominate in acha in the field.

TABLE 2.1

FUNGI ISOLATED FROM ACHA (DIGITARIA EXILIS STAPF) SAMPLES COLLECTED IN PLATEAU STATE AT DIFFERENT SEASONS OF THE YEAR.

Sampling period Months	season	No. of Acha samples examined	Fungus	No of samples infected
Nov .-Feb.	Dry, cold (harmattan season)	9	Aspergillus ochraceus	1
			A. sydowii	1
			A. terreus Thom	1
			Chaetomium spp.	1
			Curvularia lunata	2
			Fusarium solani	1
			F. moniloforme Sheld	4
			Helminthosporium spp.	1
			Phoma sorghina (Sacc.)Boerema	7
March-May	Hot,dry (dry season)	7	A. cremeus	1
			A.flavus	2
			A. nidulans (Eidam) Winter	1
			A. niger	1
			A. quadrilineatus Thom & Raper	2
			Bipolaris coici	2
			Cunninghamella elegans	1
			Drechsclera rostrata	1
			F. oxysporum Schlecht	1
			F. roseum	2
			F. solani	1
			Phoma sorghina (Sacc) Boerema	3
			Syncephlastrum racemosum	2
			Trichoderma harzianum	1

*Continue on next page

June - Oct. Hot, Humid (rainy season)	12	A. flavus	3
		A. nidulans (Eidam) Winter	2
		Aspergillus spp.	1
		Chaetomium spp.	1
		Curvularia lunata (Wakker)*	5
		Drechslera rostrata	1
		Fusarium equiseti (Corda) Sacc.	1
		F. moniliforme	2
		F. oxysporum Schlecht	2
		F. Semitechtum Berk & Rav.	1
		Phoma sorghina (Sacc.) Boerema	9
		Syncephlastrum racemosum	1

* The Boredijn state of Cochobolus lunatus

TABLE 2.2

OCCURRENCE OF AFLATOXINS, ZEARALENONE (ZEA), OCHRATOXIN A (OA) AND VOMITOXIN (Vm) IN MOULDY ACHA SAMPLES COLLECTED AT DIFFERENT SEASONS OF THE YEAR IN PLATEAU STATE

Sampling period		Main type of sample	Incidence * of:						
			Aflatoxins						
Months	Season		B_1	B_2	G_1	G_2	ZEA	OA	Vm
Npv-Feb.	Dry, cold (harmattan season)	Stored	0/9	0/9	0/9	0/9	4/9	0/9	0/3
March-May	Hot, dry (dry season)	Stored	4/7	2/7	0/7	0/7	3/7	0/7	3/3
June-Oct.	Hot, humid (rainy season)	Field	0/8	0/8	0/8	0/8	1/8	0/8	-

* No. of samples containing the toxin/total No. of samples analysed. Data on levels of toxins in individual samples are given in Table 3.

TABLE 2.3

CONCENTRATIONS OF AFLATOXINS, ZEARALENONE (ZEA), OCHRATOXIN A (OA) OR VOMITOXIN (vm) DETECTED IN MOULDY ACHA SAMPLES

Months	Season,	Code No.	Source*	Aflatoxins (μg/kg) B1	B2	G1	G2	ZEA	OA	Vm
Nov.-Feb	Dry, cold (Harmattan season)	7	Kuru/Jos	ND	ND	ND	ND	241*	ND	ND
		22	Du/Jos	ND	ND	ND	ND	200	ND	ND
		23	Zawan/Jos	ND	ND	ND	ND	309	ND	ND
		24	Kuru/Jos	ND	ND	ND	ND	241	ND	ND
March-May	Hot, dry (dry season)	2	Jengre/Bassa	2.4	ND	ND	ND	ND	ND	
		4	Mangu/Mangu	16.0	8.0	ND	ND	241	ND	58.0
		5	Bokkos/Bokkos	20.0	12 .0	ND	ND	200	ND	37.6
		12	Kuru/Jos	9.6	ND	ND	ND	602	ND	7.08
June-Oct	Hot, humid (rainy season)	14	Shere/Jos	ND	ND	ND	ND	18.0	ND	-

* Town/Local Government Area. ND - Not detected - = Not assayed

CHAPTER III

THE MYCOFLORA AND SOME MYCOTOXINS FOUND IN MAIZE (*Zea mays*) IN PLATEAU STATE

3.1 INTRODUCTION

Maize is one of the predominant cereal crops of Nigeria. In the past, its cultivation was mainly concentrated in the southern part of the country; millet and sorghum being the predominant cereal crops of the north. Recently, because of the increased demand for maize for human and animal food, maize is now grown practically throughout the country and widely used.

The fresh grains are eaten roasted or boiled on the cob. The ripe grains are cooked in combination with pulse or millet and boiled as porridge "Ogi" or "Eko" in Yoruba. In the north maize is milled with rice and used for a type of stiff porridge called "Tuwo" in Hausa.

The shortage of maize during the 1983/84 production year contributed to almost 100% increase in cost of poultry and animal feeds. Thus, the usual preference for yellow and clean maize was ignored by feed manufacturers and any maize including mouldy grains were used to compound animal feeds.

The mycoflora and mycotoxins found in maize have been documented (Uraguchi and Yamazaki, 1978). It is known that mycoflora and mycotoxins found in crops of

a particular area of a country depend on the available plant hosts, environmental conditions like temperature and humidity. Little is known of the mycoflora and mycotoxins found in maize in Nigeria and information is lacking especially about the situation in the Plateau State of Nigeria, which has a different climate from the rest of the country. The weather conditions of Vom representing most part of the Plateau proper has been described by Knudsen and Sohael (1970).

The average daily mean minimum temperature is 17^{o}C while the average daily mean maximum temperature is 28.6^{o}C. The average daily mean relative humidity at noon varies between 14% and 17%. The corresponding figures for Ibadan in the southern part of Nigeria are as follows:- average daily mean minimum temperature is 21.6^{o}C and average daily mean maximum temperature is 33.5^{o}C. Plateau State has also been described as a distinct ecological zone (Njike, 1981).

The objective of carrying out this study is to investigate the type of fungi which can infect maize and some mycotoxins which these fungi can produce in maize under natural conditions in Plateau State.

3.2 MATERIALS AND METHODS

3.2.1 Collection of Samples

Samples of maize grains that were visibly mouldy were collected during the harmattan (November - February), at the end of the rains (September - October) and during the hot dry season (March-May) from nine local Government areas (LG) of Plateau State: Bokkos, Jos, Barkin-Ladi, Pankshin, Shendam, Bassa, Lafia, Langtang, and Mangu. The dry season samples were representative of stored samples and were collected from houses in some towns and villages situated in the seven local government areas. The mouldy samples collected were mostly stored in the locally built mud storage barns called "rumbu" in Hausa. The field samples were collected from the farms during harvest. About two kilograms of the samples were collected and divided into two. One half was stored at $-20^{\circ}C$ for mycotoxin analysis and the other half was used immediately for fungal isolation studies.

3.2.2 Isolation of Fungi

About 10g of the grains were washed with 10 successive 100ml volumes of sterile distilled water and surface sterilized using 5.25% sodium

hypochlorite solution (Clorox, the Clorox Company, 1978 Oakland, CA94612). Ten grains were placed into each of 10 petri-dishes containing potato dextrose agar (PDA) and 100mg/litre of chloramphenicol. The dishes were incubated at 27^{o}C and examined daily for 7-10 days and fungi from plated grains were transfered to PDA slant media bottles and fresh PDA in petri dishes for identification.

3.2.3 Identification of Fungi

Identification of isolates was done by the Crop Protection Department of Ahmadu Bello University, Zaria, the USDA-Southern Research Centre, New Orleans and the Common Wealth Mycological Institute, Kew, London.

3.2.4. Analysis of mycotoxins

The field and stored samples were screened and analysed for aflatoxins B_1, B_2, G_1, and G_2; zearelenone, and ochratoxin A. Aflatoxins and ochratoxin A standards were obtained from Makor Chemical Ltd. Jerusalem, Israel. Zearalenone standard was obtained from USDA-Southern Regional Research Centre , New Orleans USA. A multimycotoxin assay method developed and used in U.S. Department of Agriculture, Southern Regional Research Center, New Orleans was used (Ehrlich and Lee, 1984).

The method utilizes methylene chloride and phosphoric acid for the simultaneous extraction of aflatoxin, ochratoxin A and zearalenone. A separate portion of the initial methylene chloride and phosphoric acid is subjected to a specific clean up procedure for each mycotoxin. Each of the procedure is a modification of a published procedure for each toxin.

3.2.5 Extraction, Identification and Quantitation of mycotoxin 50g of Hammermill pulverized samples were weighed into 500ml Erlenmeyer flask and 25ml of 1M phosphoric acid and 25ml methylene chloride were added.

The flask was shaken for 30 minutes and the contents filtered under reduced pressure on Buchner funnel fitted with 18.5cm circle rapid filter paper. At least 200ml of filtrate was collected. 50ml aliquots were placed into separate 100ml Erlenmeyer 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 Association of Official Analytical Chemists methods (1980). The plates were developed in ether-methanol-water (96:3:1) and quantitated densitometrically using Schoeffel SD 300 densitometer.

Ochratoxin assay was by the modified method of Paulsch et al. (1982). One molar phosphoric acid was substituted for 4M and methylene chloride was substituted for chloroform. The intensities of the standards and samples were compared densitometrically.

For zearalenone assay, except for the use of phosphoric acid and methylene chloride in place of chloroform as extracting solvent, the procedure was that of Ware and Thorpe (1978). Aflatoxin was confirmed by spraying the thin layer chromatographic plate with aqueous sulphuric acid (H_2So4) (50/50 v/v) dried and viewed at 365 nm when the spots flouresced yellow. Ochratoxin was confirmed by spraying the plate with alcoholic aluminium chloride ($Alcl_3$) (20g/100ml) alcohol and also by exposure of plate to NH_3 vapour and viewed at 365 nm when the spots of ochratoxin A flourescence changed from blue green to bright blue. Zearalenone was also confirmed by spraying the plate with alcoholic $Alcl_3$ and viewed at 365 nm when the flourescence intensity increased.

RESULTS

3.3 Table 3.1 shows the list and incidence of fungi isolated from stored and field mouldy maize samples collected at the three different seasons of the year.

One hundred and twenty-four fungal isolates were made and identified. During the harmattan period (November - February) Fusarium spp. were the most common fungi (7/14) followed by Aspergillus (5/14) and Phoma spp. (4/14). While during the warm humid wet period Trichoderma spp. (16/19), Fusarium and Neurospora spp. (6/19) were most common. During the hot dry season Fusarium spp. (15/23) predominated followed by Aspergillus spp. (8/23) and Phoma sorghina, Neurospora and Trichoderma spp. were of equal occurrence (5/23).

During the harmattan, samples from Langtang, contained the highest amount of aflatoxin and zearalenone (960 ug/kg and 17,500 ug/kg respectively). Ochratoxin A was found only in samples from Jos (50 ug/kg) (Table 3.2).

During the hot dry season samples from Pankshin LGA contained the highest amount of aflatoxins B_1 (192 ug/kg) and B_2 (144 ug/kg), and the highest amount of zearalenone was from Barkin Ladi and Mangu LGA (350.0 and 700 ug/kg, respectively, Table 3.3). During the warm, humid and wet season samples from Bokkos had the highest aflatoxin B_1 and B_2 (607 and 114 ug/kg respectively).

The highest zearalenone and ochratoxin A level were found in the samples from Jos (17,500 and 150 µg/Kg respectively) (Table 3.4).

Six of the total samples collected contained both aflatoxins and zearalenone. Six samples have both aflatoxin and ochratoxin A and five of the total samples have all the three mycotoxins.

3.4 DISCUSSION

The results of this study showed that there is a high incidence of zearalenone and ochratoxin A in mouldy maize samples found in different parts of the Plateau State. Both have been presumed to occur mainly in grains in the temperate countries with some reports from Zambia where up to 4.6mg of zearalenone/liter was found in maize beer (Scott, 1978, Lovelace and Nyathi 1976). But a cold spell, combined with the rains which occurred in 1973 - 74 when the samples were collected was said to provide favourable conditions for Fusarium growth and zearalenone production in the corn. In the Plateau State a temperature as low as $4^{o}C$ is not unusual during the harmattan and at times during the rainy season. Thus, Plateau climate at certain times of the year can be temperate like.

This might be responsible for the high incidence of Fusarium spp. and thus the wide occurrence of zearalenone. However, no ochratoxin-producing fungus was isolated in this study. This may either be due to the fact that the fungi had died leaving the mycotoxins or that the incubating temperature (27°C) was too high for ochratoxin-producing fungi. Van Walbeck was able to culture Penicillium viridicatum only after incubation at 12°C. Another possibility is that some of the fungi isolated from maize might be unrecognised producers of ochratoxin A. In a similar study on "Acha" (Digitaria exilis, Stapf), a staple grain food in the Plateau, both zearalenone and ochratoxin A had a high incidence (Gbodi et al., 1986). Among the fungi that produce aflatoxin and zearalenone, A. flavus and F. moniliforme were the common isolates from field and stored mouldy maize samples. The high incidence of these fungi correlated with the levels of aflatoxins and zearalenone in the samples.

Considering the high incidence of Phoma sorghina in field samples one should expect its metabolites like cytochalasins A and B and tenuazonates to be present, but these were not screened for in this study. In the course of our sampling it was discovered that the

villagers were reluctant to part with their mouldy maize. They either fed them to livestock or washed and used them for human consumption. The public health significance of mycotoxins has been highlighted by various studies carried out previously (Bullerman, 1979). The use of mouldy feedstuffs for human and animal food should be of great concern to veterinarians and Public Health Officials especially as mouldy samples may be contaminated by more than one mycotoxin as shown by this study and other reports (Ehrlich and Lee, 1984). It would be important therefore to educate farmers on the best ways of storing grains to prevent them from getting mouldy.

TABLE 3.1 TYPES OF FUNGI ISOLATED FROM MAIZE (Zea Mays) AT THREE DIFFERENT SEASONS OF THE YEAR AND THEIR INCIDENCE IN PLATEAU STATE.

Dry Harmattan season (November-February)		Hot Humid Rainy season (June-October)		Hot Dry season (March-May)	
Fungi	Incidence	Fungi	Incidence	Fungi	Incidence
Aspergillus flavus	4/14*	Aspergillus flavus	3/19*	Aspergillus flavus	5/23*
Aspergillus nidulans	1/14	Aspergillus niger	2/19	Aspergillus fumigatus	1/23
Cephalosporium spp.	1/14	Cunninghamella spp.	1/19	Aspergillus niger	2/23
Drechslera rostrata	1/14	Fusarium moniliforme	5/19	Cephalosporium spp.	1/23
Fusarium moniliforme (Sheld)	3/14	Fusarium spp.	1/19	Chaetomium globosum	2/23
Fusarium spp.	4/14	Neurospora spp.	6/19	Cunninghamella spp	1/23
Helminthosporium spp.	1/14	Penicillium funiculosum	1/19	Curvularia spp.	1/23
Neurospora spp.	2/14	Rhizopus spp	1/19	Drechslera sativus	1/23
Penicillium spp.	1/14	Trichoderma spp.	16/19	Fusarium moniliforme	11/23
Phoma sorghina	2/14			Fusarium spp.	4/23
Phoma spp.	2/14			Helminthosporium	1/23
Rhizoctonia solani	1/14			Nigrospora spp.	3/23
Rhizopus spp.	2/14			Neurospora spp.	5/23
Syncephalasrum racemosum	1/14			Phoma sorghina	5/23
				Rhizoctonia solani	1/23
				Rhizopus spp.	2/23
				Syncephalastrum racemosum	1/23
				Trichoderma spp.	5/23

* Number of samples collected during the period.

TABLE 3.2 OCCURRENCE OF AFLATOXINS, ZEARALENONE AND OCHRATOXIN A IN MOULDY MAIZE (*Zea mays*) DURING THE COLD DRY HARMATTAN SEASON (NOVEMBER - FEBRUARY) IN PLATEAU STATE (CONCENTRATIONS IN ug/kg or ppb)

Source LGA		Aflatoxins B_1	Aflatoxins B_2	Aflatoxins G_1	Aflatoxins G_2	Zearalenone	Ochratoxin[A]
Bokkos		ND(1)	ND	ND	ND	ND	ND
Jos	Range	0.0-20.0(1/4)	0.0-6.0(1/4)	ND	ND	0.0-250.0(1/4)	0.0-50.0(3/4)
	Mean	5.0	1.50			62.50	23.75
Langtang	Range	8.0-960.0(3/6)	0.0-543.0(3/6)	0.0-83.39(3/6)	0.0-23.53(1/6)	0.0-17500(2/6)	ND
	Mean	372.49	113.02	13.89	3.92	2958.53	
Shendam	Range	0.0-8.88(2/3)	ND	ND	ND	0.0-250.0(1/3)	ND
	Mean					83.33	

(1/4) = One sample out of four contained the mycotoxin.

LGA = Local Government Area.

ND = Not detected.

TABLE 3.3 OCCURRENCE OF AFLATOXIN, ZEARALENONE AND OCHRATOXIN A IN SAMPLES OF MOULDY MAIZE (Zea mays) COLLECTED DURING THE HOT DRY PERIOD(MARCH - MAY) IN THE PLATEAU STATE (CONCENTRATIONS IN ug/kg OR ppb)

Source LGA		Aflatoxins B_1	B_2	G_1	G_2	Zearalenone	Ochratoxin A
Bassa	Range	0.0-0.8(1/3)	ND	ND	ND	0.0-100(13)	0.0-20.0(2/3)
	Mean	0.27				33.33	11.67
B/Ladi	Range	ND	ND	ND	ND	350(1)	35(1)
Bokkos	Range	0.0-100(1/2)	0.0-30.0(1/2)	ND	ND	0.0-250.0(1/2)	ND
	Mean	50.0	15			125.0	
Lafia	Range	0.0-100(2/3)	0.0-100(2/3)	0.0-15.0(2/3)	0.0-20.0(2/3)	0.0-350.0(1/3)	0.0-20(1/3)
	Mean	66.76	66.76	10	13.33	116.67	6.67
Mangu	Range	ND	ND	ND	ND	0.0-700.0(1/2)	ND
	Mean					350.0	
Pankshin	Range	0.0-192.0(1/6)	0.0-144.0(1/6)	ND	ND	ND	0.0-8.0(2/6)
	Mean	32.0	24.0				2.17
Shendam	Range	0.0-23.20(4/9)	0.0-2.73(2/9)	ND	ND	0.0-200.0(1/9)	0.0-20.0(1/9)
	Mean	4.80	0.57			22.22	2.22

(1/3) = One sample out of three contained the mycotoxin

LGA = Local Government Area

ND = Not detected

TABLE 3.4 OCCURRENCE OF AFLATOXINS, ZEARALENONE AND OCHRATOXIN[A] IN SAMPLES OF MOULDY MAIZE (Zea mays) COLLECTED DURING THE HOT MUMID RAINY SEASON (JUNE - SEPTEMBER) IN THE PLATEAU STATE (CONCENTRATIONS IN ug/kg OR ppb)

LGA		Aflatoxins				Zearalenone	Ochratoxin A
		B_1	B_2	G_1	G_2		
Bassa	Range	0.0-41.63(1/3)	0.0-7.71(1/3)	ND	ND	0.0-350(1/3)	ND
	Mean	13.86	2.57			116.67	
Barkin-Ladi	Range	0.0-18.56(2/4)	0.00-4.80(3/4)	0.0-8.0(1/4)	0.0-2.40(1/4)	ND	0.0-110.0(2/4
	Mean	8.85	2.75	2.00	0.60		38.33
Bokkos	Range	245.0-607.01(2/2)	36.0-114.38(2/2)	ND	ND	ND	ND
	Mean	426.0	75.19				
Jos	Range	0.0-245.0(4/11)	0.0-36.0(4/11)	ND	ND	0.0-17,500(5/11)	0.0-150.0 (4/11)
	Mean	43.97	5.98			1640.92	18.64
Lafia	Range	0.0-10.0(1/2)	ND	ND	ND	ND	ND
	Mean	5.00					
Mangu	Range	0.0-8.0(1/3)	0.0-2.40(1/3)	ND	ND	ND	ND
	Mean	2.67	0.80				
Shendam	Range	0.0-20.20(1/3)	ND	ND	ND	10.0-100.0(3/3)	0.0-5.0(1/3)
		6.67				53.33	1.67

(1/3) = One sample out of three contain the mycotoxin

LGA = Local Government Area

ND = Not Detected

CHAPTER IV

THE MYCOFLORA AND MYCOTOXINS FOUND IN STORED COTTON SEED (Gossypium spp.) IN PLATEAU STATE

4.1 INTRODUCTION

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 to cattle farmers to be used as feed, and distributed free to farmers for planting. In some parts of Nigeria like Sokoto state the cottonseed is said to be used in place of beans for making fried bean cake "akara" and in some parts of Plateau and Kwara states, cottonseed is used for a special spice for soup in preference to the more popular condiment made from locust beans (Ikurior, 1982).

Because cottonseed products have found wide use in Nigeria for Livestock feed and for human food, and because little has been done on the mycoflora and

mycotoxins in cottonseed it was thought necessary to investigate these in Plateau state, though the state is not a major cotton producing area of Nigeria.

4.2 MATERIALS AND METHODS

4.2.1 Collection of Cottonseed Samples - About one kilogram each of samples of visibly mouldy cottonseed were collected from three Local Government Areas (Bassa, Jos and Keffi) of Plateau State during the harmattan (November - February), hot dry season (March - May) and the wet humid season (June - October). All the samples were collected from storage places. The samples were collected in sterile polythene bags and divided into two: one half was ground and stored at -20^{o}C until analysed for mycotoxin, and the other half was used immediately for fungal isolation.

4.2.2 Isolation of Fungi

The cottonseed samples used for fungal isolation were delinted mechanically using scarpel blade. They were then washed with 10 successive 100ml volumes of sterile distilled water and surface sterilized using 5.25% sodium hypochlorite solution (Clorox, the Clorox Company, 1978 Oakland, CA94612). Ten seeds were placed into each of 10 petri-dishes containing potato dextrose agar (PDA) and 100mg/litre of chloramphenicol.

The dishes were incubated at 27°C and examined daily for 7-10 days and fungi from plated grains were transferred to PDA slant media bottles and fresh PDA in petri-dishes for identification.

4.2.3 Identification of Fungi - Identification of isolates was done by the Crop Protection Department of Ahmadu Bello University, Zaria, the USDA-Southern Research Centrer, New Orleans and the Common Wealth Mycological Institute, Kew, London.

4.2.4 Analysis of Mycotoxins

The procedure outlined in the AOAC (1980) for cottonseed and cottonseed products were used.

4.2.5 Identification of Mycotoxins

Aflatoxin was confirmed by spraying the thin layer chromatographic plate with aqueous sulphuric acid (50/50 v/v) dried and viewed at 365nm when the spots of ochratoxin A flourescence changed from blue green to bright blue. Zearalenone was also confirmed by spraying the plate with alcoholic $AlCl_3$ and viewed at 365nm when the flourescence intensity increased.

4.3 RESULTS

4.3.1. Sampling

Only stored samples were used because only few areas in Plateau State grew cotton in 1981 when the

samples were collected. In these few areas it was difficult to collect mouldy samples. Ten samples were collected: one during the harmattan; three during the hot dry season and six during the rainy season.

4.3.2 Fungi Isolated

Phoma sorghina was the only fungus isolated during the harmattan (Table 4.1) Cunninghamella spp. were isolated from all the three samples collected during the hot dry season. One sample was also contaminated with Trichoderma spp. Of the six samples collected during the wet and humid period, Trichoderma spp was isolated from four, Aspergillus flavus, Phoma sorghina, Curvularia spp. from two and Nigrospora sppand Cunninghamella spp. from one.

4.3.3 Mycotoxins Isolated

Six mycotoxins were screened for in mouldy cotton-seed. Only aflatoxins were found in three out of ten samples: in one of two samples collected from Keffi LGA; two of six samples from Jos LGA; no mycotoxin was found in any of the two samples collected from Bassa LGA. The Keffi sample had the highest aflatoxin B_1 content of 271ug/kg and B_2 content of 36.6 ug/kg. The highest B_1 content found in Jos was 139.0 ug/kg while that of B_2 was 30 ug/kg.

4.4 DISCUSSION

Uraguchi and Yamazaki (1978) reported the incomplete knowledge of the mycoflora of cottonseed. They gave a list of fungi isolated from both field and stored cottonseeds mainly from the U.S.A. In a study of fungi and bacteria infecting cottonseed in Mozambique, De'Mendonca and Alves (1973) isolated many fungal species including *Trichoderma* spp., the main fungus isolated in this study, and which is an advanced decay fungus. Almost all the samples were badly stored.

Other fungi isolated included *Cunninghamella* spp, *Aspergillus flavus*, *Phoma sorghina*, *Curvularia* spp and *Nigrospora* spp. Some *Trichoderma* spp. are known to produce toxic metabolites. Brian (1944) isolated *gliotoxin* from the filtrate of *T. viride*. *Aspergillus flavus* strains produce many types of mycotoxins including aflatoxins, oxalic acid and tremorgens (Hesseltine, 1974). Aflatoxin contamination of cottonseed is said to be a world-wide problem (Bullerman, 1979). Most of this contamination occurs in the field and can result in contaminated cottonseed oil and meal.

Abalaka and Elegbede (1982) found high levels of aflatoxins in both cottonseed pellets (480 ug/kg), crude oil (65ug/kg) and refined cottonseed oil (24ug/kg) from an edible oil extracting plant in Nigeria. The hazards of aflatoxins to human and animal health has been fully recognised. Of great concern is the finding that aflatoxin is one of the most potent naturally occurring carcinogen known to date. The hazards of aflatoxins to Public Health and the linking of the frequency of contamination of local diets with incidence of certain cancers in different parts of the world have been adequately reviewed (Wilson, 1978, Bullerman, 1979).

The cytochalasins produced by Curvularia spp. are known for their cytostatic activity: they inhibit cell motility, and reversibly inhibit uptake of 2-deoxy-D-glucose by fibroblasts and cultured liver cells (Wells et al., 1981). Martin et al.(1971) in their work on incidence of fungi in feedstuffs in Eastern Transvaal and Swaziland isolated seventeen spp. of Nigrospora oryzae; eight produced toxic metabolites. Steyn and Rabie (1976) implicated a mycotoxin from Phoma sorghina in the aetiology of a disease called Onyalia and characterized the toxic constitutent to be magnesium and calcium tenuazonate. Thus most of the fungi isolated

from mouldy cottonseed in this study are potential toxin producers, but only aflatoxins were detected. Aflatoxins G_1 and G_2 which were said to be rare in cottonseed (AOAC, 1980) were found and positively identified in two of the samples. Scott (1978) in his review of mycotoxins in feed ingredients reported the occurrence of only aflatoxins in cottonseed. The reason 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. Another possible reason may be that little or no attempt has been made to identify these mycotoxins in cottonseed and cottonseed products.

TABLE 4.1

TYPES OF FUNGI ISOLATED FROM STORED COTTONSEED IN THE PLATEAU STATE AT THREE DIFFERENT SEASONS OF THE YEAR AND THEIR INCIDENCE

	Harmattan Season (November-February)	Hot Dry Season (March - May)	Hot Humid Season (June - October)
Fungi and Incidence	Phoma sorghina (1/1*)	Cunninghamella sp. (3/3*)	Aspergillus flavus (2/6*)
		Trichoderma sp. (1/3)	Cunninghamella sp. (1/6)
			Curvularia sp. (2/6)
			Nigrospora sp. (1/6)
			Phoma sorghina (2/6)
			Trichoderma sp. (4/6)

*Number of samples collected during the season.

TABLE 4.2

OCCURRENCE OF AFLATOXINS IN SAMPLES OF MOULDY COTTONSEED (GOSSYPIUM SP LINN) FROM SOME LOCAL GOVERNMENT AREAS OF PLATEAU STATE

Source LGA	Aflatoxins Level ug/kg of Cottonseed			
	B_1	B_2	G_1	G_2
Jos	Range 0.0-139.0(2/6)*	0.0-30.0(2/6)	0.0-183(1/6)	-
	Mean 24.5	5.20	30.5	
Keffi	Range 0.0-271.0(1/2)	0.0-36.6(1/2)	0.0-122.0(1/2)	0.0-9.1(1/2)
	Mean 135.5	18.3	61.0	4.5
Bassa	ND ND	ND	ND	ND

* 2/6 = Two samples out of six contained aflatoxin

LGA = Local Government Area

ND = Not Detected

CHAPTER V

TOXICITY FOR MICE OF EXTRACTS OF FUNGI ISOLATED FROM ACHA (*Digitaria exilis* Stapf)

5.1 INTRODUCTION

In a previous study (Gbodi *et al.*, 1986) 96 fungi were isolated from acha. Amongst these fungi *Aspergillus ochraceus*, *A. sydowii*, *A. terreus*, *A. niger*, *A. nidulans*, *A. flavus*, *A. cremeous*, *Fusarium solani*, *F. moniliforme*, *F. oxysporum*, *F. roseum*, *F. equiseti*, *F. semitectum*, *Phoma sorghina* et-cetera, are known to produce mycotoxins; others such as *A. quadrilineatus*, *Syncephlastrum racemosum*, *Trichoderma harzianum* and *Cunninghamella elegans* have not been reported to produce mycotoxins. There is not enough information on the types of fungi infecting foods and feedstuffs and the extent of mycotoxins produced by these fungi in Nigeria. Various workers like Elegbede (1978) and Dada (1979) worked on the fungi infecting sorghum crop under field and storage conditions. Only Dada made some effort to culture a strain of *Phoma sorghina* on potato dextrose agar and extracted some metabolites from the culture but never screened the extract for toxicity.

Many syndromes of unknown aetiology are now suspected to be caused by mycotoxins. For example,

some metabolites of Phoma sorghina have been implicated in a haematologic disorder called "Onyalia" disease which occurs widely amongst the black African population South of the Sahara (Rabie et al., 1975). Also in Western Nigeria a seasonal disease known as "Ijeshashakes" is said to be a fairly common problem amongst the populace. The cause is unknown but a tremorgenic mycotoxin is suspected (Wright and Morely, 1958).

Many of the moulds that are capable of producing mycotoxins are also frequent contaminants of food and feedstuffs. The objective of this study is to investigate the mycotoxin producing fungi found in Acha (Digitaria exilis Stapf) and thus to have an insight into the possible mycotoxins which can be produced in acha in nature and their roles in food safety and human and animal health.

5.2 MATERIAL AND METHODS

5.2.1 Culturing of fungi from Acha

To 20g of uncle Ben rice, 8ml of distilled water was added and mixed thoroughly and left overnight for moisture equilibration. The rice substrate was then autoclaved for 20min. at 121^{o}C and 15 psi pressure.

After cooling, it was aseptically inoculated with conidia or mycelia of four to seven-day old pure cultures grown on Potato Dextrose Agar (PDA) slant tubes and incubated for 12-14 days at 28°C.

Eight fungi (A. quadrilineatus, A. nidulans, Curvularia lunata, Fusarium equiseti, F. moniliforme, F. semitectum, F. solani and Phoma sorghina) isolated from stored and field acha samples and found to produce the most toxic fungal metabolites on screening were selected. Fresh PDA tubes were inoculated with each selected fungus from stock cultures maintained on PDA tube and incubated for 5-7 days at 28°C.

For mass culturing of the eight fungi producing the most toxic metabolites, 5ml of Triton X-100 (Searle Hopkin and Williams, Chalwell Health, Essex, England) treated sterile distilled water (1 drop Tritox X-100 to 200ml sterile distilled water) was added to each culture tube and the surface of the culture scratched with sterile inoculating wire to suspend the spores. The suspensions were then used to inoculate a 2.5l Fernbach flask containing 250g of uncle Bens rice, the moisture content of which had been equilibrated as earlier described. The flasks were autoclaved for 25 minutes under pressure of 15 psi. The cooled flasks

were inoculated with the suspended spores and maintained in an incubator at 28°C as a static culture for 21 days.

5.2.2 Extraction of Toxins

The 20g rice cultures were homogenised for 2 min. in 100ml methylene chloride using explosion proof homogeniser or blender. It was then filtered through fast fluted filter paper. Two ml of corn oil was added to the filtrate in a round bottomed flask and concentrated in a rotatory evaporator at water bath temperature of 55°C. When the methylene chloride was distilled off, clean empty distillate flask collector was used to replace the methylene chloride collector and rotatory evaporator turned on for another ten minutes to ensure complete removal of methylene chloride vapour. The corn oil-toxin extract was transferred into a dram vial and kept in deep freezer at -25°C till used for toxicity testing in mice.

For extraction of the most toxic fungal isolates 750 ml of chloroform were added to the Fernbach flask with the mouldy rice and after one hour the solvent soaked rice was chopped into small fragments and blended for three minutes. It was filtered through a bed of Hyflosuper-cell in a Buchner funnel fitted with fast

flow filter paper. The filtrate was again filtered through anhydrous sodium sulphate to remove the moisture and clarify the extract. The clarified clear solvent was removed at 50°C by rotatory vacuum evaporator.

The oily viscous residue was added to chilled swirling petroleum ether (1:15 residue/petroleum ether v/v) and the mixture kept overnight in a deep freezer at -15°C to complete the precipitation of the toxins. The crude toxin was recovered by filtration and dried in a fan oven at 50°C for three hours before being used for pilot toxicity studies.

5.2.3 Experimental Animals

Four to five week old male white mice weighing 20-25g were used for the screening of toxin-producing fungi isolated from acha. They were purchased from King Animal Laboratory Inc. Oregon, Winsconsin, U.S.A. Animals were housed in conventional plastic wire cages with synthetic bedding. They were given mice pellets and tap water ad-libitum. The air conditioned room was kept at 20°C and no extra lighting was provided in the night. The animals were randomly alloted to cages and five animals were kept in a cage for seven days prior to commencement of the toxicity testing. The cages were washed daily and bedding changed.

5.2.4 Screening acha culture extracts for toxicity in mice.

Five mice each received by IP injection 0.2ml of the corn oil extract of fungal culture. The mice were observed for signs of toxicity for 14 days. Five mice which received corn oil were used as controls. The toxicity of the extracts was arbitrarily classified into four categories viz;-

1. Very toxic (if all five of the extract treated mice died)
2. Moderately toxic (if three or four of the mice died)
3. Mildy toxic (if one or two of the five mice died) and
4. Nontoxic (if none of the mice died)

5.2.5 Acute Toxicity Testing with Eight Extracts

A pilot test was carried out using eight selected fungi producing very toxic metabolites from the screening test. To determine the dose of different extracts that will cause 0% to 100% death in mice, five groups of three mice each were used for each extract. The extract was dissolved in dimethyl sulphoxide. Log intervals of 0.60 was used to select a range of IP doses, 40, 160, 640 and 2560 mg/kg body weight. A group of five mice which served as control received the highest dose of the

DMSO administered to extract treated mice. The animals were observed for 14 days for signs of toxicity.

5.3 RESULTS

5.3.1 Toxicity screening of Extracts of fungi isolated from Acha in mice

Table 5.1 shows the results of toxicity screening of extracts, incidence and types of fungal isolates from acha found to produce toxic metabolites and those found not to produce metabolites. Of the 96 isolates, 50 (or 52%) produced toxic metabolites. Of these 50, 20 (40%) were mildly toxic, thirteen (34%), moderately toxic and 17 (26%) were highly toxic. Of the 20 isolates that produced mildly toxic metabolites 6 were Aspergillus spp. (30%), 6 Phoma spp. (30%), and 4 Fusarium spp. (20%). Of the 13 moderately toxic isolates, 7 were Fusarium spp. (54%) and 2 each (total of 15.4%) were Phoma and Trichoderma spp. Of the 17 very toxic isolates, five (29.5%) were Phoma sorghina and 4 each were Aspergillus and Fusarium spp., constituting 23.5%.

5.3.2 Pilot Acute Toxicity Testing

Table 5.2 shows the results of a pilot acute toxicity testing conducted on 8 selected extracts. At 160 mg/kg, three of the extracts (from Aspergillus quadrilineatus, Fusarium solani and Phoma sorghina) produced death in the mice, at 640mg/kg only one (from A.quadrilineatus) was found to be toxic, killing all three test mice. At 2560mg/kg three of the extracts (from A. quadrilineatus, A. nidulans and F. solani) were found

toxic: two (A. quadrilineatus and A. nidulans) killed all the three test mice, and one killed one of three.

The most toxic extracts were from A. quadrilineatus , A. nidulans, F. solani . A. quadrilineatus was selected for further study because not much information about its toxicity is available in the literature.

5.4 DISCUSSION

The results of the toxicity screening tests showed that fungal isolates from acha produced toxins that were lethal to mice. In earlier studies on mycotoxins in field and stored acha, zearalenone and vomitoxin thought to constitute problem only in temperate countries were found to be common in acha samples (Gbodi et al., 1986). These findings agree with the screening studies in which a good number of the toxin producing fungi were from Fusarium spp, which are known producers of zearalenone and vomitoxin. Phoma spp were also significantly present among toxin producing species in this study but none of the mycotoxins such as cytochalasins A and B produced by Phoma was analysed for in the previous study.

Of the toxic fungal isolates the Aspergillus spp are known to produce a host of mycotoxins, some of which are the aflatoxins, ochratoxins, citrinin, sterigmatocystin, penicillic acid, secalonic acid

kojic acid, palmotoxins,fumigillin and helvolic acid (Uraguchi and Yamazaki, 1978). The aflatoxins and sterigmatocystin are known to be naturally occurring carcinogens and have been associated with high incidence of liver cancer in certain parts of the world where foods are frequently contaminated with aflatoxins (Keen and Martin, 1971, Linsell and Peers, 1977). The Fusarium spp. are known to produce the trichothecenes like T-2 toxin, moniliformin, zearalenone, diacetoxyscirpenol, nivalenol, emetic factors and neosolaniol naturally in cereals (Hesseltine, 1974). All these mycotoxins have been linked with mycotoxicoses in different domestic animals (Mirocha et al., 1968; Ueno, 1977; Mc'Erlean, 1952; McNutt, 1928).

In this study A. quadrilineatus produced one of the most toxic metabolites although search in literature has not revealed what mycotoxin it produces. It will be worthwhile to investigate the nature of the toxin(s)produced by this fungus.

Fourty six other fungi were found not to produce toxic metabolites. Thirteen of these could not be subcultured for identification after the toxicity studies, because they would not grow again but all those identified are known from literature to be potential mycotoxin producers.

It is known that not all strains of a fungus elaborate toxins. Some strains are tamed by nature or from subculturing, since fungi are known to mutate and thus, loose the ability to produce toxins. Also the conditions under which they were cultured could have been unsuitable for mycotoxin elaboration.

This study shows that Aspergillus, Fusarium and Phoma spp. are the likely fungal spp. that can infect and elaborate mycotoxins in poorly stored or mouldy acha. During the collection of samples for this study it was noted that mouldy acha were freely used for human and animal food, thus exposing both animal and humans to the risk of mycotoxicosis.

TABLE 5.1

TOXICITY SCREENING OF EXTRACTS AND INCIDENCE OF FUNGI ISOLATED FROM STORED ACHA SAMPLES IN MICE.

Mildy Toxic[1] (20	Incidence	Moderately Toxic[2] (13)	Incidence	Very Toxic[3] (17)	Incidence	Non Toxic[4]	Incidence
A. flavus	2/20	Chaetomium spp.	1/13	Aspergillus flavus	1/17	Aspergillus flavus	4/46
A. ochraceus	1/20	Cylindrocarpon spp.	1/13	A. nidulans	1/17	A. nidulans	1/46
A. quadrilineatus	2/20	Fusarium equiseti	1/13	A. quadrilineatus	1/17	A. spp.	2/46
A. sydowii	1/20	F. moniliforme	4/13	A. terreus	1/17	Chaetomium spp.	2/46
Chaetomium spp.	1/20	F. oxysporum	1/13	Chaetomium spp.	1/17	Curvularia spp.	4/46
Cunninghamella spp.	1/20	F. solani	1/13	Curvularia lunata	1/17	Fusarium moniliforme	3/46
Curvularia spp.	1/20	Phoma sorghina	1/13	fusarium equiseti	1/17	F. spp.	2/46
Fusarium equiseti	1/20	P. spp.	1/13	F. moniliforme	2/17	Phoma sorghina	12/46
F. moniliforme	1/20	Trichoderma		F. semitectum	1/17	Phoma spp.	3/46
F. oxysporum	1/20	longibrachitum	1/13	Phoma sorghina	5/17	Unidentified	13/46
F. spp.	1/20	T. spp.	1/13	Rhizopus microsporus	1/17		
Penicillium citrinium	1/20			Trichoderma			
Phoma sorghina	4/20			harizianum	1/17		
P. spp.	2/20						

1. Mildy Toxic = 1-2 mice killed (20)
2. Moderately Toxic = 3-4 mice killed (13)
3. Very Toxic = All five mice killed (17)
4. Non Toxic = No mice killed (46)

Number of Isolates

TABLE 5.2

PILOT ACUTE TOXICITY* (I.P) OF EIGHT SELECTED FUNGAL EXTRACTS FROM ACHA

Fungi Tested	40mg/kg No. Dead	160mg/kg NO.Dead	640mg/kg NO.Dead	2560mg/kg NO. Dead
Aspergillus quadrilineatus Thom & Raper	0/3	0/3	3/3	3/3
Aspergillus nidulans (Eidam) Winter	0/3	1/3	0/3	3/3
Curvularia lunata (Wakker) Boredijin state of Cochliobolus lunatus (Nelson & Hoasis)	0/3	0/3	0/3	0/3
Fusarium equiseti (Corda) Sacc.	0/3	0/3	0/3	0/3
F. miniliforme Sheld	0/3	0/3	0/3	0/3
F. semitectum Berk & Rav.	0/3	0/3	0/3	0/3
F. solani (Mart) Sacc.	0/3	2/3	0/3	1/3
Phoma sorghina (Sacc.) Boerema, Dorenbosch and V. Kest.	0/3	1/3	0/3	0/3

* I.P. = Intraperitoneal route.

CHAPTER VI

ACUTE TOXICITY OF THE CRUDE EXTRACTS OF ASPERGILLUS QUADRILINEATUS (THOM & RAPER) IN MICE, CHICKS AND CALF.

6.1 INTRODUCTION

Various species of the genus Aspergillus are known to cause mycotoxicoses in animals and man. Aflatoxicosis devastated the turkey industry in U.K. in 1960. This disease also afflicted calves (Loosmore and Markson 1961) cattle (Clegg and Bryson, 1962; Gopal et al., 1968) and pigs (Hurter, 1966). An outbreak of acute liver cirrhosis due to aflatoxicosis in children was reported by Amla et al., (1970, 1971) in India. In all animals aflatoxin is known to have particular effects on the liver. It causes centrilobular necrosis, proliferation of bile ducts and fibrosis and haemorrhages in the intestines (Detroy, Lillehoj and Ciegler, 1971). Variations in symptoms depend on dosage: in one-day-old ducklings, a high dosage produces haemorrhagic necrosis; a medium dosage slight bile duct proliferation; and a low dosage extensive degenerative changes in liver cells and bile duct proliferation (Purchase, 1967). Acute symptoms in many animals including chickens and rats comprise haemorrhagic necrosis of the liver.

Ochratoxicosis caused by ochratoxins A and B which are metabolites of A. ochraceus, A. sulphureus, A. sclerotiorum, A. alliaceus, A. ostianus and A. melleus (Ciegler, 1972) is associated with widespread hyaline degeneration of liver cells with focal necrosis (Theron et al., 1966). Doupnik and Peckham, (1970) reported haemorrhages in proventriculi, emaciation, dehydration, dry, hard and firm gizzard linings and hepatic injury with fatty changes or necrotic foci, when cockrels were fed moist corn contaminated with ochratoxin.

Sterigmatocystin is a mycotoxin isolated from A. nidulans, A. regulosus and A. versicolor. (Hesseltine, 1974). This toxin is known to cause necrosis of liver and kidneys in albino rats. High dosages are known to cause hepatoma and cholangiosarcoma in rat (Purchase and van der Watt, 1968).

Patulin is another mycotoxin of the Aspergillus species. It has been isolated from A. clavatus, A. giganteus and A. terreus and associated with many disease outbreaks in France (Moreau and Moreau 1960), Bulgaria and East Germany (Schultz,1968) and Russia (Tomov, 1966). Symptoms included fever, in co-ordination and hepatic degeneration. Blyth and Lloyd (1971)

produced focal necrosis and granulomata in the liver and pancreas, tubular degeneration and metaplasia in kidney cortices and nervous symptoms in mice after intramuscular or intraperitoneal injection of patulin.

A tremorgenic neurotoxicosis which decimated a herd of cattle in northern Transvaal of South Africa was reported by Kellerman *et al.* (1976). *A. clavatus* was the fungus associated with this disease. In 1964, Aust, reported the death of 20 out of a pen of 29 heifers and steers due to haemorrhagic syndrome caused by contamination of a corn by a species of *Aspergillus*.

In the pilot study reported in Chapter V, *A. quadrilineatus*, one of the fungal isolates from acha produced toxic metabolites which have hetherto not been reported. The present study was carried out to confirm the acute toxicity of *A. quadrilineatus* extracts in mice and chick and the cultured fungus in a calf.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Animals

6.2.1.1 Mice

White male and female Balb-C mice, 4-5 weeks old

and weighing 20-25g purchased from Nigerian Institute for Trypanosomiasis Research, Vom were used for this study. Males and Females were housed seperately in conventional plastic wire cages with synthetic beddings or wood shavings. They were given mice pellets and tap water ad-libitum. No extra lighting was provided in the night. They were kept for seven days before commencement of the experiment. The cages were washed daily and beddings changed.

6.2.1.2 Chicks

The day-old chicks were the Apollo (White Leghorn) got from Poultry Division of the National Veterinary Research Institute, Vom. They were housed in groups of fives, in wire battery cages used for brooding chicks. They were fed balanced diet of chick ration prepared in the Poultry Division and were given tap water ad-libitum. The cages were cleaned daily and no extra light was provided but the room was kept at 25°C throughout the three week experimental period, using electric heaters.

6.2.1.3 Calf

The calf used in this experiment was housed in

a covered barn. The concrete floor was divided by wooden rafters into pens measuring 2.4 by 2.4 meters and about 1.52 meters high with wooden hay racks and lockable gates. The calf was screened for blood parasites and dewormed with thiabendazole (MsD, Hoddesdon, Herforshire, England) two weeks before they were used. Acha hay and water were supplied ad-libitum and supplemented with 1 kg of concentrate per day. The concentrate consisted of 48% maize, 35% wheat offals, 15% groundnut cake, 1% bone meal, 1% commonsalt and 0.01% vitamin mixture.

6.2.2 Extracts of A. quadrilineatus

A. quadrilineatus was cultured and the crude toxin extracted as described in section 5.2.1. The petroleum ether filtrate was poured back into the flask and the petroleum ether removed at 50°C on rotatory vacuum evaporator. When there was no more distillate condensing in the distillate collecting flask it was assumed that the petroleum ether had been removed but to ensure complete removal of petroleum ether fresh clean and dry flask was substituted for the distillate flask and rotatory evaporator turned on for another 5 minutes with the water bath temperature at 50°C.

The oily petroleum ether residue (PER) was preserved for toxicity screening. It was suspended in corn oil for administration to animals. Petroleum ether defatted crude residue (PEDCR) was also used for toxicity, it was dissolved in DMSO/70% ethanol (3: 1 v/v) before administering to animals.

6.2.3 Petroleum Ether Residue (PER) Toxicity in chicks

To determine the toxicity of PER, 3000, 6000, 9000 and 12000mg/kg were administered, orally using a stomach tube, to four groups of five day-old chicks; the fifth group received petroleum ether extracted corn oil alone. The animals were observed for fourteen days and post mortem examinations were carried out on dead chicks.

6.2.4 Acute Toxicity of PER in Mice.

From the result of the Pilot test, a range of doses were selected between 750 and 1500mg/kg at log intervals of 0.06. Thus, the IP doses chosen were 771.3, 887, 1027, 1201, 1405 and 1615mg/kg. Seven groups of 10 mice per group (5 females + 5 males) were used, the seventh group served as the control

receiving the highest dose of vehicle (corn oil) administered to the experimental mice IP. Thompson moving average (Thompson, 1947) and the graphical method of Miller and Tainter (1944) were used to estimate the LD_{50} of PER in mice. Postmortem examinations were carried out on dead mice.

6.2.5 Acute Toxicity of PEDCR in day-old chicks

From the result of the pilot test a range of doses were selected between 80 and 1280mg/kg at log intervals of 0.31 to estimate the acute toxicity of PEDCR in day-old chicks. Five groups of ten chicks each (five females and five males) were dosed IP with 80, 160, 320, 640 and 1280mg/kg of PEDCR dissolved in DMSO/ethanol (3:1v/v). A control group received the highest dose of the solvent. The moving average method of Thompson (1947) was used to estimate the LD_{50} of PEDCR. Chicks dying were subjected to postmortem examinations.

6.2.6 Acute intraperitoneal toxicity of PEDCR in mice

From the pilot test, a range of IP doses of PEDCR: 13.33, 16.0, 19.2, 23.04, 27.65 and 33.18mg/kg were given to six groups of ten mice per group (5 males and 5 females). A control group of 10 mice received the highest dose of the solvent (DMSO/70% ethanol 3:1).

Both Thompson Moving Average (Thompson 1947) and graphical method of Miller and Tainter (1944) were used to estimate the LD_{50} of PEDCR in mice. Mice dying in any group were subjected to post mortem examinations including histopathologic studies.

6.2.7 Acute oral toxicity of PEDCR in mice

From the results of a pilot test a range of doses were selected between 180 and 911.25mg/kg at a log interval of 0.1761. Five oral doses of PEDCR at 180, 270, 405, 607.5 and 911.25mg/kg were administered to five groups of ten mice per group (5 males + 5 females) using a polyethylene cannula attached to cut 26 x 3/8 hypodermic needle. The sixth control group received the highest dose of the solvent (DMSO/70% ethanol in a ratio of 3:1). Both Thompson moving average method (Thompson, 1947) and graphical methods of Miller and Tainter (1944) were used to estimate the oral LD_{50} of PEDCR in mice. Postmortem examinations were carried out on mice that died and samples of organs were collected for histopathologic studies.

6.2.8 Acute Toxicity of cultured A. quadrilineatus in a calf

A. quadrilineatus was cultured as described in section 5.2.1, except that rice was replaced by maize.

The culture was in large Fernbach 2.5 litre flasks kept in a small dark room at 28 -30°C for a period of three weeks, after which the cultured material was ground using a Hammer mill, and stored in large polythene bags at -25°C till used.

About five, kilograms of the cultured material was blended with water and given by drenching to a bull weighing 122kg. The animal was observed for about six hours for signs of acute toxicity, and thereafter everyday for two weeks. Control cattle was similarly drenched with water slurry of ground maize and observed for two weeks.

6.2.9 Preparation of tissues for histopathologic studies

6.2.9.1 Tissue processing and sectioning - Fresh samples of liver, lung, kidney, pancreas , brain, ovary, testis, stomach, duodenum and intestine were fixed in 10% formol-saline. 3-4mm thick tissue was cut from each organ for processing. The cut tissues were transferred to the automatic tissue processor where the tissues were further fixed in 10% buffered formol-saline for two hours and dehydrated for 2 hours in each ascending grades of alcohol (70%, 90%, 100% v/v). The dehydrated tissues were then cleared in xylene for two hours and

the tissues impregnated in molten paraffin was for another two hours and left to cool. The sections were then trimmed and sectioned on the microtome at 5 microns. The sections were floated out in a warm water bath, then attached to slides, and dried on a hot plate and stained.

6.2.9.2 Staining of the Sectioned Tissues

The sections were dewaxed in xylene, and hydrated in descending grades of alcohol - 100%, 90% and 70%. They were stained with haematoxylin for 10 minutes, differentiated in1% acid alcohol, blued in Scotts tap water and stained in eosin for 3 minutes. They were then rinsed, dehydrated in ascending grades of alcohol- 70%, 90% and 100% and finally cleared in xylene and mounted in PDX.

6.3 RESULTS

6.3.1 Preparation of Toxic Extract for Administration

Petroleum ether residue (PER) formed homogenous suspension in corn oil. Petroleum ether defatted crude residue (PEDCR) was insoluble in water, corn oil, aqueous ethanol, propylene glycol, and dilute dimethyl sulphoxide (DMSO/0.IM $NaHCo_3$)

6.3.2 Toxicity Screening of PER given orally in chicks

Table 6.1 shows the results of oral administration of PER to four groups of five chicks at high dosages.

Three of the five chicks that received 6000mg/kg of PER died within five days after administration of the extract; all five chicks given either 9000mg/kg or 12,000mg/kg died within six days. The clinical signs observed, tachypnea and tachycardia occurred two hours after treatment. This was followed by decreased activity, anorexia and somnolence about 24 hours later. When treated birds were proded to move, ataxia was evident; diarrhoea was also noted in some, followed by coma and death within one to five days depending on dosage. The post mortem findings in dead chicks were heamorrhagic enteritis and slight congestion of the liver and lungs. The internal surfaces of the stomachs of dead animals were sloughed off. The main histopathologic findings were extensive degeneration and necrosis of hepatocytes and multifocal areas of severe necrosis in the parenchyma of the lungs.

6.3.3 Acute oral Toxicity of PER in mice

Table 6.2 shows the oral LD_{50} (1148 $\pm$ 170 mg/kg

of petroleum ether residue (PER) of A. quadrilineatus in mice as determined by Thompson moving average method.

The extract caused tachypnea and tachycardia, followed by anorexia as in chicks. The postmortem findings included heamorrhagic enteritis and congestion of the liver and lungs. Using the graphical method of Miller and Tainter,(1944) a linear regression line of effect of dose on percent death of PER in mice was plotted on a logarithmic percentage probability paper as shown in Fig. 6.1. The LD_{50} represented by the dosage value at 50% is 1190mg/kg with standard deviation of $\pm$36.40mg/kg. The correlation coefficient is 0.99.

6.3.4 Acute oral Toxicity of PEDCR in chicks

Table 6.3 shows oral LD50 determination of PEDCR in chicks, using the Thompson moving average method, $PEDC^R$ DMSO/70% ethanol had an oral LD_{50} of 556 $\pm$ 80mg/kg. Using the graphical method of Miller and Tainter (1944) (Fig. 6.2) PEDCR had an LD_{50} value of 540 + 39.44mg/kg. The correlation coefficient of the regression line was 0.99. Signs of toxicity which occurred in most chicks before death included tachypnea and tachycardia. These signs occurred thirty minutes to one hour after administration of the toxin, and were followed by anorexia, somnolence, diarrhoea and coma.

Death was recorded from about the eighteenth hour to five days after treatment, the onset of which was dose dependent. Histopathologic findings included oedema and mild degeneration of the myocardium. Tubular epithelial cells of the kidney showed severe necrosis and degeneration of some glomerular tufts. There was severe necrosis of the hepatocytes and extensive congestion of liver sinusoids. The lungs were congested and showed severe haemorrhages, especially at 405mg/kg and above. Also necrosis and destruction of bronchioles were very extensive. No significant changes were seen in control chicks.

6.3.5 Acute Intraperitoneal Toxicity of PEDCR in mice.

Table 6.4 shows the IP LD_{50} determination of PEDCR in mice. By the Thompson Moving Average method (1947) the LD_{50} was calculated to be 21.0 $\pm$ 11.69 mg/kg with the graphical method of Miller and Tainter (1944), it was 20.87 $\pm$ 11.69 mg/kg (Fig.6.3); correlation coefficient of the regression line was 0.98.

The clinical signs of toxicity included tachypnea tachycardia, anorexia, somnolence and coma. Death was recorded ten hours after administration of 27.65mg/kg and 33.18mg/kg; the last death were recorded six days

after administration. The only post-mortem findings were congestions of the heart, lungs and liver.

6.3.6 Acute oral Toxicity of PEDCR in mice

The oral LD50 of PEDCR in mice was 566 ± 16mg/kg by Thompson method (Table 6.5), or using the graphical method, it was 507 ± 23.83 mg/kg (Fig. 6.4). The correlation coefficient was 0.95. No deaths were recorded in the control group dosed with DMSO/70% ethanol (3:1). At lower doses, 405mg/kg and below, the main postmortem findings were mild congestions of the heart, lungs and liver. There was severe cortical and medullary congestion and capsular haemorrhage of the kidney. Acute haemorrhagic enteritis was noticed in some and mucoid enteritis in others, with gas trapped in the stomach and small intestine. The muscles of the oesophagus and skeletal muscles were mildy degenerated.Necrosis of the mucosal glandular cells of the stomach were quite extensive and severe in some cases. The liver was congested and few focal areas of hepatocytic cellular degeneration and necrosis were seen in the parenchyma (Plate 6.1).The myocardium was congested and degeneration and necrosis was marked in the cells. These were characterized by granular cytoplasm and occasional loss of nuclei in the cells. The lungs were congested and interstitial pneumonia were seen.

Mild meningeal congestion of cerebrum and cerebellum were seen.

Mice given higher doses, 607mg/kg and above showed most of the histopathologic signs described above but in some cases more severe. Severe submucosal and mucosal gastric oedema were seen. The liver showed severe centrilobular necrosis of the hepatocytes. The skeletal muscle showed severe and extensive chronic degenerative myopathy characterized by degeneration; clumping and loss of cross striations in some fibres. Muscle nuclei proliferation and fibres with intense eosinophilic staining were seen. Tubulo-nephrosis was quite marked in the kidneys. Some of the small blood vessels contained fibrinous thrombi. Parenchymal congestion was quite marked in the cerebellum including significant meningeal congestion and haemorrhage. No significant changes were seen in control mice.

6.3.7 Acute toxicity of culture of A. quadrilineatus in a calf

The only toxic manifestations observed about one hour after drenching the calf with an aqueous fungal culture were whole body tremors, tachycardia, and profuse salivation covering the hairs around the mouth.

The tremors subsided about two hours later. No other signs were noticed during the observation except for a slight decrease in feed consumption for the first two days. When the animal was sacrificed no significant gross post-mortem lesions or histopathologic changes were found.

6.4 DISCUSSION

The mycotoxin(s) produced by A. quadrilineatus was extracted by both petroleum ether and chloroform, but the toxin appeared to be more soluble in chloroform. This can be seen from the amount of crude toxin precipitated from chloroform extract. PER at 3000mg/kg did not produce death in day old chicks. In mice the oral LD_{50} of PER was 1148mg/kg, that of PEDCR was 560mg/kg, showing the toxin of A. quadrilineatus extracted in chloroform to be more potent. In estimating the LD_{50} of both extracts, the graphical method of Miller and Tainter did agree closely with the Thompson moving average method.

The toxic principle(s) in both PER and PEDCR seemed to be the same as they produced similar toxic signs and histopathologic effects in both chicks and mice. Such toxic effects as tachypnea suggest

stimulation of respiratory center in the brain, and tachycardia and diarrhoea may suggest effect of the extract on the autonomic nervous system. Somnolence suggests effect on the sleeping centre of the CNS.

Mycotoxins are known to have deleterious effects on various body organs and functions. Many of them have affinity for certain tissues and mycotoxins have thus been classified as hepatotoxins, nephrotoxins, dermatoxins et-cetra. The liver is the organ most commonly affected by mycotoxins. The most potent hepatotoxic mycotoxins are luteoskyrin and aflatoxin (Allcroft et al., 1961). From the lesions produced by the toxins of A. quadrilineatus in chicks and mice it would appear that the toxins have wide-spread effects on different body organs and tissues. A single oral dose of 180-1280mg/kg showed marked degenerative and necrotic effects on cells and tissues of kidneys, liver, lungs, pancreas, spleen, skeletal muscle, stomach, brain and heart muscle.

No significant effects were noticed when five kilogram of the corn culture of the fungus was administered to a calf. It is possible that the toxin (s) of A. quadrilineatus was detoxified by the rumen microflora and enzymes. This may account for the high tolerance of ruminants to mycotoxins.

TABLE 6.1

SCREENING OF PETROLEUM ETHER RESIDUE (PER) FOR TOXICITY IN CHICKS

Groups	Oral dose (mg/kg)	Number of Dead/Treated chicks
1	3000	0/5
2	6000	3/5
3	9000	5/5
4	12,000	5/5
5	Corn oil	0/5

TABLE 6.2

ORAL LD50 OF PETROLEUM ETHER RESIDUE EXTRACT OF ASPERGILLUS QUADRILINEATUS IN MICE

Groups	Dose (mg/kg)	Number of dead/Treated Mice	$LD_{50} \pm$ SD
1	771.3	0/10	
2	887.0	1/10	
3	1027.0	3/10	1148 ± 170mg/kg (Thompson method)
4	1201.0	8/10	
5	1405.0	8/10	
6	1615.0	10/10	
7	Control	0/10	

TABLE 6.3

ORAL LD50 DETERMINATION OF PETROLEUM ETHER DEFATTED CRUDE RESIDUE EXTRACT OF ASPERGILLUS QUADRILINEATUS IN CHICKS

Groups	Dose mg/kg	No. of dead/treated chicks	$LD_{50} \pm$ SD
1	120	0/10	
2	180	1/10	556 ± 80mg/kg
3	270	2/10	(Thompson method)
4	405	4/10	
5	607.5	5/10	
6	911.25	10/10	
7	Control	0/10	

TABLE 6.4

INTRAPERITONEAL LD50 DETERMINATION OF PETROLEUM ETHER DEFATTED CRUDE RESIDUE OF EXTRACT OF ASPERGILLUS QUADRILINEATUS IN MICE

Groups	Dose mg/kg	Number of dead/Treated Mice	$LD_{50} \pm SD$
1	13.33	0/10	
2	16.00	2/10	21.0 ± 11.6mg/kg
3	19.20	5/10	(Thompson method)
4	27.65	6/10	
5	33.18	10/10	
6	Control	0/10	

TABLE 6.5

ORAL LD50 DETERMINATION OF PETROLEUM ETHER DEFATTED CRUDE RESIDUE OF ASPERGILLUS QUADRILINEATUS IN MICE

Groups	Dose mg/kg	No of dead/treated Mice	$LD_{50} \pm SD$
1	270.00	0/10	
2	405.00	4/10	566 ± 16mg/kg
3	607.50	8/10	(Thompson method)
4	911.25	10/10	
5	Control	0/10	

Fig 5.1, Least Square Regression Line of Miller and Tainter Method of LD_{50} Estimation of Petroleum Ether Residue (PER) of *A. quadrilineatus* in Mice.

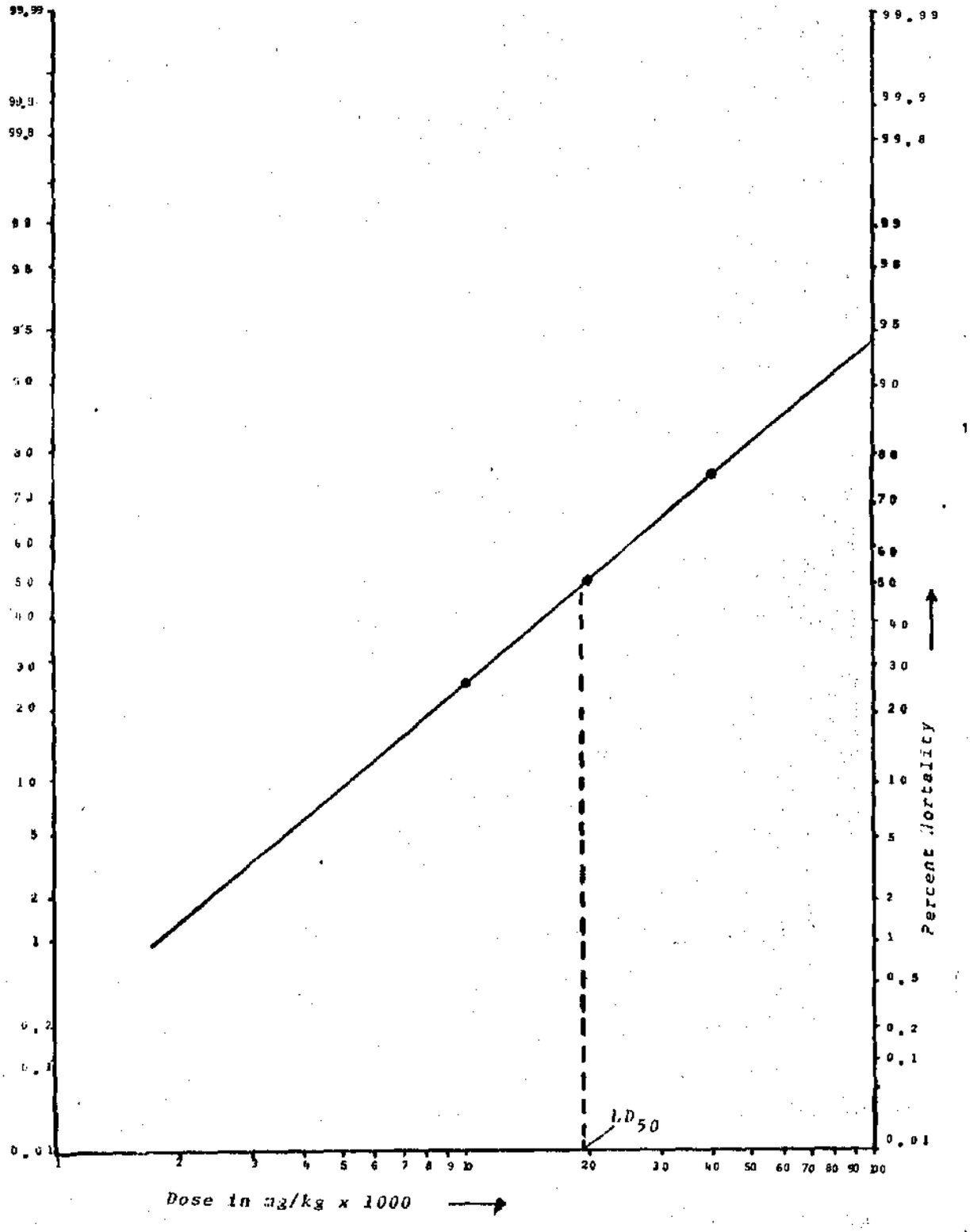


Fig.62 Least Square Regression Line of Miller and Tainter Method of LD_{50} Estimation of Proteleum Ether Defatted Crude Residue (PEDCR) of A. quadrilineatus in chicks (Oral).

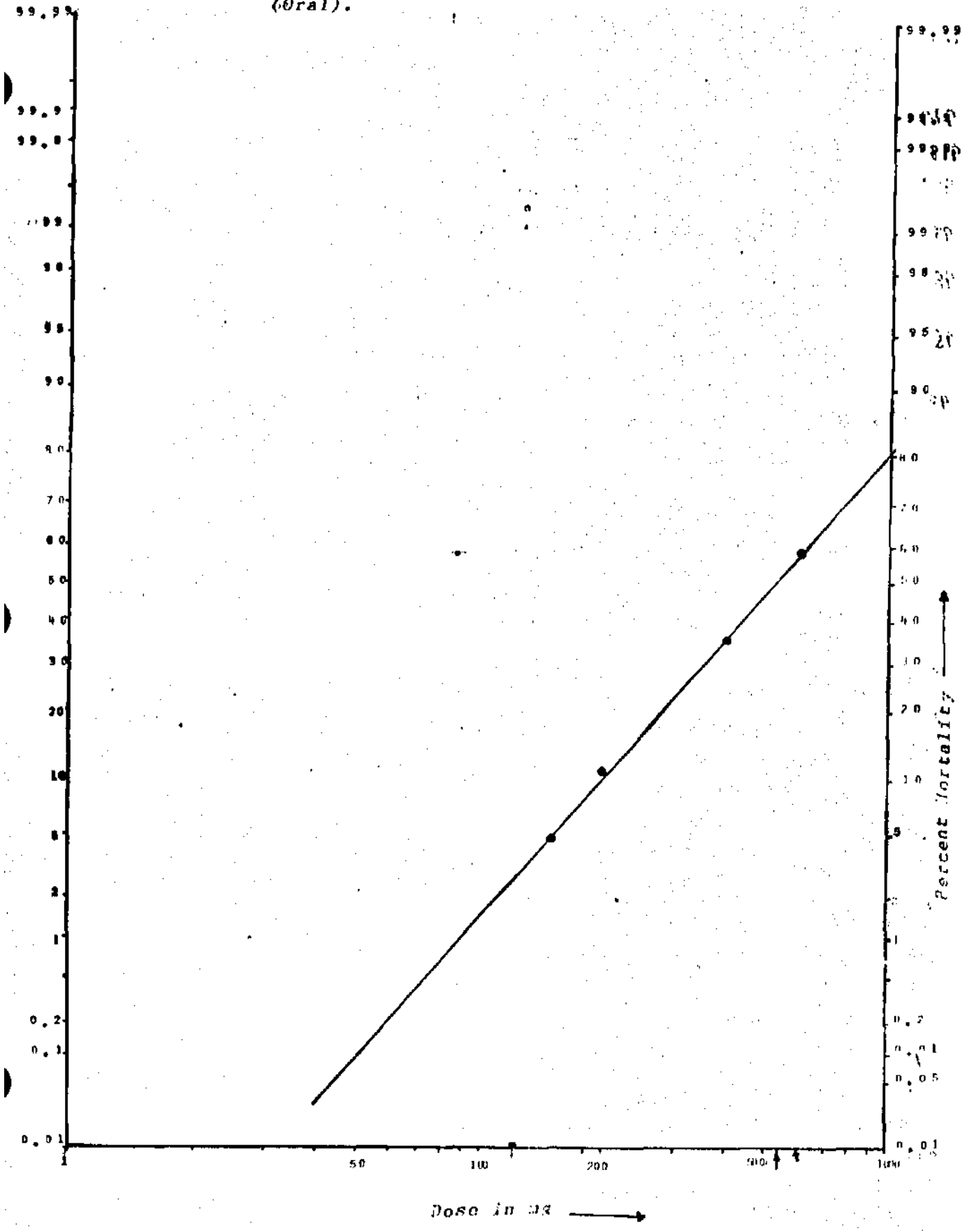


Fig. 6.3 Graphical Calculation of Miller and Tainter Method of LD_{50} Estimation of Petroleum Ether Defatted Crude Residue (PEDCR) of *A. quadrilineatus* in Mice.

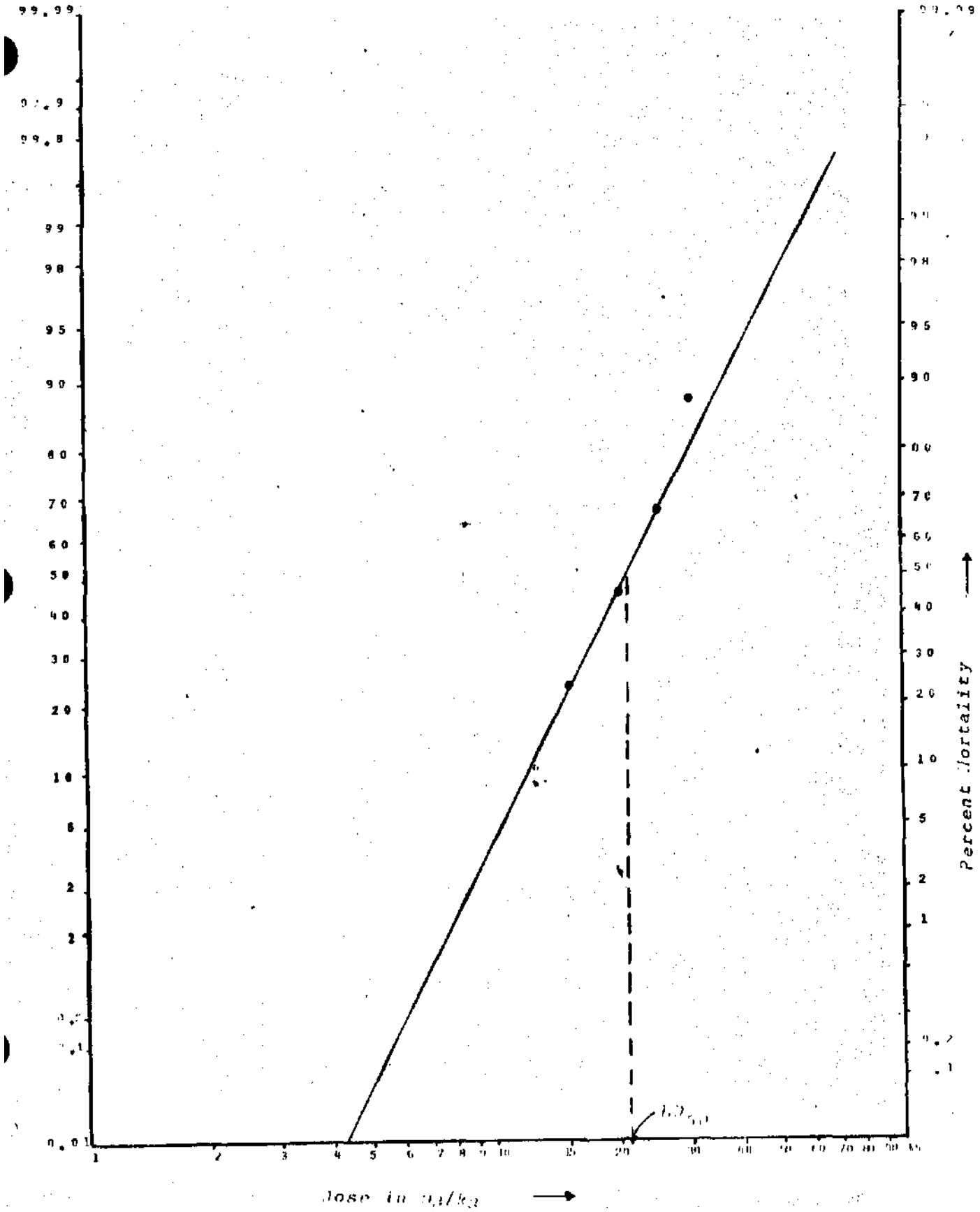


Fig. 6.4 Graphical Calculation of Miller and Tainter Method of LD_{50} Estimation of Petroleum Ether Defatted Crude Residue (PEDCR) of A. quadrilineatus in mice (Oral)

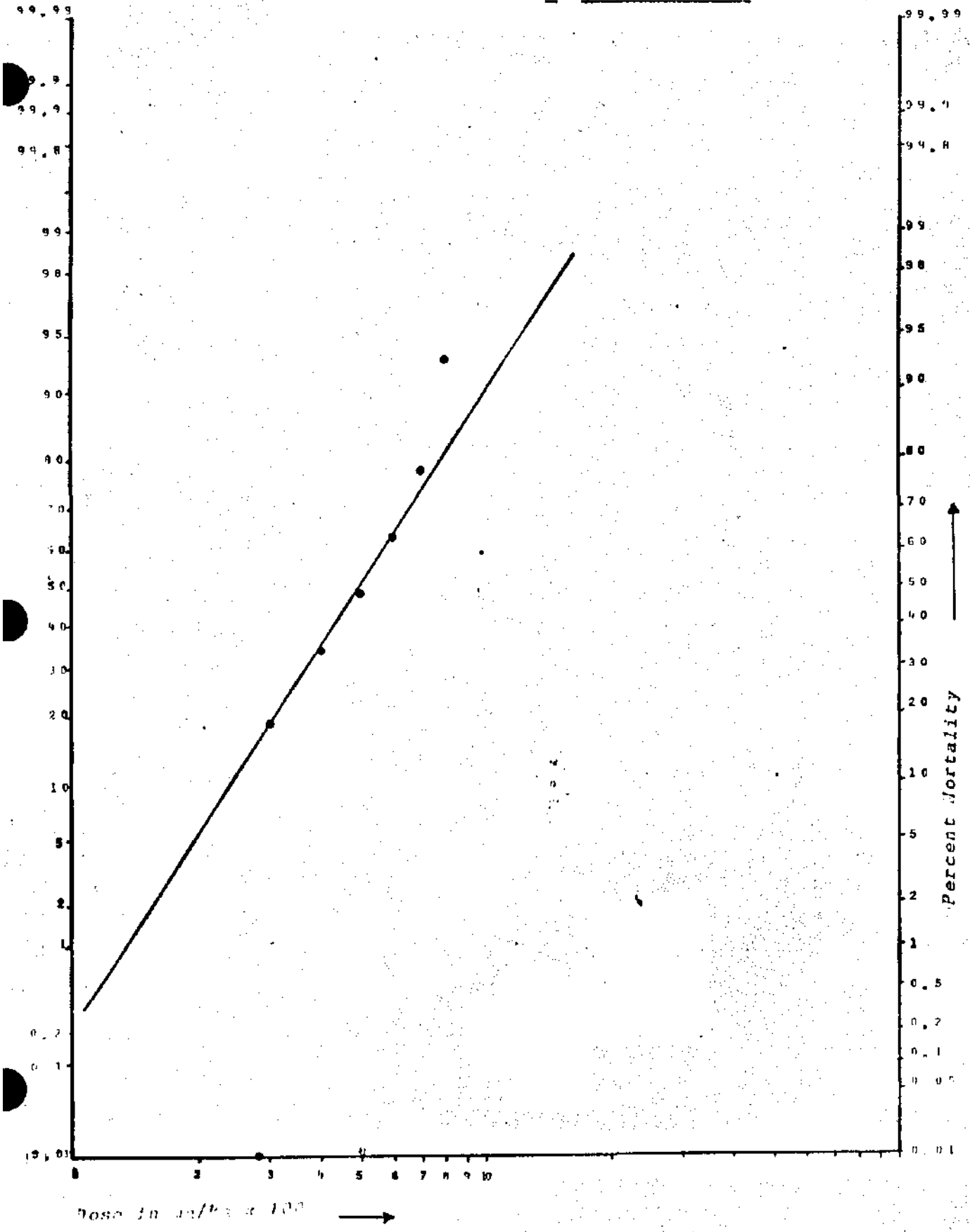
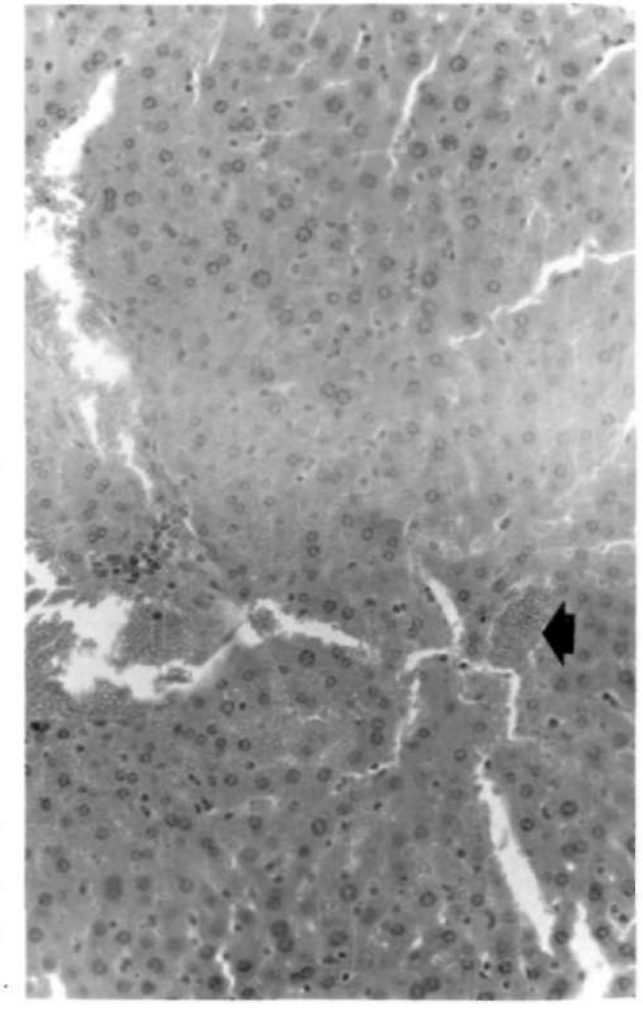


Plate 6.1. Photomicrograph of liver of mouse acutely dosed 405mg/kg of petroleum ether defatted crude residue, extract of Aspergillus quadrilineatus. Note the congestion of the liver x 160



CHAPTER VII

CHRONIC TOXICITY OF ASPERGILLUS QUADRILINEATUS IN RATS AND CATTLE

7.1 INTRODUCTION

Aspergillus quadrilineatus (Thom and Raper) used in this study was isolated from *acha* (*Digitaria exilis*) a popular cereal crop used for human food on the Plateau.

Only mouldy *acha* grains are fed to domestic animals like goats, sheep and cattle. But *acha* straw is used as fodder for animals at the National Veterinary Research Institute, Vom, where this experiment was carried out. Parcels of the Institute's lands are allocated to local farmers to cultivate *acha* and the Institute gets the straw in return.

The incidence of acute toxicoses caused by moulds and their toxins in animals is limited, but of great concern to many public health workers, scientists and agriculturists are their long term economic and health effects on animals and man exposed to minute quantities of mould toxins as they occur in nature. Thus, when a mould is isolated from feedstuffs and shown to elaborate toxins, it is necessary to study the acute and chronic effects of the toxin in animals in order to evaluate its hazard potential. Some scientists

have suggested that any mould metabolite that is not toxic when administered orally should not be considered to be a mycotoxin (Cole, 1978). Extracts of A. quadrilineatus have been shown to possess acute toxic effects. The present study was undertaken to determine the chronic toxic effects of A. quadrilineatus in rats and cattle.

7.2 MATERIALS AND METHODS

7.2.1 Experimental Animals

7.2.1.1 Rats - The rats used were weanling Wister Albino rats purchased from Nigerian Institute for Trypanosomiasis Research, Vom. The rats were three to five weeks old weighing 24 - 63g. Two males or two females were kept per cage and were given formulated diet consisting of 25% groundnut cake, 25% fish meal and 50% maize. Both the diet and water were supplied ad-libitum before the commencement of the experiment.

7.2.1.2 Cattle - Nine cattle were used. They were 1½ - 2½ year-old pure breed white Fulani cattle weighing 114 - 159kg, and were from Livestock Investigation Division of N.V.R.I. . They were housed in covered cattle barn. The floor was divided by wooden rafters

into pens measuring 2.4 by 2.4 metres and about 1.52 metres high with a wooden lockable gate. Each animal was placed individually in such a pen. The floor was of concrete with drainage system to allow urine to run off. Wooden boxes were constructed attached to the wooden rafters to hold the hay supplied. Cattle were screened for blood parasites and dewormed with Thiabendazole (80 mg/kg) MSD, Hoddesdon, Herforshire, England) two weeks before they were placed on the experimental diet consisting of acha hay and water ad-libitum and 1kg of concentrate per head per day before commencement of the experiment. The concentrate consisted of 48% maize, 35% wheat offal, 15% groundnut cake, 1% bone meal, 1% common salt and 0.01% vitamin mixture.

7.2.2 Culturing of A. quadrilineatus on maize and Preparation of Culture Material

Fresh Potato Dextrose agar tubes were inoculated with spores from pure culture of A. quadrilineatus and maintained in the incubator for 3-5 days at 28^{o}C. Five ml. of Triton X-100 (Searle Hopkin and Williams, Chalwell Health, Essex, England) treated sterile distilled water (1 drop Tritox X-100 to 200ml sterile distilled water) was added to the culture tubes and

surface of the culture scratched with sterile inoculating wire to suspend the spores. The suspension was then used to inoculate a 2.5 litre Fernbach flask containing 300g of yellow maize. The maize had been prepared by adding 250ml of distilled water and shaking the flask several times and left overnight for moisture equilibration. The flasks were autoclaved at 15psi for 25 minutes. The cooled flasks were inoculated with the suspended spores and maintained as a static culture at 28^{o}C in an incubator for 21 days. Cultures were made in a large number of Fernbach flasks (up to 250 flasks at a time). The cultures were then placed in fan extracted oven at 50^{o}C for four hours to remove the moisture and the cultured material in the flasks removed and ground using a Hammer mill (Christy and Norris Ltd., Chemford England). The ground culture was stored in large sterile polythene bags at -20^{o}C until used.

7.2.3 Preparation of Substituted Diets

Normal concentrate feed fed to cattle consisted of 48% maize, 35% wheat offal, 15% groundnut cake, 1% bone meal, 1% common salt and 0.01% vitamin mixture. Three other rations were prepared in which 100%, 60% and 20% of the maize constituent was replaced by maize on which A. quadrilineatus was cultured. Commercial

rat feed was purchased and fed to rats. The diet was substituted with 60% or 30% A. quadrilineatus cultured maize.

7.2.4 Chronic Toxicity of A. quadrilineatus in rats

Rats were alloted into five groups of five females and five males each. Group 1 received pure maize on which A. quadrilineatus was cultured; group 2 received normal diet substituted 60% with fungal maize culture; group 3 received 30% substituted diet, group 4 was given pure ground yellow maize, and the control group 5 received normal diet. The rats were weighed at the beginning of the experiments and thereafter weekly for twelve weeks.

7.2.5 Chronic Toxicity of A. quadrilineatus in cattle

Nine cattle were alloted into four groups. Group 1, made up of two males, received normal diet substituted 20% by maize culture of A. quadrilineatus; group 2 of two males received 60% substituted diet; group 3 of two males received pure A. quadrilineatus cultured maize (100%) and group 4 (control) made up of two males and one female received normal diet.

7.2.6 Histopathologic Examination

Postmortem examinations were carried out on dead and euthanatised animals by exposing them to ether fumes at the termination of the experiment. Samples of liver, kidney, lung, brain, stomach, intestines spleen, testis, oviduct and thigh muscles were collected in 10% formalin/saline solution for histopathologic studies.

7.2.6.1 Tissue processing and sectioning

Fresh samples of organs were fixed in 10% formol saline. 3-5mm of each tissue was cut from each organ for processing. The cut tissues were transferred to the automatic tissue processor where the tissues were further fixed in 10% bufferred formol-saline for two hours and dehydrated in ascending grades of alcohol: 70%, 90%, 100% for two hours in each of the alcohol solutions. The dehydrated tissues were then cleared in xylene for two hours and the tissues impregnated in molten paraffin wax for another two hours and left to cool down. The sections were then trimmed and sectioned on the microtome at 5 microns. The sections were floated out in a warm water bath, attached to slides, dried on a hot plate and stained.

7.2.6.2 Staining of the Sectioned Tissues

The sections were dewaxed in xylene, and re-hydrated in descending grades of alcohol: 100%, 90% and70%. They were stained in haematoxylin for 10 minutes, differentiated in 1% acid alcohol, blued in Scotts tap water and stained in eosin for 3 minutes. They were then rinsed, dehydrated in ascending grades of alcohol: 70%, 90% and 100% and finally cleared in xylene and mounted in DPX.

7.2.7 Determination of Organ Body Weight Ratio in Rats

Rats on experimental fungal cultured diet and control rats that survived throughout the experimental period were euthanatised with ether. They were subjected to postmortem examinations and the following organs were removed from each rat; brain, heart, kidneys, liver, lungs, spleen, stomach, testis or oviduct and intestines. Excessive blood was blotted off with filter paper and the tissues weighed. The ratio of organ weight in gram per 100 gram total body weight of rat were calculated.

7.2.8 Collection of Blood and Serum from cattle for Haematological, Serum Mineral and Serum Biochemical Studies.

Cattle were bled by jugular venipuncture. The blood for haematological parameters were collected fortnightly for five months using disodium ethylene diamine tetracetic acid (Na_2EDTA) as anticoagulant; blood for mineral and other biochemical determinations was collected into dilute nitric acid washed, sterile plain universal bottles. The blood was allowed to clot at room temperature and centrifuged. Serum was separated within three hours after collection. The serum samples were stored at $-25^{o}C$ in deep freezer until assayed.

7.2.9 Serum Mineral Determination

Calcium, copper, magnesium, iron, sodium, potassium, and zinc were determined by atomic absorption spectrophotometric method, using Pye Unicam SP 1900 (Pye Unicam, York Street, Cambridge England CB12PX). Sodium and potassium were determined in flame emission mode. Inorganic phosphate was determined by the method of Delsal and Mahouri (1958) and read in the SP500 Pye Unicam spectrophotometer.

Chloride was determined by the method of Schales and Schales (1941).

7.2.10 Determination of Serum Biochemical Parameters

Ames Sera - Pak diagnostic Kits were used (Ames Division, Miles Laboratories Limited. Stokes Pages, Slough S124LY, England). Alkaline phosphatase was determined at wavelength of 405nm, albumin at 628nm, glucose at 505nm, urea nitrogen at 545nm, total protein at 546nm, triglyceride at 340nm, bilirubin at 578nm, lactic acid dehydrogenase (LDH) at 340nm, GOT at 340nm and cholesterol at 510nm. One centimetre Pye Unicam glass cuvettes and spectrophotometer Model SP6-300 were used for all determinations.

7.2.11 Haematological Determinations

Haemoglobin was determined by the cyanomethemoglobin method using Ames blood analyzer and packed cell volume (PCV) by the microhaematocrit method. Total white blood cell count was determined by the microscopic method in haemocytometer counting chamber after 1 to 20 dilution of blood in Turks solution. Blood smears were stained with Leishmans stain for differential leucocyte counts.

7.2.12 Statistical Analysis of Results

For statistical analysis of the data, the method used was as recommended by Gad and Well (1982). This basically uses Barlett's test or F - test to check for homogeneity of variance. Then analysis of variance was carried out using the Cochran T - test to compare two or more groups of data. The levels of significance were recorded. Also an Olivetti M24 computer with Epistat statistical package was used to run one way analysis of variance on the data. The level of significance were derived from F - table.

7.3 RESULTS

7.3.1 Clinical Observations

7.3.1.1 Rats

No remarkable clinical signs were seen in animals on 30% substituted diet during the experimental period. The faeces of animal on substituted diet were softer in texture. Rats on 100% substituted diet became lethargic about the second week of commencement of the experiment. Some had sideways tilting of the head. One rat had arched back. The hair coat in many rats in this group were rough and the faeces

softer in texture than that of control rats. Death was first recorded in this group by the second week when two rats died out of ten. By the sixth week nine of the ten rats had died and only one survived the experimental period. Rats on pure maize did not show any remarkable clinical signs.

Fig. 7.1 shows group mean weekly weight fluctuations in rats on, 30%, 60%, 100%, substituted diets, and rats on pure maize and control rats on normal diet. By the end of the fourteen week experimental period, rats on 30%, 60%, 100%, maize and control had the following weight changes: 141.25 $\pm$ 42.86gm, 138.43 $\pm$ 34.26gm, -16.70gm, -4.42 $\pm$ 5.87 and 150.41 $\pm$ 32.41gm respectively. Control rats had superior weight gain as compared with the other four groups. The mean weight change in both rats on pure maize and 100% substituted diets were highly significantly different from that of the other three groups ($P < 0.005$). That of the rats on 30%, 60% and control were not significantly different ($P < 0.05$).

7.3.1.2 Cattle

No abnormal clinical signs were seen in all cattle on 30%, 60%, 100% substituted diets and the control group.

One animal in 100% substituted diet group was uncontrollable at the start of experiment and had to be culled. Fig. 7.2 shows the fortnightly weight fluctuations in the four groups of cattle on the experimental diets. The group mean weekly weight gain of cattle on 20% and 60% substituted diets were significantly lower (0.90 $\pm$ 0.64 and 0.70 $\pm$ 0.71 kg)/ week respectively) than that of control animals (1.68 $\pm$ 0.20 kg/week at $P < 0.005$). That of animals on 100% substituted diet was not significantly different from that of the controls ($P < 0.05$).

7.3.2 Postmortem Findings

7.3.2.1 Rats

Six rats on 30% substituted diet exhibited hyperaemia of the mucosa of the stomach and intestines Rats on 60% substituted diet showed sloughing of the mucosa of the stomach, petechial haemorrhages on the liver and gastro-enteritis. Rats on 100% substituted diet had the epithelium of the stomach and duodenum sloughed off. The large intestine was filled with dark brown faeces.

7.3.2.2 Cattle

No significant postmortem findings were observed in animals on 20%, 60% substituted diets and in control animals. The mucosa of the stomach and duodenum of animals on 100% substituted diet were sloughed off.

7.3.3 Histopathologic Findings

7.3.3.1 Rats

The liver of rats on 30% substituted diet were congested. Mild hepatocellular degeneration and necrosis were seen in two out of 6 rats examined There was periacinar distribution of the degenerating hepatocytes. Some liver showed cloudy swelling and hydropic degeneration of the hepatic cells (Plate 7.1).

The spleen had highly congested venous sinuses in two out of 6 cases and some were characterized by mild to moderate lymphocyte depletion of the periarteriolar lymphatic sheath.

The lungs showed varying degrees of intestitial inflammation with peribronchiolitis and atelectasis. In two cases the lungs showed severe alveolar oedema, congested interstitial tissues and foci of interstitial pneumonia were observed.

In one case the cerebellar meninges was congested. Submucosal oedema of the stomach were observed in two animals. Three animals showed granular degeneration of the tubular epithelial cells particularly of the medulla. There was extensive congestion of the cortical area in some cases (Plate 7.2). Other organs did not show significant lesions.

Animals on 60% and 100% substituted diet showed similar but more severe lesions than those seen in animals on 30% substituted diet. One of 5 rats examined in the group on 60% substituted diet had severe degeneration of the myocardium.

The brain stem of one of the5 rats examined in the group on 60% substituted diet showed very severe meningeal congestion, haemorrhage and sub-meningeal degeneration. Perivascular cuffing in some parenchymal vessels were seen (Plate 7.3).

One out of three control rats examined showed mild degeneration of some fibres of myocardium and interstitial pneumonia. Apart from these observations no significant lesions were seen in the other organs.

7.3.3.2 Cattle

Cattle on 20% and 60% substituted diet showed hepatic degeneration and necrosis. The lesions in those on 60% substituted diet were more severe.(Plate 7.4). Cattle on 100% substituted diet showed multifocal areas of haemorrhage and necrosis in the liver and severe and extensive tubular necrosis and degeneration of glomerular tufts (Plate 7.5). No significant lesions were seen in control animals.

7.3.4 Haematological Determinations

7.3.4.1 Packed Cell Volume (PCV)

Fig. 7.3A shows group mean PCV values of cattle on the different substituted diets and those of the controls. Within the first four weeks animals on 20% and 60% substituted diet showed an increase in mean PCV values from 29.5 $\pm$ 2.12% to 32.00 $\pm$ 2.83% for 20% substituted diet. That of 60% was from 37.00 $\pm$ 4.24% to 40.00%. Animals on 100% substituted and control diets maintained constant PCV values for the first four weeks (32% and 35%, respectively).

After the sixth week the mean PCV values of all animals on substituted diets were lower than that of control for the 24 week experimental period. The 24 week mean PCV values (Table 7.1) of all the animals on substituted diets were lower than that of control (20%, 29.13 ± 1.04%;60%, 32.86 ± 4.28; 100%, 31.75 ± 1.48 and control, 36.35 ± 3.24) but the differences were not statistically significant ($P<0.05$).

7.3.4.2 White Blood Cell (WBC)

Fig. 7.3B shows the monthly group mean WBC values of the four treatment groups of cattle. Baseline values of animals on 60% substituted diets could not be determined because their blood clotted and were unsuitable for determinations. Animals on 20% and 100% substituted diets showed a marked decrease in WBC by the sixteenth week from 10350 ± 1202 to 8600 ± 3676 for 20% and from 9600 to 6000 for 100%. The 24 week mean values of WBC of animals on substituted diets were lower than that of control (for 20%, 9715 ± 1101; 60%, 9633 ± 3.77; 100%, 8657 ± 16 and control 10,575 ± 1284) but the differences were not statistically significant ($P<0.05$). Also the cumulative means of neutrophil, lymphocyte, monocytes

and eosinophil counts (Table 7.1) of animals on substituted diets were not statistically significantly different when compared with control value ($P<0.05$).

7.3.4.3 Haemoglobin (Hb)

Table 7.1 and Fig. 7.3C show 24 week mean haemoglobin values and the fortnightly fluctuations over the experimental period. The mean Hb value of the control animals was 12.27 $\pm$ 1.5gm/dl; the corresponding values of animals on 20%, 60% and 100% substituted diets were 9.12 $\pm$ 0.93, 10.14 $\pm$ 1.09 and 9.40 $\pm$ 0.84 gm/dl. The haemoglobin values of the three treatment groups were significantly lower than that of control group ($P<0.05$).

7.3.5 Serum Mineral Determinations

7.3.5.1 Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K), Copper (Cu), Zinc (Zn), Iron (Fe), Inorganic phosphate (IPO4) and chloride (Cl)

Table 7.2 and Fig. 7.4A, B, C, and 7.5A - F show means and the fluctuations over the 24 week experimental period of serum Ca, Mg, Na, K, Cu, Zn, Fe, IPO4 and Cl. No significant differences were seen in all serum minerals determined in animals on substituted and control diets.

7.3.6 Serum Biochemical Determinations

7.3.6.1 Blood Urea Nitrogen (Bun), Glucose (Glu) cholesterol (CHL), Bilirubin (BIL), Triglyceride (TG), Total protein (TP) Globulin (GLO) and Albumin (ALB).

Table 7.3 and Figs. 7.6 and 7.7 show mean serum BUN, GLu, CHL, BIL, TG, TP, GLO and ALB, values and their fortnightly fluctuations over the 24 week experimental period. Blood Urea nitrogen averaged 3.82 $\pm$ 0.15 Mmol/I in the control animals. In animals on 20%, 60% and 100% substituted diets the BUN values were 3.80 $\pm$ 1.24, 4.60 $\pm$ 0.80 and 3.76 $\pm$ 1.15 Mmol/l, respectively. There was a significantly higher BUN values in animals on 60% substituted diet as compared to control animals ($P<0.05$). The others were not significantly different.

Serum glucose was determined over a 10 week period. (Fig. 7.6B; Table 7.3). The mean value for control animals was 3.92 $\pm$ 0.65 Mmol/l. For animals on 20%, 60% and 100%, the means were 3.07 $\pm$ 0.50; 3.37 $\pm$ 0.71 and 3.71. $\pm$ 0.73 Mmol/l, respectively. Animals on 20% substituted diet had significantly lower values than the controls ($P<0.01$).

Serum cholesterol was determined over a 20-week period (Fig. 7.6C; Table 7.3). The mean values for control animals and those on 20%, 60% and 100% substituted diets were 3.86 $\pm$ 0.19; 3.91 $\pm$ 0.20; 2.96 $\pm$ 0.10 and 2.73 $\pm$ 0.40, respectively. The mean serum cholesterol values of animals on 20% substituted diet were significantly higher than those of control ($P<0.01$).

Fig. 7.6D and Table 7.3 show the fortnightly fluctuations and mean values of serum bilirubin (SB) in control animals and those on 30%, 60%, and 100% substituted diets. Very high values were recorded in SB of animals on 60% (0.072 Mmol/l) and 100% (0.097 Mmol/l) substituted diets as compared with control (0.052 Mmol/l) by the eighth week. The mean SB values of animals on 20% substituted diet (0.027 $\pm$ 0.006 Mmol/l) was significantly lower than that of control animals (0.041 $\pm$ 0.01 Mmol/l) at $P<0.05$.

Fig. 7.6E and Table 7.3 show the fortnightly fluctuations and mean values of Triglyceride (ST) over a 12 week experimental period. The mean ST values of control animals was 0.39 $\pm$ 0.025 Mmol/l and those of animals on 20%, 60% and 100% substituted diets were 0.775 $\pm$ 0.11, 0.37 $\pm$ 0.23 and 0.31 $\pm$ 0.20 Mmol/l, respectively. The mean ST value of animals on 20%

substituted diet was significantly higher than that of control animals ($P<0.05$).

The fortnightly and mean serum total protein (TP), albumin (AL) and globulin (GB), in control and animals on 30%, 60% and 100% substituted diets are shown in Fig. 7.7A, 7.7B and 7.7C and Table 7.3. The mean AL value for control animals was 33.55 $\pm$ 0.48g/l where as those of animals on 20%, 60% and 100% substituted diets were: 29.54 $\pm$ 1.02, 30.65 $\pm$ 1.26 and 30.92 $\pm$ 4.61 g/l respectively. The mean AL of animals on 20%, 60% and 100% substituted diets were significantly lower than that of control animals ($P<0.05$). Mean TP and GB of animals on substituted diets were not significantly different. Table 7.3 also shows the mean values of albumin/globulin ratio.

7.3.7 Serum Enzyme Determinations

7.3.7.1 Serum Glutamate Oxalacetate transaminase (SGOT), Serum Alkaline Phosphatase (SAP) and Serum Lactate Dehydrogenase (SLD)

Fig. 7.7D, E and F and Table 7.4 show the fortnightly fluctuations in SGOT, SAP and SLD of control and animals on 30%, 60% and 100% substituted diets.

Although higher values of SGOT, SAP and

SLP were recorded for animals on substituted diets, the differences were not statistically significant ($P<0.05$).

7.3.8 Organs/Body Weight Relationship Determinations

Table 7.5 shows the means and standard deviations of the ratio of different organ weights per 100gm total body weight (R) for all groups of rats. The table also shows the mean starting and mean terminal weights of the different groups of rats on experimental diets. There were no significant differences in these values between animals on 30% and 60% substituted diet and control animals. Only one rat out of ten survived the experimental period on 100% substituted diet and thus the values could not be statistically tested.

7.4 DISCUSSION

In toxicologic experiments, change in body weight is an indicator ofan adverse effect (Gad and Well, 1982). In this study A. quadrilineatus diet was shown to have dose-dependent weight decreasing effects indicating adverse effects of the fungus in the rat. In cattle the control animals had superior weight gain as compared to treated animals However, the weight gain depressing effect of the toxin of A quadrilineatus did not appear to be dose related.

For example, cattle on 20% substituted diet had the least weight gain.

The weight decreasing effect of fungal metabolites has been confirmed by the work of Pencic et al.(1973) who fed grains contaminated with Nigrospora oryzae to piglets and noted reduced rate of growth of about 26%. A similar effect was noted by Zimmermann et al. (1979) in pigs fed A. ochraceus contaminated ration.

Results of this study also show that A. quadrilineatus produced metabolites which were toxic to rats. 100% substituted diet caused death in 9 out of 10 rats; it also caused significant weight loss, and roughened hair coat.

The histopathologic findings include damage particularly to the liver and kidneys, as well as spleen, stomach and brain. No clinical signs nor death were observed in cattle on A. quadrilineatus cultured diet, but the slight increased LDH, SGOT and BUN values indicate damage to the heart, liver and kidney. This was confirmed by degeneration and necrosis of the liver and kidney. Another significant lesion seen was haemorrhage, indicating that the toxin has effect on blood vessels.

In a similar study, Schultz et al. (1969) fed two

6 - month old calves a daily ration which included 1kg of maize experimentally contaminated with A. clavatus they found an increase in SGOT and SGPT values. El - Sheriff et al.(1975) fed 38 buffaloes with cotton seed contaminated with A. flavus, A. glaucus and Penicillium spp; and they noted that all the animals had increased SGOT levels, whereas Zimmermann et al. (1979) fed rice culture of a toxigenic strain of A. ochraceus at a concentration of 25% to weanling pigs for 10 days and reported increase in urinary lactic dehydrogenase, glutamate exalacetate transaminase and isolactate dehydrogenase but not in serum enzyme levels.

Mycotoxins are known to exhibit their deleterious effects on various organs and tissues, and some have affinities for some target organs. Because of this they have been grouped as hepatotoxins, nephrotoxins dermatoxins; and some affect the digestive tract, haematopoietic organs like bone marrow, lymphnodes, spleen, thymus, bursa of fabricius, endocrine organs like adrenal body, islet of Langerhans, reproductive organs like ovary, vulva, mammary glands and testis. Some also affect the nervous system, circulatory organs like the heart and respiratory organs like the lung (Uraguchi and Yamazaki, 1978).

The feeding of A. quadrilineatus contaminated diets had a lowering effect on PCV, WBC and Hb. A dose - dependent effect was observed in the case of WBC and Hb values. Thus the toxin of the fungus can cause anaemia in exposed animals. Of the WBC, neutrophil and eosinophil counts were higher, lymphocyte count was lowered by A. quadrilineatus contaminated diet. Among the mycotoxins known to affect the haematopoetic organs are ochratoxin A and 12, 13 - epoxytrichothecene which affects the bone marrow in mice, rats and chickens. Aflatoxin B_1 and rubratoxin B are known to affect the lymph nodes; aflatoxin B_1, cyclopiazonic acid, rubratoxin B, ochratoxin A affect the spleen of various laboratory animals including chickens; fusarenon - X is known to affect the thymus in mouse (Uraguchi and Yamazaki, 1978). With respect to the studies on serum mineral status, no significant differences were found between treated and control animals. Pencic et al. (1973) fed grains contaminated with Nigrospora oryzae to six piglets for 26 days starting two weeks after weaning. He found that when compared with controls there were no differences in blood Na, K, Ca and P. Also Zimmermann et al. (1979) fed rice

culture of a toxigenic strain of A. ochraceus at a concentration of 25% to weanling pigs for 10 days. He observed no change in serum concentration of K, Na and Cl but concentrationsof these electrolytes were reduced in the urine. El-Sheriff et al. (1975) on the other hand fed 28 male buffalo mouldy cotton seed cake from which they isolated A. flavus, A. glaucus and Penicillium spp. and found a decrease in serum calcium, magnesium phosphorous and iron.

Serum biochemical parameters did not reveal clear cut dose related response to toxins of A. quadrilineatus. For example, serum levels of cholesterol and triglyceride were significantly higher in animals on 20% substituted diets as compared with control animals and animals on 60% and 100% substituted diets for most duration of the experimental period. It was also found that serum urea was significantly higher in animals on 60% substituted diet than in control animals and serum triglyceride value was significantly higher in animals on 20% substituted diet than in control animals. A lower serum albumin was recorded for all the animals on substituted diets and a higher serum globulin levels were recorded for animals on substituted diets as compared to control animals. Most mycotoxins have

target organs of attack and since the organs are responsible for the metabolism of blood chemical parameters it should be expected that the blood chemical parameters could be affected. The degree to which they are affected will depend largely on the toxicity of the administered mycotoxin, duration of exposure, susceptibility of the animal to the toxic agent, constituents of the diet and stress to which the animal is subjected. All these can interplay to modulate an animal's response to a toxic agent.

In a similar study, Schultz et al.(1969) fed two calves with a daily ration of A. clavatus contaminated feed and noticed signs of intoxication in about four days, they noticed increase in serum glucose and bilirubin level in exposed animals. Some of the animals had altered values of blood urea nitrogen. In this study both serum glucose and bilirubin were not significantly altered but very high values were recorded for animals on 60% and 100% substituted diets, by the eighth week. Increase in blood urea is indicative of damage to the kidney. Even though there were some differences in both globulin and albumin levels between treated and control animals the total protein values of all the groups of animals were similar.

Increase in serum globulin together with increase in neutrophil counts in animals on substituted diets will suggest an immunological response as seen in infection. Also the increase in eosinophil counts suggest the body's response to the presence of toxic agents in the body.

TABLE 7.1.

HAEMATOLOGICAL VALUES FOR CATTLE CHRONICALLY FED DIETS CONTAINING 20%, 60% AND 100% A. QUADRILINEATUS CULTURED MAIZE

	20%	60%	100%	Control
PCV%	29.13 ± 1.04	32.86 ± 4.28	31.75 ± 1.48	36.35 ± 3.24
Hb gm/dl	9.12* ± 0.93	10.14* ± 1.09	9.40* ± 0.89	12.27 ± 1.54
WBC	9715.47±1101	9633.33±377.12	8657.00±16.28	10,575.77±1284.46
NEUTROPHIL	35.77±1.73	35.39 ± 3.90	29.12 ± 11.19	29.33 ± 6.20
LYMPHOCYTES	54.19 ± 1.45	53.82 ± 1.48	54.50 ± 8.78	63.18 ± 3.26
MONOCYTES	2.89 ± 1.03	3.10 ± 1.27	3.50 ± 2.72	1.38 ± 0.42
EOSINOPHILS	7.25 ± 0.35	7.19 ± 2.93	7.00 ± 14.57	5.35 ± 2.61
BASOPHILS	ND	ND	ND	ND

*Significantly different from control at $P \leq 0.05$

ND = Not determined.

TABLE 7.2: SERUM MINERAL VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH DIFFERENT LEVELS OF *A. QUADRILINEATUS* CULTURED MAIZE

	20%	60%	100%	Control
Calcium mmol/l	2.62 ± 0.01	2.80 ± 0.21	2.78 ± 0.41	2.67 ± 0.03
Magnesium mmol/l	1.35 ± 0.09	1.32 ± 0.03	1.29 ± 0.24	1.39 ± 0.20
Potassium mmol/l	3.51 ± 0.30	3.50 ± 0.86	4.23 ± 0.47	3.91 ± 0.37
Sodium mmol/l	135.06 ± 2.25	140.10 ± 1.62	148.93 ± 6.28	143.76 ± 2.38
Copper mmol/l	15.79 ± 0.79	14.92 ± 0.65	15.78 ± 4.29	14.58 ± 2.72
Zinc mmol/l	33.94 ± 1.05	34.33 ± 0.39	32.41 ± 8.84	37.98 ± 4.32
Iron mmol/l	43.35 ± 0.56	45.51 ± 3.65	40.91 ± 9.43	46.78 ± 1.18
Inorganic phosphate mmol/l	2.44 ± 0.02	2.48 ± 0.27	2.27 ± 0.18	2.42 ± 0.21
Chloride mmol/l	101.18 ± 0.73	100.18 ± 1.10	102.18 ± 4.28	100.00 ± 1.88

TABLE 7.3: BIOCHEMICAL VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH DIFFERENT LEVELS OF A. QUADRILINEATUS CULTURED MAIZE.

	20%	60%	100%	Control
Urea Mmol/l	3.80 ± 1.24	4.60 ± 0.80*	3.76 ± 1.15	3.82 ± 0.15
Glucose Mmol/l	3.07 ± 0.50*	3.37 ± 0.71	3.71 ± 0.73	3.92 ± 0.65
Cholesterol Mmol/l	3.91 ± 0.20*	2.96 ± 0.10	2.73 ± 0.40	3.86 ± 0.19
Bilirubin Mmol/l	0.027±006*	0.037 ± .007	0.038 ± 0.038	0.04 ± 0.01
Triglyceride Mmol/l	0.775 ± 0*.11	0.37 ± 0.23	0.31 ± 0.20	0.39 ± 0.025
Total Protein g/l	69.99 ± 1.48	67.60 ± 8.00	75.83 ± 11.67	69.28 ± 1.28
Albumin g/l	29.54 ± 1.02*	30.65 ± 1.26*	30.92 ± 4.61*	33.55 ± 0.48
Globulin g/l	40.84 ± 2.12	36.86 ± 9.25	44.90 ± 12.21	36.79 ± 4.57
Albumin/Globulin Ratio	0.72 ± 0.042	0.76 ± 0.134	0.64 ± 0.18	0.78 ± 0.11

*Significantly different from that of control at $P < 0.05$

TABLE 7.4: ENZYME VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH DIFFERENT LEVELS OF A. QUADRILINEATUS CULTURED MAIZE.

	20%	60%	100%	Control
Glutamate oxalacetate transaminase IU/L	20.19 ± 1.84	22.95 ± 3.41	22.00 ± 12.28	19.19 ± 2.68
Lactate Dehydrogenase IU/L	78.50 ± 10.60	55.20 ± 14.85	61.67 ± 16.16	47.38 ± 16.40
Alkaline Phosphatase IU/L	236.97 ± 29.49	233.59 ±110.11	246.82 ± 77.27	212.97 ± 53.51

TABLE 7.5: RATIO OF ORGAN WEIGHT-TO-TOTAL BODY WEIGHT OF RATS FED DIETS SUBSTITUTED WITH DIFFERENT LEVELS OF A. QUADRILINEATUS CULTURED MAIZE.

	30%*	60%*	100%**	Control
Brain	0.86 ± 0.22	0.85 ± 0.17	1.90	0.84 ± 0.13
Heart	0.36 ± 0.05	0.35 ± 0.04	0.55	0.37 ± 0.05
Intestine	7.92 ± 1.51	8.11 ± 1.13	9.42	7.48 ± 0.63
Kidneys	0.87 ± 0.11	0.81 ± 0.08	1.13	0.93 ± 0.05
Liver	4.59 ± 0.50	4.63 ± 0.48	5.33	4.77 ± 0.38
Lungs	0.68 ± 0.27	0.66 ± 0.09	1.82	0.69 ± 0.18
Spleen	0.35 ± 0.07	0.32 ± 0.04	0.30	0.31 ± 0.08
Stomach	2.25 ± 1.35	2.23 ± 1.09	1.55	2.05 ± 0.91
Testis	1.11 ± 0.23	1.25 ± 0.33	1.43	1.09 ± 0.20
Ovary and Oviduct	0.47 ± 0.11	0.34 ± 0.03	-	0.43 ± 0.08
Mean starting Wt±SD(gm)	186.92 ± 52.26	184.37 ± 41.37	73.5	192.14 ± 36.61

* 10 Rats (5 males + 5 females)

** 1 rat that survived the experimental period.

Wt - Weight

SD - Standard deviation

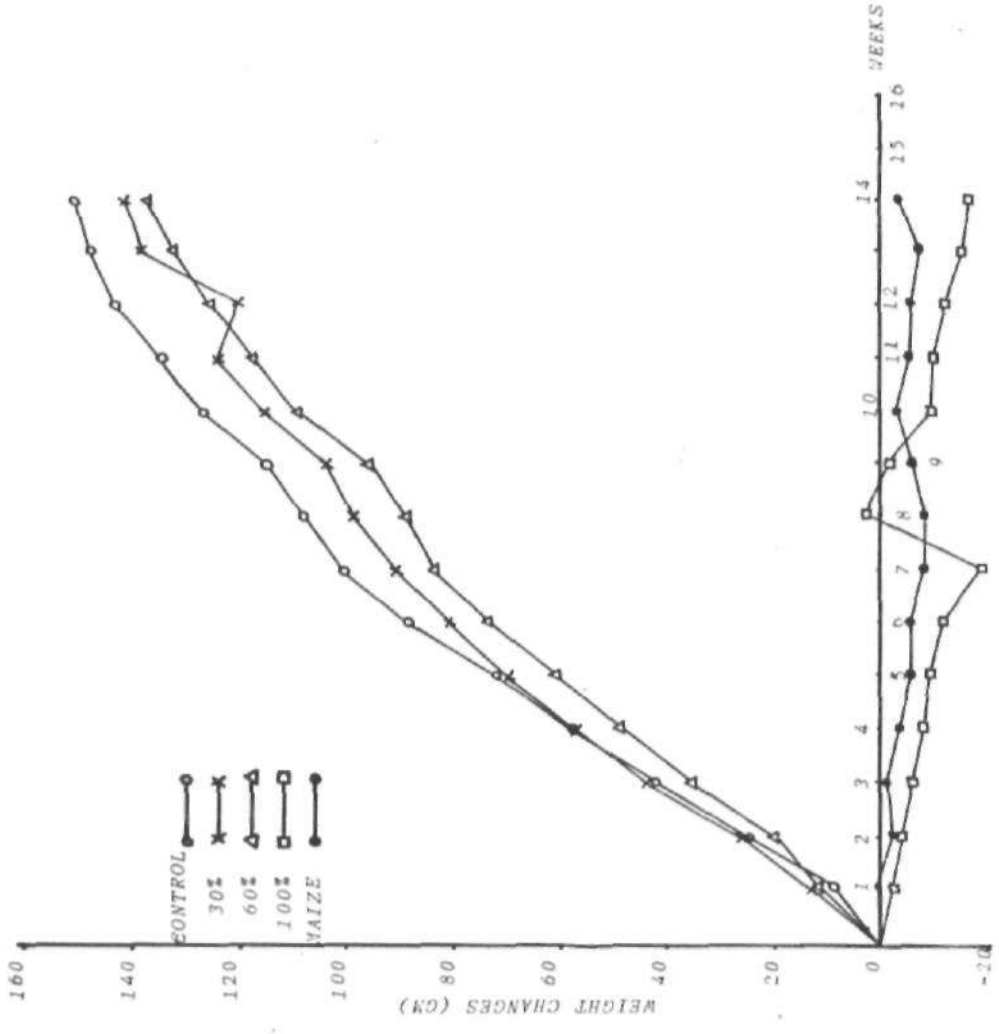


FIG. 7.1 GROUP MEAN WEEKLY WEIGHT CHANGES OF RATS FED 30%, 60%, 100 DIETS SUBSTITUTED WITH ASPERGILLUS QUADRILINEATUS CULTURED MAIZE AND THAT OF NORMAL DIET.

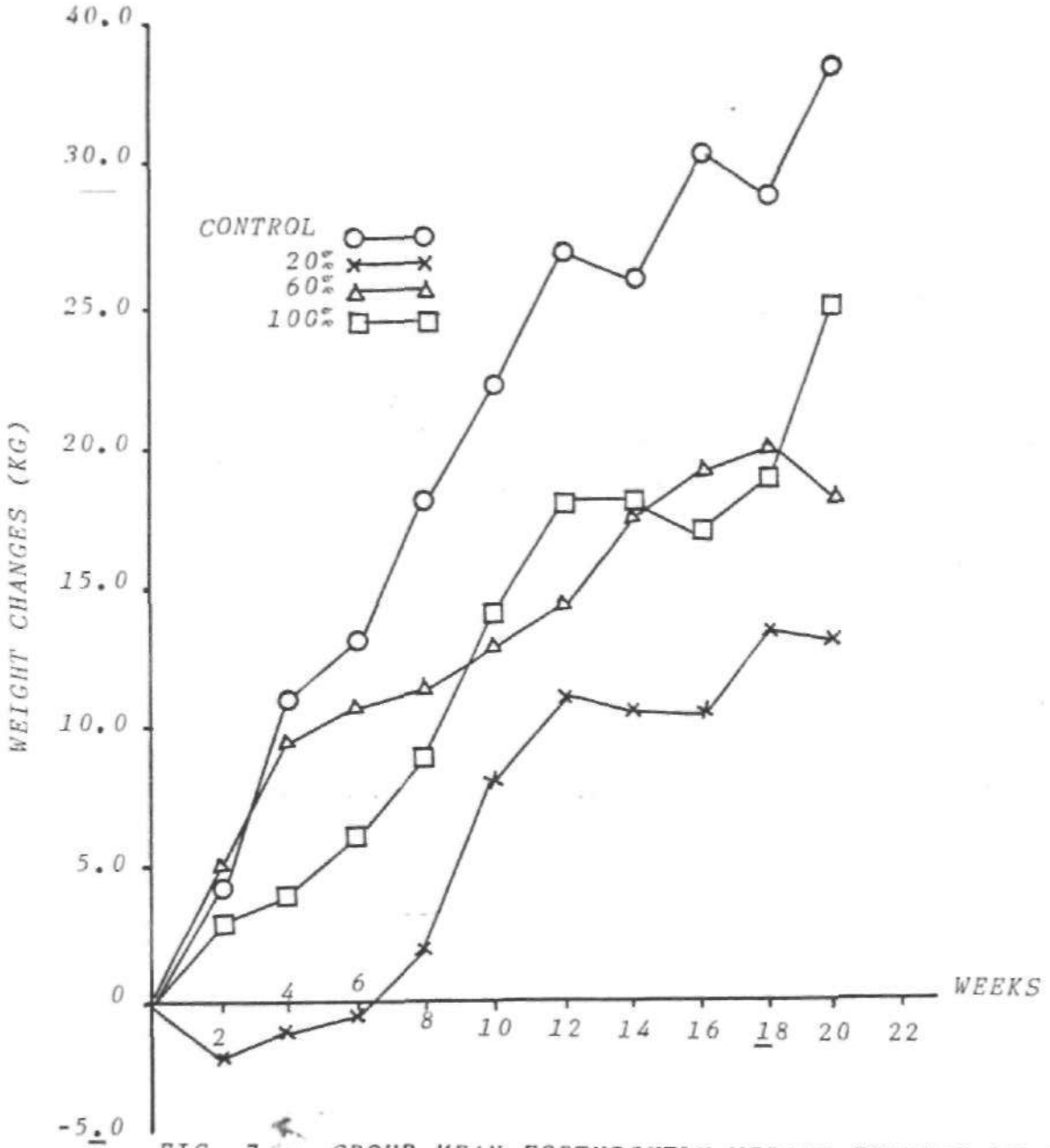


FIG. 7.2 GROUP MEAN FORTNIGHTLY WEIGHT CHANGES OF CATTLE FED 20%, 60% AND 10
DIETS SUBSTITUTED WITH ASPERGILLUS CULTURED MAIZE AND THAT
OF NORMAL DIET.

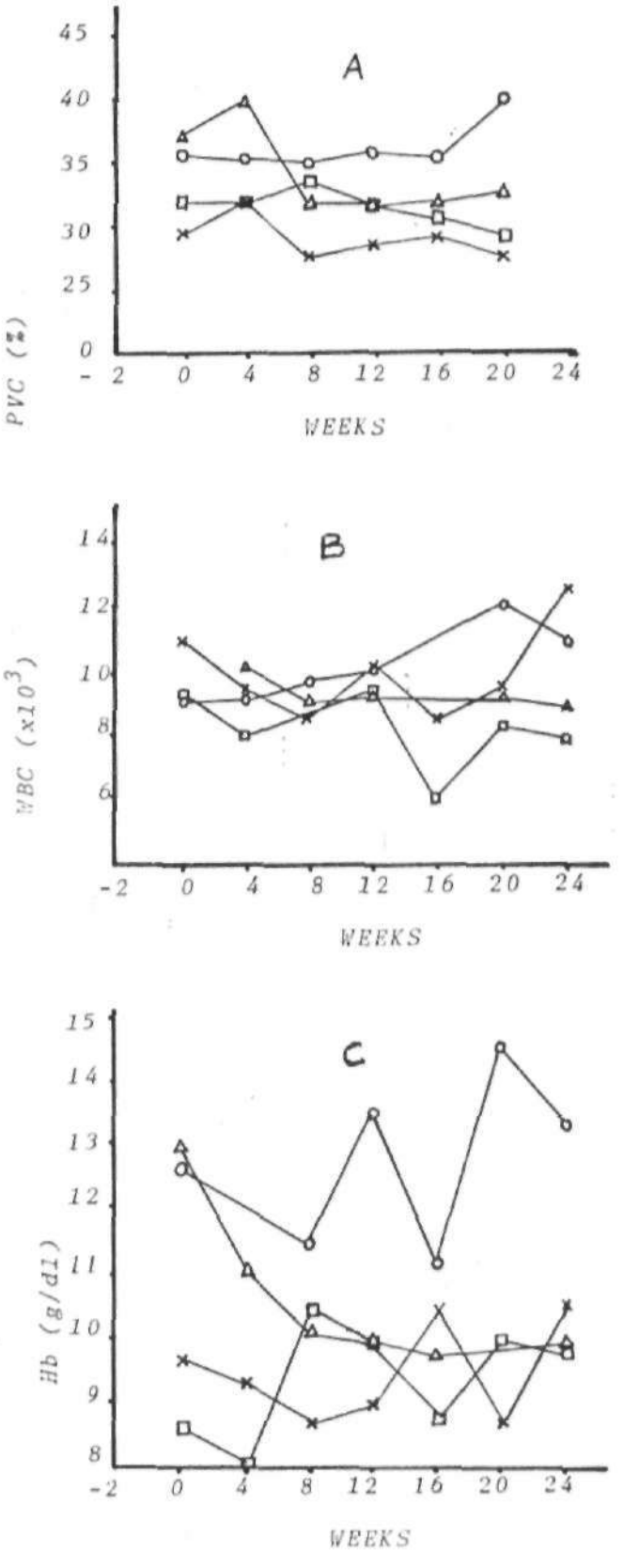


Fig 7.3 Mean values of PCV, WBC and Hb of cattle orally fed 20%(X); 60% (Δ); 100% (□) diet substituted with Aspergillus quadrilineatus culture maize and that of normal diet (O).

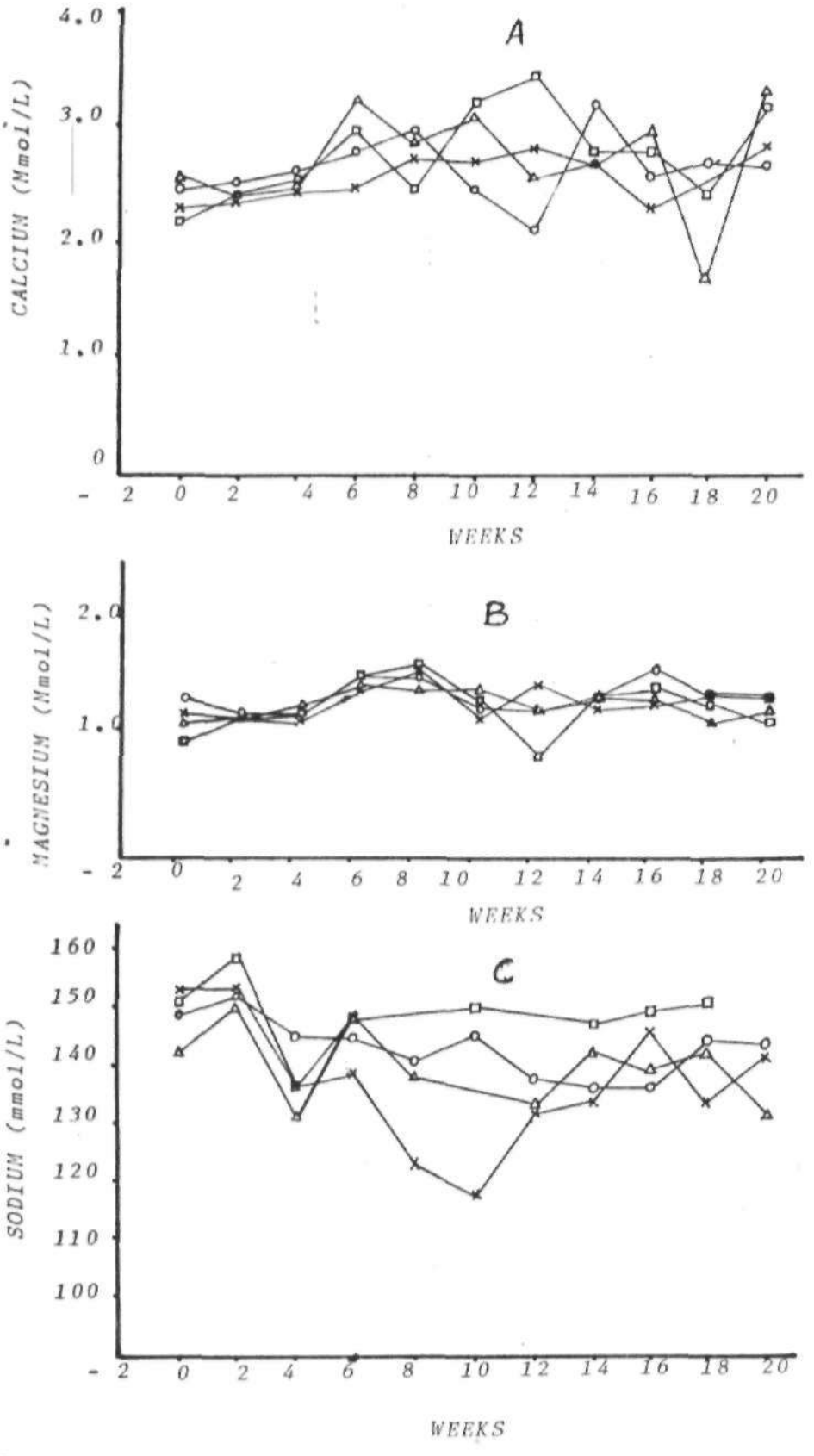


Fig 7.4 Mean values of Calcium, magnesium and sodium of cattle orally fed 20% (X); 60%(Δ); 100% (□) diet substituted with Aspergillus quadrilineatus cultured maize and that of normal diet (0)

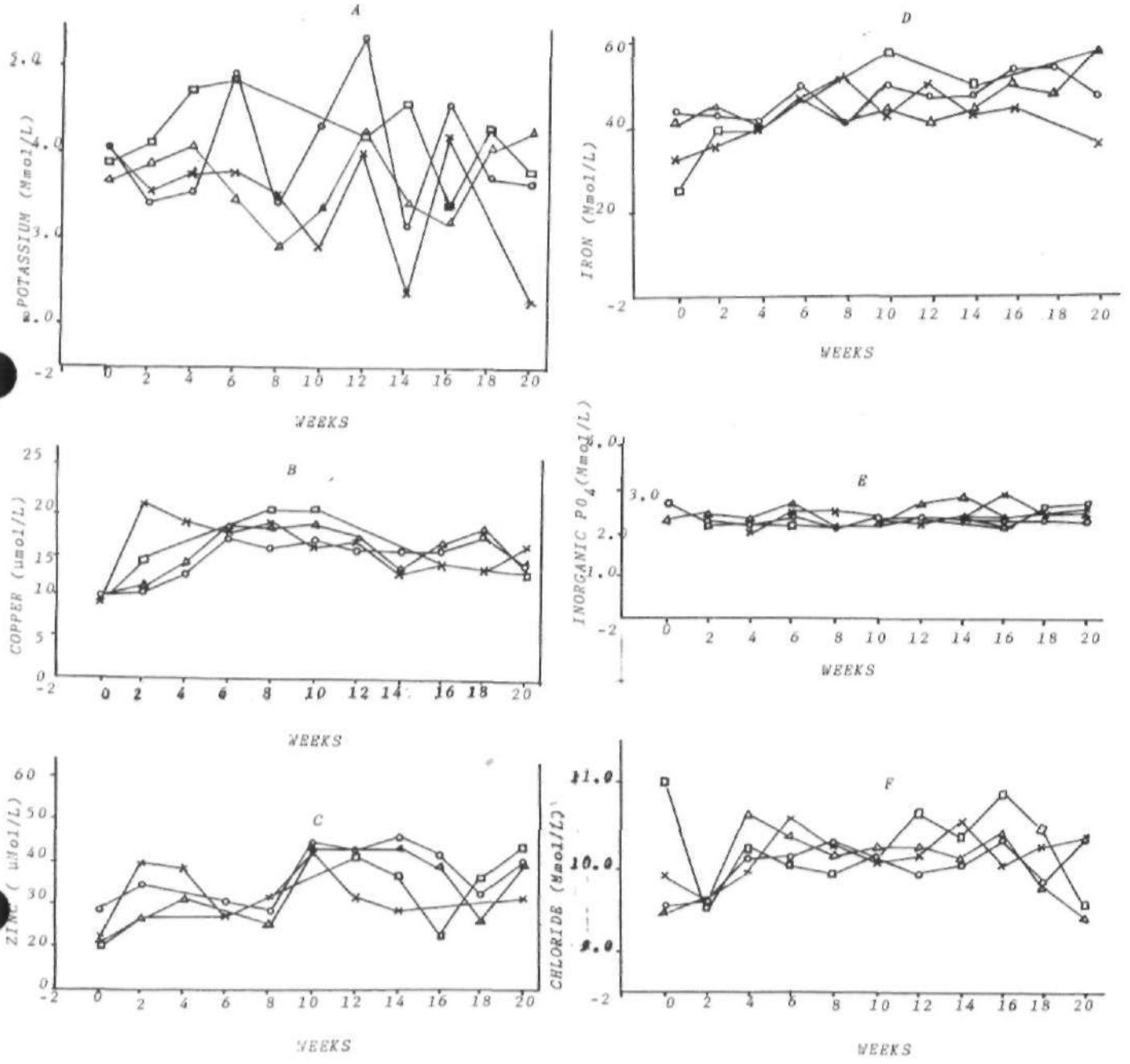
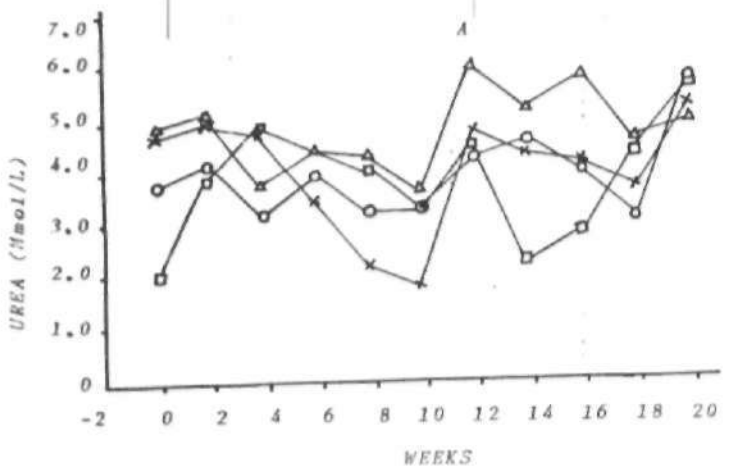
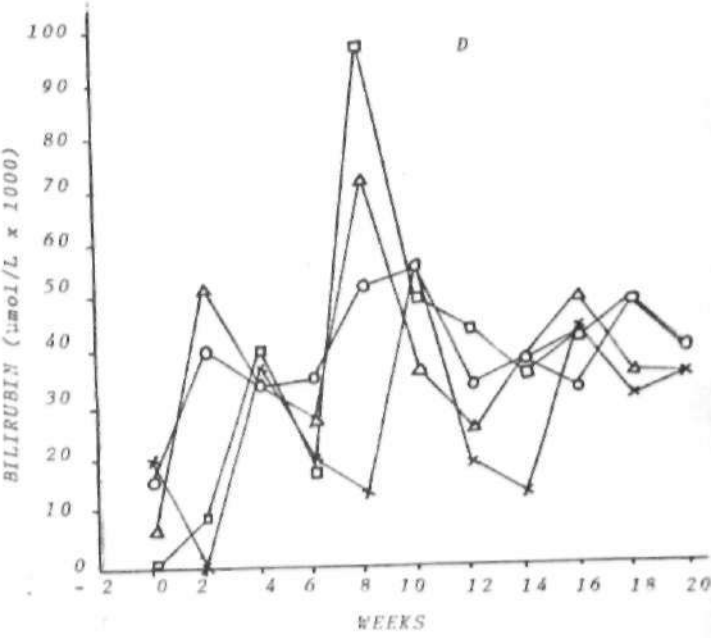
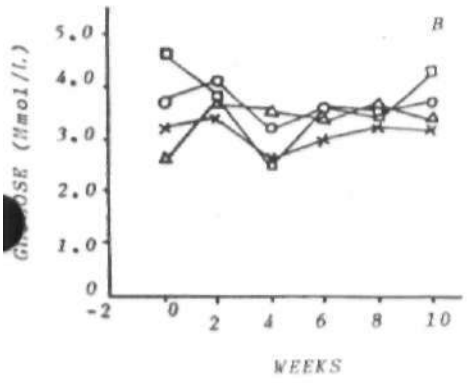


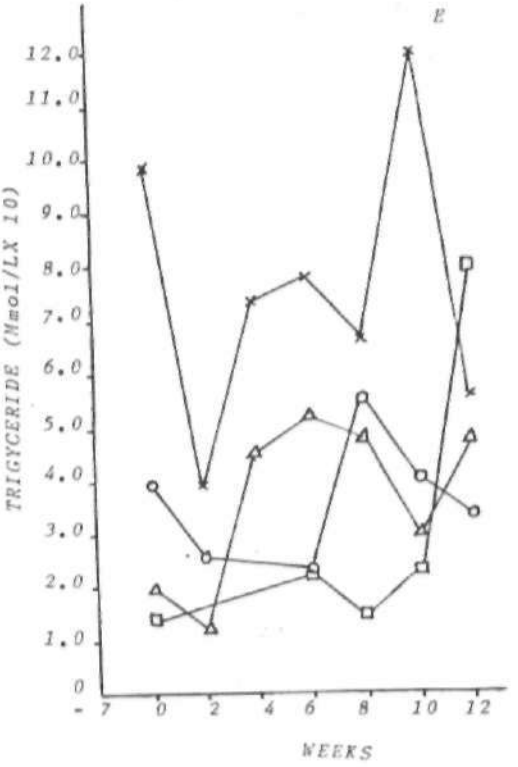
Fig. 7.5 Mean values of Serum Potassium, Copper, Zinc, Iron, Inorganic phosphate and Chloride of cattle orally fed 20%(X); 60% (A); 100%(D) diet substituted with Aspergillus quadrilineatus cultured maize and that of normal diet (0).

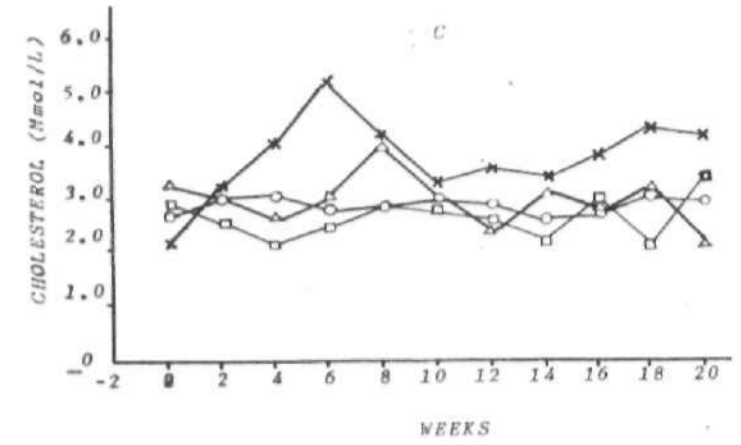
Fig. 7.6 - Mean values of serum urea, glucose, cholesterol, bilirubin and triglyceride of cattle fed 20% (X); 60%(Δ); 100% (□) ; diet substituted with *Aspergilus quadrilineatus* cultured maize and that of normal diet (0).











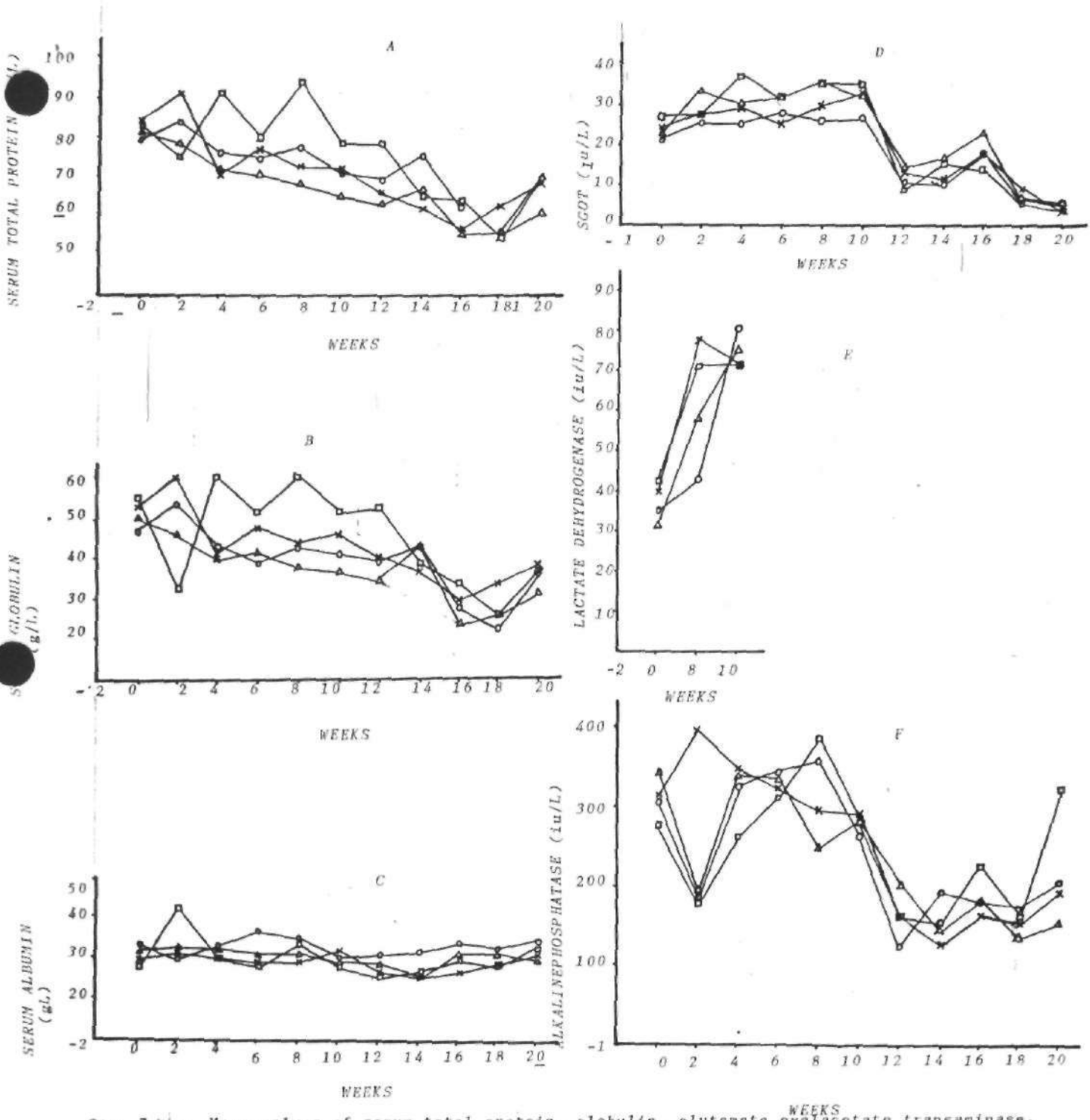


Fig. 7.7 - Mean valves of serum total protein, globulin, glutamate oxalacetate transaminase, lactate dehydrogenase and alkaline phosphatese of cattle orally fed 20%(X); 60% (Δ); 100 (□) diet substituted with Aspergillus quadrilineatus cultured maize and that of normal diet (0).

Plate 7.1 Photomicrograph of liver of rat chronically fed 30% Aspergillus quadrilineatus cultured maize. Note the cloudy swelling of the hepatic cells. X 160

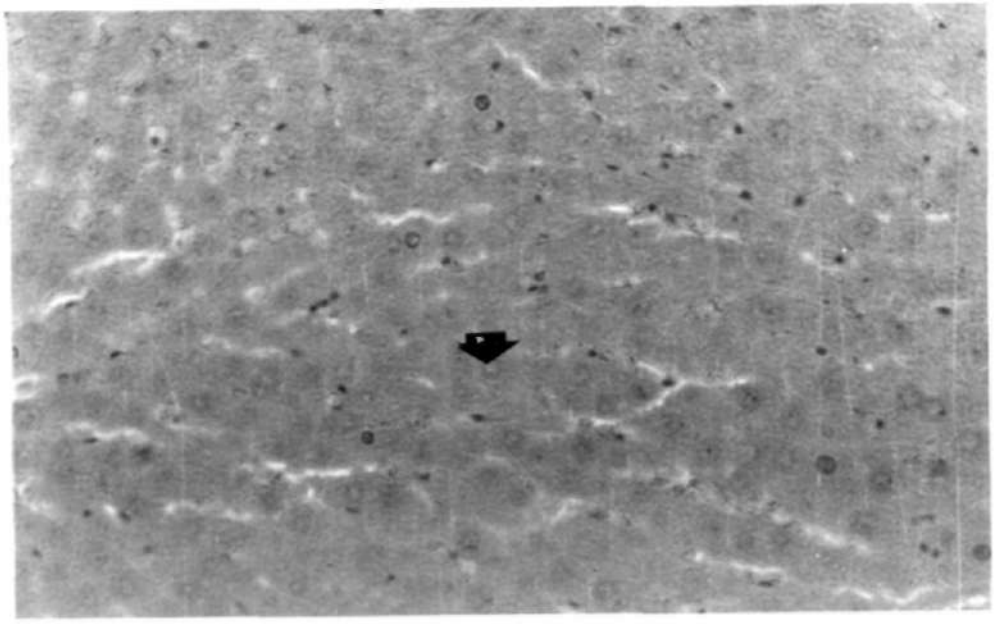


Plate 7.2 Photomicrograph of kidney of rat chronically fed 60% Aspergillus quadrilineatus cultured maize. Note the congestion at the cortico-medullary junction. X 160

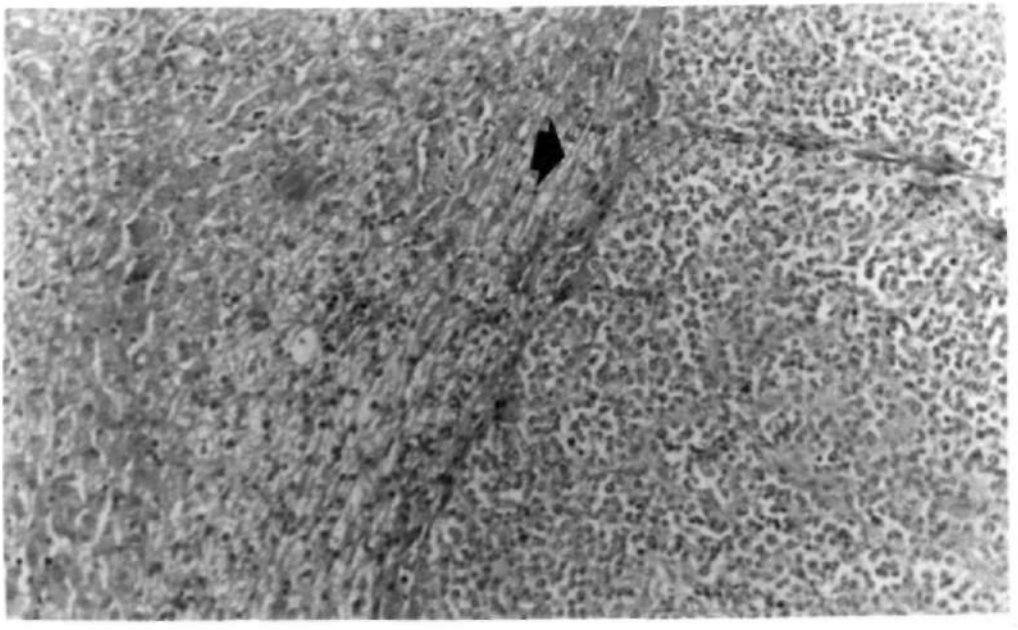


Plate 7.3. Photomicrograph of brain of rat chronically fed 60% Aspergillus quadrilineatus cultured maize. Note the perivascular cuffing in some parenchymal vessels. X 160

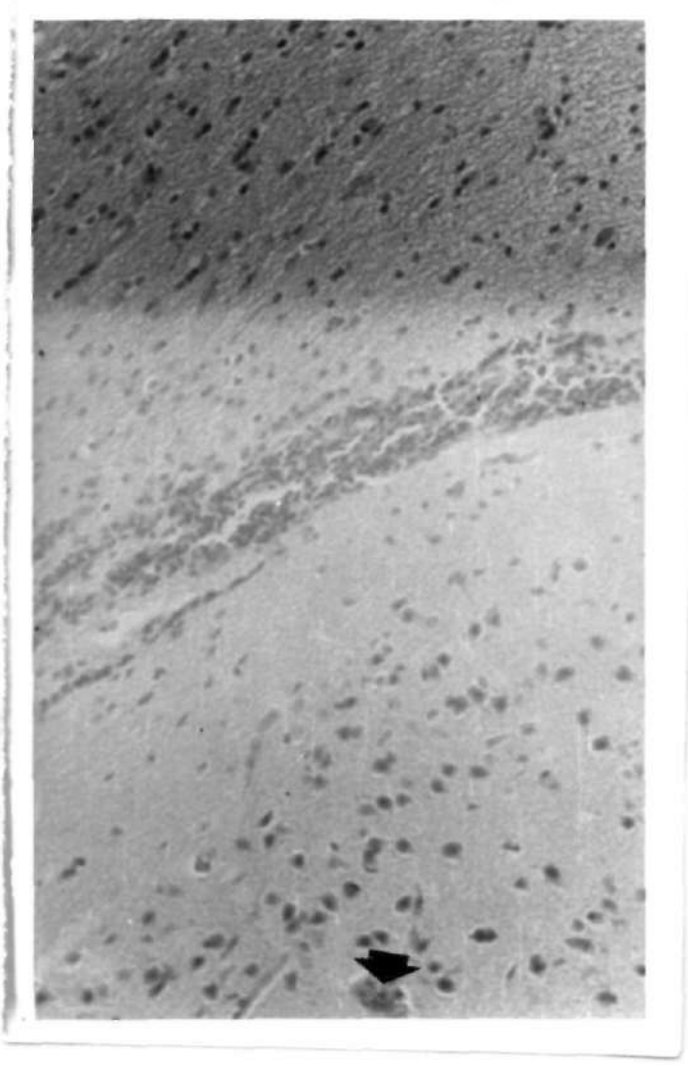


Plate 7.4. Photomicrograph of liver of cattle chronically fed 20% Aspergillus quadrilineatu cultured maize. Note the accumulation of eosinophils with nectrotic cells of the hepatocytes X 250

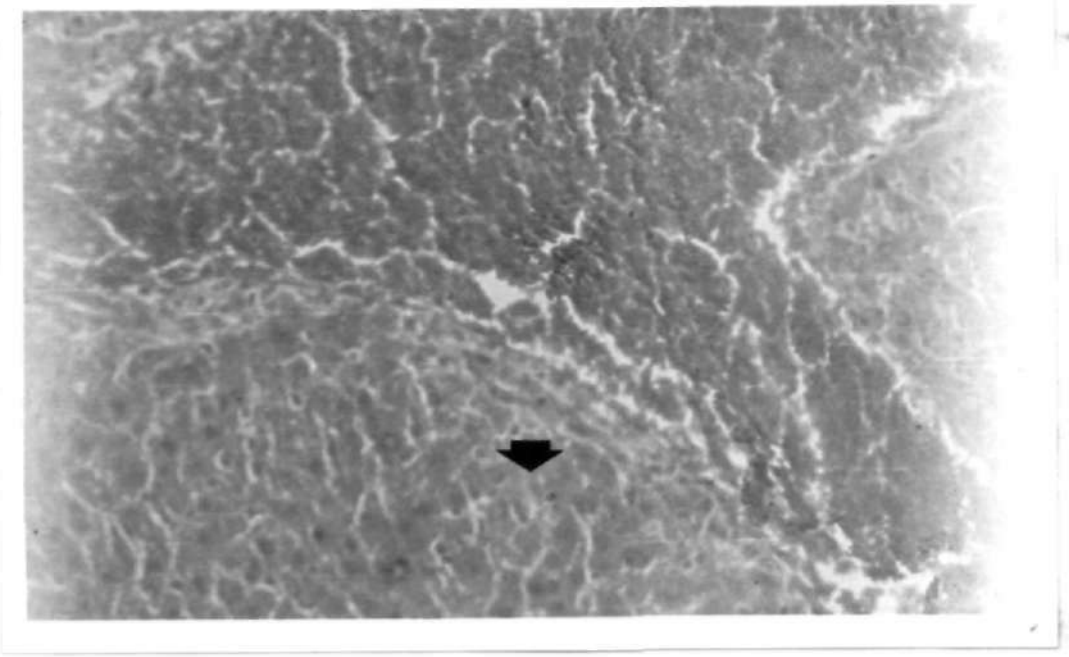
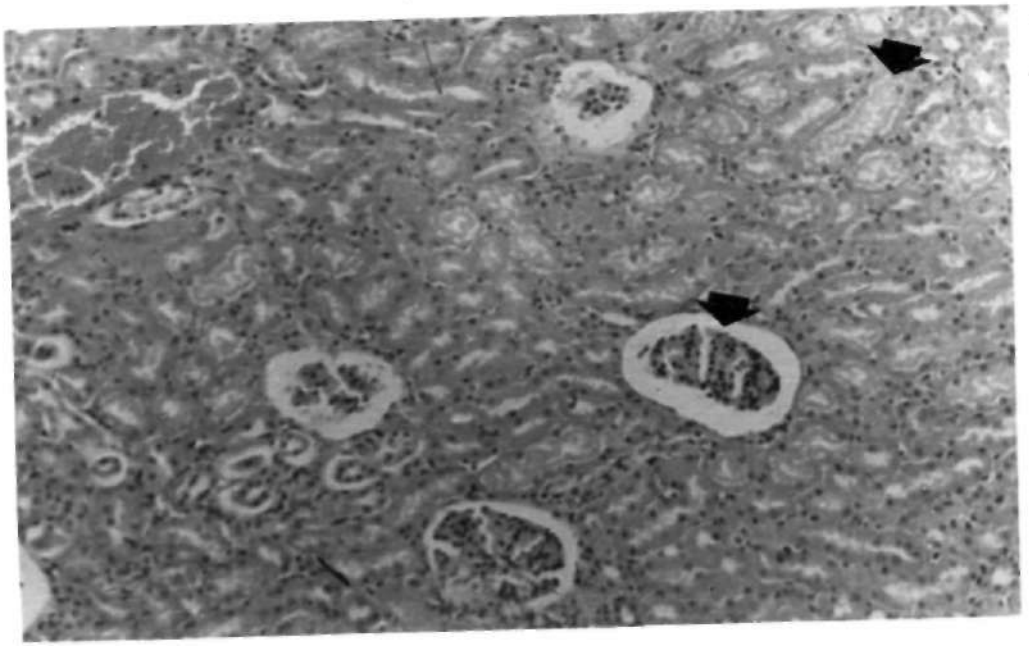


Plate 7.5. Photomicrograph of kidney of cattle chronically fed 60% of Aspergillus quadrilineatus cultured maize. Note glomerular shrinkage and tabular necrosis. X 160



CHAPTER VIII

ISOLATION, PURIFICATION AND PHYSICO-CHEMICAL PROPERTIES OF MYCOTOXIN(S) PRODUCED BY ASPERGILLUS QUADRILINEATUS (THOM AND RAPER)

8.1 INTRODUCTION

Aspergillus quadrilineatus produces metabolites that are toxic to mice, rats, chicks and cattle. However, no information is available in literature on the types of mycotoxins it produces, although other *Aspergillus* species are known to produce several mycotoxins.

Ochratoxins A and B are produced by several strains of *A. ochraceus*, penicillic acid by *A. ochraceus* and *A. sulphureus*. Patulin is produced by *A. clavatus* and *A. giganteus*, citrinin by *A. terreus* and *A. niveus*, sterigmatocystin by *A. virsicolor*, *A. nidulans* and *A. rugulosus*. Kogic acid is produced by many species of *Aspergillus*; gliotoxin is produced by *A. fumigatus*, oxalic acid by *A. flavus* and other species. Maltoryzine is produced by *A. oryzae* and some tremorgens are produced by *A. flavus* (Hesseltine, 1974). The list is not exhaustive.

Some fungi can produce more than one metabolite when grown on a suitable medium, e.g. A. ochraceus produces ochratoxins A and B and penicillic acid.

The objective of this study is two fold: (1) to identify the toxin produced by A. quadrilineatus (Thom and Raper) cultured on rice medium,using some mycotoxin standards as reference; (ii) to isolate, purify and perform some physicochemical analysis of the most toxic isolate.

8.2 MATERIALS AND METHODS

8.2.1 Culturing of A. quadrilineatus on rice

Conidia or mycelia of four day old pure cultures of A. quadrilineatus grown on Potato Dextrose Agar (PDA) slant tubes were used to inoculate rice. Five millilitres of Triton X-100 (Searle Hopkin and Williams, Chalwell Health, Ezzex, England) treated sterile distilled water (1 drop Triton X-100 to 200ml sterile distilled water) was added to the culture tubes and the surface of the culture scratched with sterile inoculating wire to suspend the spores. The suspensions were then used to inoculate a 2.5 litre Fernbach flask containing 250g of uncle Bens rice. The rice had been prepared by adding 200ml of distilled water, shaking the

flask several times, and left over night for moisture equilibration. The flasks were autoclaved at 15psi for 25 minutes. The cooled flasks were inoculated with the suspended spores and maintained as a static culture at 28°C in an incubator fof 21 days.

8.2.2 Extraction of Toxins

Seven hundred and fifty ml of chloroform was added to the flask containing the cultured rice. After one hour, the solvent soaked rice was chopped into small fragments using a large stainless steel spatula. The rice-solvent mixture was poured into a waring blender, blended for three minutes and filtered through a bed of Hyflosuper-cell in a Buchner funnel fitted with a fast flow filter paper. The filtrate was then passed through anhydrous sodium sulphate to remove the moisture and clarify the extracts. The solvent was removed at 50°C in a rotatory vacuum evaporator.

The oily viscous residue was added to chilled swirling petroleum ether (1:15 residue/petroleum ether V/V) and the mixture kept in a deep freezer at -15°C overnight to complete precipitation. The crude toxin was recovered by filtration and dried in a fan oven at 50°C for three hours.

8.2.3 Preparation of Thin Layer (Chromatographic plates)

One hundred gram of Kieselgel G. Type 60 (E. Merck, Darmstadt, West Germany) was weighed out and transferred into 500ml round bottomed flask. 220ml of distilled water was added and hand shaken to form a slurry. The flask was then transferred to a wrist action shaken and the flask shaken for 30 minutes. The thick slurry of the silica gel was used to coat five 20 x 20cm glass plates 0.5mm thick,using thin layer coating apparatus (Quick Fit Company, England). The coated plates were air dried for one hour and in a fan oven at 100^{o}C for another one hour. The plates were stored in a vacuum oven with dessicating silica gel till used. In the case of preparative thin layer plates, glass plates were prepared as above but coated to a thickness of 1mm instead of 0.5mm.

8.2.4 Identification of some Toxins in Crude Extracts Using available mycotoxin standards

The dried residue (in vial described in 8.2.2) was dissolved in chloroform to a concentration of 40mg/ml. Ten microlitres of the solution in chloroform were spotted on thin layer plates activated for 1 hour at 100^{o}C. Standards of aflatoxins B_1, B_2, G_1, G_2, M_1,

ochratoxin A, sterigmatocystin, patulin, penicillic acid, zearalenone and citrinin (Makor Chemicals Ltd. Jerusalem, Israel) were spotted as reference standards. Also sample plus internal standard was spotted. The spotted thin layer plates were developed in chromatographic tanks with Toluene + ethylacetate + formic acid (TEF) (60+30+10) to 10cm solvent front. It was air dried, viewed under long wave uv light, sprayed with 20% (W/V) aluminium chloride in ethanol and heated at 100^{o}C for minutes.

8.2.5 Two dimensional TLC chromatography of crude extract

Two - dimensional TLC using two solvent systems as recommended by Patterson and Roberts (1979) was used for the identification of sterigmatocystin. A separate thin layer chromatographic plate was then used to spot 5ul of the crude chloroform extract solution and 5ul (10mg/ml) of sterigmatocystin standard. The plate was first developed in chloroform + methanol (98 + 2) to a solvent front of 10cm. It was dried in air and then turned at right angles and developed in TEF to a solvent front of 10cm. It was again air dried and observed in chromatovue cabinet at 365nm, after which it was sprayed with 20% aluminium chloride.

8.2.6 Defattening of Crude Extract

The crude toxin described in section 8.2.2 was defatted using a mixture of petroleum ether and excess hexane (1:15 v/v). It was filtered through a fluted No 44 Whatman filter paper and allowed to dry on the filter paper at room temperature and stored in a dark bottle at -15^{o}C till used.

8.2.7 Column chromatography of defatted Crude Extract

About one gram of the crude toxin was mixed thoroughly with 10g of silica gel (Type 60-200 mesh KOCK-LIGHT Laboratories, Coln Brook Bucks, England) and 5ml of acetone was added to the mixture and mixed thoroughly and the acetone evaporated at 40^{o}C in an oven. This dried toxin/silica gel mixture was applied to a silica gel column (4x30cm) packed in hexane, and was eluted with 500ml each of hexane, benzene, diethylether, chloroform, ethylacetate, acetone and methanol. Fractions of 500ml were collected and evaporated at 60^{o}C on vacuum rotatory evaporator. The residues were treated with small amounts of petroleum ether and excess hexane and filtered as earlier described.

A second column of silica gel was prepared as above and one gram of the crude extract (PEDCR) was

applied as described above and the column was subjected to gradient elution using 250ml chloroform (CF), 200ml each of 90% chloroform + 10% ethyl acetate (EA), 80% CF + 20% EA, 70% CF + 30% EA, 60% CF + 40% EA, 50% CF + 50% EA and pure ethyl acetate. Fractions of 20ml were collected.

8.2.8 Toxicity of Residues in mice

Forty mg/ml of the different residues from the diethyl ether, chloroform, ethylacetate, acetone and methanol toxin fractions were prepared in DMSO/70% ethanol (3:1 v/v) respectively. Each fraction was injected intraperitoneally into two mice weighing 9-15g at the dose of 40mg/kg; two control mice received the highest volume of vehicle administered to treated mice. All mice were observed for 14 days for clinical signs.

8.2.9 Thin Layer Chromatography of fractions from column chromatography

Ten microlitres of the different fractions were spotted on aluminium support sheet. Thin layer chromatographic sheets (TCL) coated with silica gel G (Woelm, Pharma CmbH and Co, D-440 Eschwege, West Germany) and developed in S-Chamber Eastman

chromatogram developing apparatus (Eastman Kodak, Compy, Rochester, N.Y. 14650, U.S.A.) using toluene + ethylacetate + formic acid (60 + 30 + 10 v/v). The dried plates were observed under UV and all fractions having corresponding spots were pooled together and evaporated on rotatory evaporator. The residues were separately treated with small amounts of petroleum ether and hexane, filtered and dried at room temperature and tested for toxicity in mice.

8.2.10 Preparative Thin Layer Chromatography of Fractions 14 - 19

Three hundred milligrams of pooled toxic fractions 14-19 were dissolved in 100ml of chloroform. Twenty millilitres of the toxin solution was applied to each of the five preparative thin layer plates coated with silica gel G as described in section 8.2.3. They were developed in presaturated tank lined with filter paper and containing toluene + ethylacetate + formic acid (60+30+10 v/v). The plates were removed and dried after the solvent front had moved about 10cm from the origin. The plates were again placed in the tank for another development. This process was repeated seven times. The different bands were marked under long wave (364 nm) ultra violet light and scraped off from the glass plate and eluted four times using

chloroform.

The chloroform eluates were evaporated under vacuum on a rotatory evaporator with water bath at 55°C. The residues were separately dissolved in little petroleum ether and excess hexane and the resultant precipitates were filtered through fluted Whatman 44 filter paper. The room, dried crystals or amorphous powders of the different bands were collected into two dram vials. Some of the crystals were dissolved in DMSO/70% alcohol (3: 1v/v) and each administered intraperitoneally to two mice to detect which band had the toxic component. One mg of the crystals of the most toxic band was dissolved in 200ml of chloroform and subjected to thin layer chromatography to test for purity.

Two hundred milligrams of the crystals of the most toxic band weredissolved in 100ml of chloroform and applied to preparative thin layer chromatographic plates as described earlier. The plates were developed to a solvent front of 18cm in a tank containing chloroform + methanol (97:3) and air dried. This process was repeated seven times. The separated bands were carefully scraped off and eluted four times with chloroform, the eluates were evaporated under vacuum in a rotatory evaporator. The residue were treated

with small petroleum ether (B.P. 40-60^{o}C) and excess hexane. Bands 3 and 4 yielded the bulk of the residues. The residues (0.5 mg each) from the two bands were dissolved in 200ml chloroform and 10ul of each was spotted on thin layer chromatogram sheet and developed in chloroform/methanol 97:3 v/v to 10cm solvent front to check for purity. They were again tested for toxicity in mice.

8.2.11 Spectral Measurements

Infra red spectrum of fraction 7/3 and 7/4 were measured in liquid paraffin with air reference using Perkin-Elmer Infrared spectrophotometer model 710B. For UV-spectrum, the sample was dissolved in chloroform and measured with a recording Pye Unicam SP - 800 UV-visible spectrophotometer (Pye Unicam Ltd., York street, Cambridge England CB1 2PX U.K.).

8. 3 RESULTS

8.3.1 Identification of some Toxins in Crude Extract

Under long and short wave length UV illumination the crude extract showed eight different flourescent spots on TLC. The thin layer chromatographic characteristics of the different spots under white,

long wave - UV and short wave-UV are shown in Table 8.1 and Fig. 8.1. None of the spots had the same Rf. as aflatoxins B_1, B_2, G_1, G_2, M_1, ochratoxin A, citrinin, patulin, penicillic acid, and zearalenone. One of the spots had the same Rf as sterigmatocystin in two dimentional TLC. It is a dull brick red flourescent spot with Rf of 0.68 when sprayed with 20% $AlCl_3$ in ethanol; the flourescence changed from dull brick red to bright yellow flourescence under long wave UV-light.

8.3.2 Column Chromatography

Table 8.2 shows the physical characteristics and toxicity of seven different solvent fractions collected from column chromatograms of crude extracts of A. quadrilineatus. The toxins came off in the diethylether, chloroform and ethylacetate fractions as shown bytheir lethality to mice. Table 8.3 shows some properties of fractions of the gradient elution of column chromatography of crude chloroform extract of A. quadrilineatus with different concentrations of chloroform and ethyl acetate. Fractions 15-19 gave the highest residues after evaporation of the solvent and had greater concentration of the toxin(s) because

all the four mice dosed with 40mg/kg died within a few hours. Lesser amount of the toxin came out in fractions 1 - 14 and only two out of the four mice dosed with 40mg/kg died.

8.3.3 Preparative Thin Layer Chromatography

Table 8.4 shows toxicity and some visual characteristics of two bands of the eight bands yielded by preparative thin layer chromatography of residues from fraction 15 - 19. From Table 8.4 band 7 had the most toxic component as 10mg/kg produced death in one of two treated mice. Further, preparative thin layer separation of band 7 yielded five bands, but only two bands (3 and 4) yielded appreciable precipitate. The visual and chromatographic characteristics of these are given in Table 8.5

8.3.4 Thin Layer chromatography of Residue of Bands 3 and 4

Thin layer chromatographic treatment of residues of bands 3 and 4 yielded only one spot in toluene + ethanol + formic acid (60 + 30 + 10) chloroform + ether + acetic acid (85 + 10 + 05), chloroform + acetic acid (93 + 7), ether + methanol (98 + 2) solvent systems. They also yielded one spot each when developed two dimensionally in toluene + ethanol,

formic acid and chloroform + acetic acid. This confirms their chromatographic purity.

8.3.5 Toxicity of Two Pure Mycotoxins

Table 8.6 shows the toxicity of the two mycotoxins derived from the crude chloroform extract of A. quadrilineatus in mice. All the mice dosed IP with 20 and 40mg/kg of the white fluffy extract died within 6 and 18 hours, respectively. Five of those given 10mg/kg IP, died within five days, two of ten given 5mg/kg died five days later.

The yellow electrostatic isolate was less toxic. Death was produced only at 20mg/kg, but not at 5 or 10 mg/kg.

8.3.6 Spectral measurements

Figs 8.2 and 8.3 show the infrared spectra of fractions 7/3 and 7/4 respectively. The two spectra were too complex to interpret readily but they looked very similar when carefully compared. There seemed to be three peaks in the carbonyl region at 17,00, 1680, and 1660 cm^{-1}. Also, the peak at 3460 cm^{-1} indicated the presence of an OH group in both spectra.Fig. 8.4 shows the infrared spectrum of the solvent (paraffin) alone. The UV spectrum of fraction 7/3 showed a broad peak at 255-265nm and another at 284nm. That of fraction 7/4 showed a broad peak at 250-260nm and another peak at 285nm.

8.4 DISCUSSION

Some fungi are known to produce more than one metabolite on the same medium and some produce different metabolites on different media. For example, A. flavus can produce aflatoxins and aspergillic acid on the same medium and some species of A. flavus produced palmotoxin Bo and Go when palm wine was the supporting medium (Bassir and Adekunle, 1968). In this study with crude extract of A. quadrilineatus isolated from acha (Digitaria exilis Stapf) it has been shown that out of 8 bands identified under UV light, one was sterigmatocystin. However, sterigmatocystin was a minor metabolite since its band yielded extremely low residue on elution of the silica gel scraping, when compared to residues from bands six and seven. This is the first report of the ability of A. quadrilineatus to produce sterigmatocystin.

Sterigmatocystin is structurally related to the aflatoxins: both contain bisdihydrofuran moiety. Sterigmatocystin may be derived biogenetically from a precursor similar to that involved in aflatoxin biosynthesis. Figure 8.7 shows the structure of sterigmatocystin and aflatoxin B_1. The structure of sterigmatocystin was deduced chemically in 1962 (Uraguchi and Yamazak, 1978). It is a pale yellow pigment and was first isolated from A. versicolor in

1954. Like the aflatoxins interest in sterigmatocystin stemed from its carcinogenic properties. The first reported case of natural occurrence of sterigmatocystin came from Canada (Scott et al., 1972) when A. versicolor was reported to contaminate Canadian wheat sample. Since then its occurrence has been reported in mouldy coffee beans in South Africa (Purchase and Pretorius, 1973) and in some food samples in Mozambique (Watt, 1974). It has also been found in long stored rice in Japan (Manabe and Tsuruta, 1975) and in feeds in Britain (Shreeve et al., 1975). Apart from A. versicolor which is the major producer of sterigmatocystin, other fungi like A. nidulans, A. regulosus, A. flavus, A. parasiticus, A. chevalieri, A. ruber, A. amsterlodami are known to produce the toxin as well (Scott, 1978).

Uraguchi and Yamazaki (1978) gave the LD_{50} of pure sterigmatocystin as 800mg/kg (p.o.) in mice and 60mg/kg (i.p) in rats. In the toxicity study of the crude extract of A. quadrilineatus in Chapter V it was found that the oral LD_{50} in mice was 566 mg/kg and the i.p. LD_{50} dose was 21.0mg/kg. In day old chicks the oral LD_{50} was 267.7mg/kg. The crude extract contained impurities and other mycotoxins hence the difference in the toxicities reported by Uraguchi and Yamazaki

(1978) and those found in this study. The lower IP LD_{50} may be due to the synergestic effect of other toxins present in the crude extract.

Two other toxic metabolites were obtained from the chloroform extract of A. quadrilineatus grown on rice and maize. The white fluffy extract (coded Band 7/3) killed all 10 mice dosed with 20mg/kg, IP. The yellow electrostatic extract (coded Band 7/4) killed 8 of 10 mice dosed at 40 mg/kg dose IP.

The ultraviolet spectra of fractions 7/3 and 7/4 in chloroform are similar. The maximal absorbancy for 7/3 is 269nm and that of 7/4 is 254nm. These values are different from that of aflatoxin B_1 in chloroform (353nm).

The infrared spectra of fractions 7/3 and 7/4 are very similar when carefully matched. When their spectra was compared to that of aflatoxins the carbonyl band is in the same range (Fig. 8.5). Further physico-chemical analysis would have to be done on fractions 7/3 and 7/4 to enable the specific

identification of their chemical structures.

A. quadrilineatus used in this study was isolated from mouldy acha collected from the field in Plateau State of Nigeria. The importance of this finding lies on its implications for public Health. Some sterigmatocystin has been found in nature contaminating feeds in other countries it is possible that it could have a natural occurrence in Nigeria also. Together with the aflatoxins which are known to have high incidence in foodstuffs in Nigeria, sterigmatocytin might play a role in the high rate of liver cancer in Nigeria. This is purely speculative at this stage but efforts should be made to screen food and feedstuffs for mycotoxins including sterigmatocystin.

TABLE 8. 1: SOME THIN LAYER CHROMATOGRAPHIC CHARACTERISTICS OF CRUDE EXTRACTS OF *A. QUADRILINEATUS*

Spot	Rf	Colour		
		White Light	Long UV	Short UV
1	0.79	Yellow Spot	Deep yellow	Dark Spot
2	0.70	Faint Yellow	Light brown	Light brown
3	0.68	Brick brown	Deep brown	Deep brown
4	0.63	Dark yellow	Black	Light brown
5	0.59	Purple	Light blue	Dark spot
6	0.40	Street Brown	Shining yellow	Light yellow
7	0.17	Streak brown	Light blue	Light blue
8	0.0	Brown	Light yellow	Light green

Table 8.2: CHARACTERISTICS OF DIFFERENT SOLVENT ELUTION FRACTIONS COLLECTED FROM COLUMN CHROMATOGRAPHY OF A. QUADRILINEATUS TOXIN

Solvent fraction	Colour of fraction	Cystals Colour after evaporation	Mortality at 40mg/kg in mice
Hexane	Colourless	No residue or Crystals	-
Benzene	Deep Yellow	Not crystallizable	0/2
Diethylether	Chocolate Brown	Deep Yellow Crystals	1/2
Chloroform	Light Yellow	Shining Yellow Crystals	2/2
Ethylacetate	Brown	Yellow amorphous powder	2/2
Acetone	Light Brown	Light grayish crystals	0/2
Methanol	Purple	Deep purple crystals	0/2

TABLE 8.3 GRADIENT ELUTION OF COLUMN AND PHYSICAL AND BIOLOGICAL EFFECTS OF FRACTIONS COLLECTED FROM A.QUADRILINEATUS TOXIN

Solvent		Fractions of 20ml collected	Colour of fraction	Colour of crystal	Mortality 40mg/kg in mice
Pure chloroform - 200ml 90% chloroform (CF)+10% **ethylacetate** (EA) 200ml		1-14	Deep brown	Light brown	*2/4
80% CF + 20% EA	(200ml)	15-19	Deep brown	Light Yellow	4/4
70% CF + 30% EA	(200ml)		Deep brown	Light Yellow	
60% CF + 40% EA	(200ml)	20-37	Deep Yellow	Deep Yellow	0/4
50% CF + 50% EA	(200ml)				
100% EA	(200ml)	38-50	Light Yellow	Light Yellow	0/4

*2/4 - Two of the four treated mice died.

TABLE 8.4: SCREENING OF TOXICITY OF BANDS 6 AND 7 OF PREPARATIVE THIN LAYER CHROMATOGRAPHIC PREPARATIONS OF RESIDUE OF FRACTIONS 15 - 19 OF COLUMN CHROMATOGRAPHIC TOXINS OF A. QUADRILINEATUS IN MICE

PTLC	Colour of Residues	Lethality			
		5mg/kg	10mg/kg	20mg/kg	40mg/kg
6	Orange Yellow	*0/2	0/2	0/2	2/2
7	Light Yellow	0/2	1/2	2/2	2/2

0/2 - No death recorded for two treated mice.

TABLE 8.5: VISUAL AND CHROMATOGRAPHIC CHARACTERISTIC OF TWO PURE FRACTIONS OF BAND 7 RESIDUE OF CHLOROFORM EXTRACT OF CULTURE OF A. QUADRILINEATUS

	Visual Appearance	Appearance under Long UV Wave length	Appearance under short UV Wave Length	Appearance under White Light	Rf chloroform/ methanol (97:3 v/v)
Band 7 Crystals	White fluffy residue	Faint Blue flourescence	Black Spot	Not visible	0.38
	Yellow Electri-static residue	Bright Yellow flourescence	Dull Yellow flourescence	Yellow spot	0.32

TABLE 8:6 TOXICITY OF TWO PURE TOXINS ISOLATED FROM CULTURE OF A.QUADRILINEATUS IN MICE (IP)

Toxin Isolates	Lethality			
	5mg/kg	10mg/kg	20mg/kg	40mg/kg
White fluffy Isolate Band 7/3	2/10*	5/10	10/10	10/10
Yellow Electrostatic Isolate Band 7/4	0/10	0/10	3/10	8/10

* No.dead/no. treated

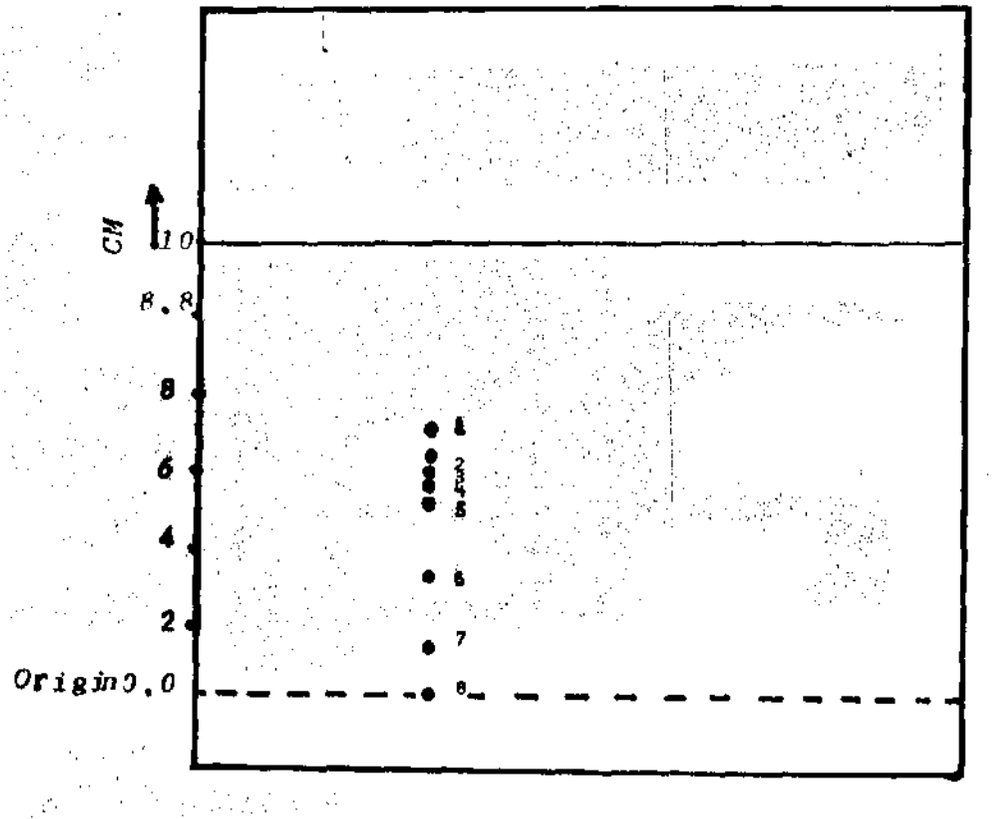


Fig. 8.1 *Diagram of Thin Layer Chromatograhic Separation of crude chloroform extract of A. quadrilineatus developed with Toluene + Ethylacetate + Formic Acid (60+30+10:v:v)*

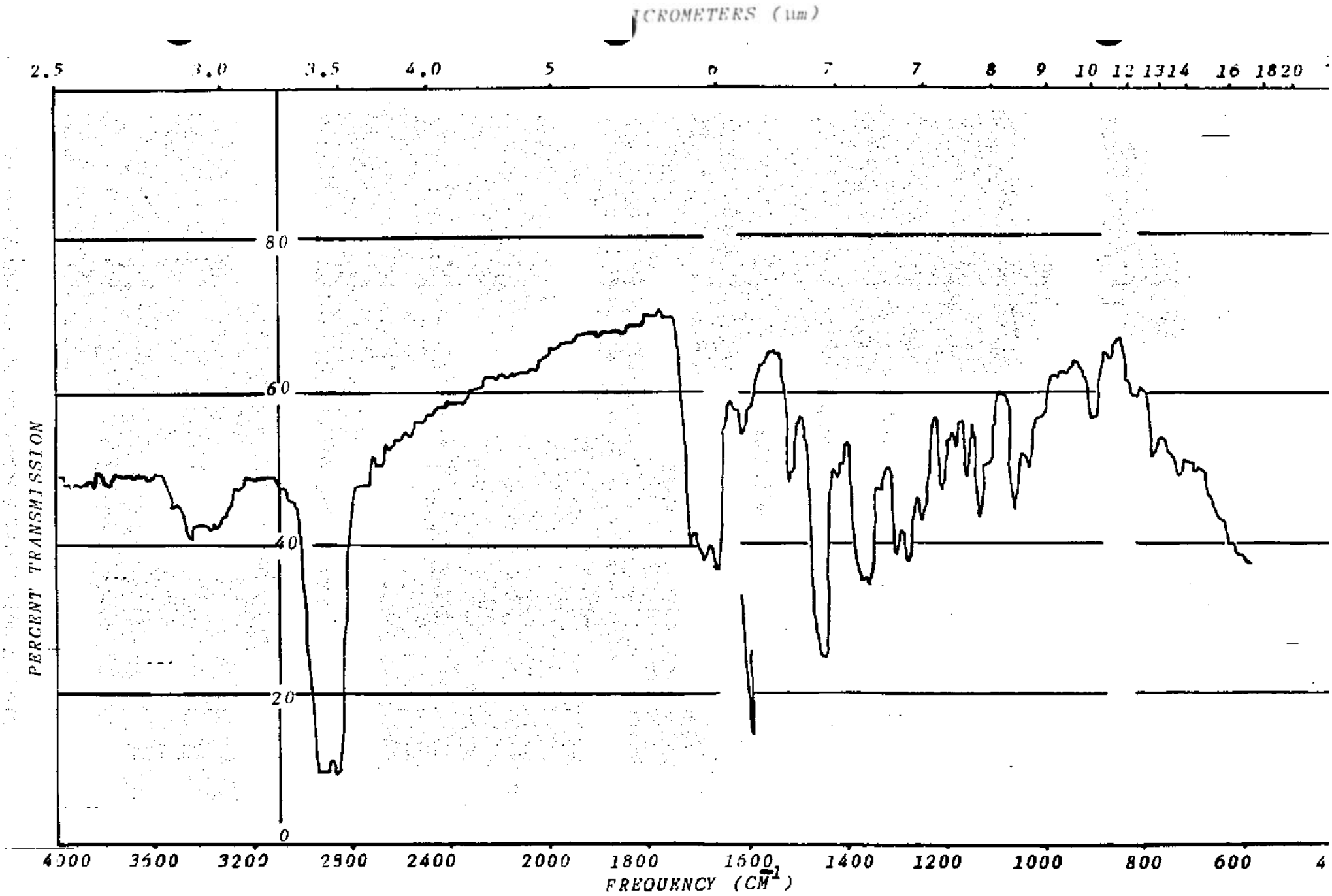


Fig. 8.2 Infrared spectrum of fraction 7/3 in phase solid + liquid paraffin and air reference.

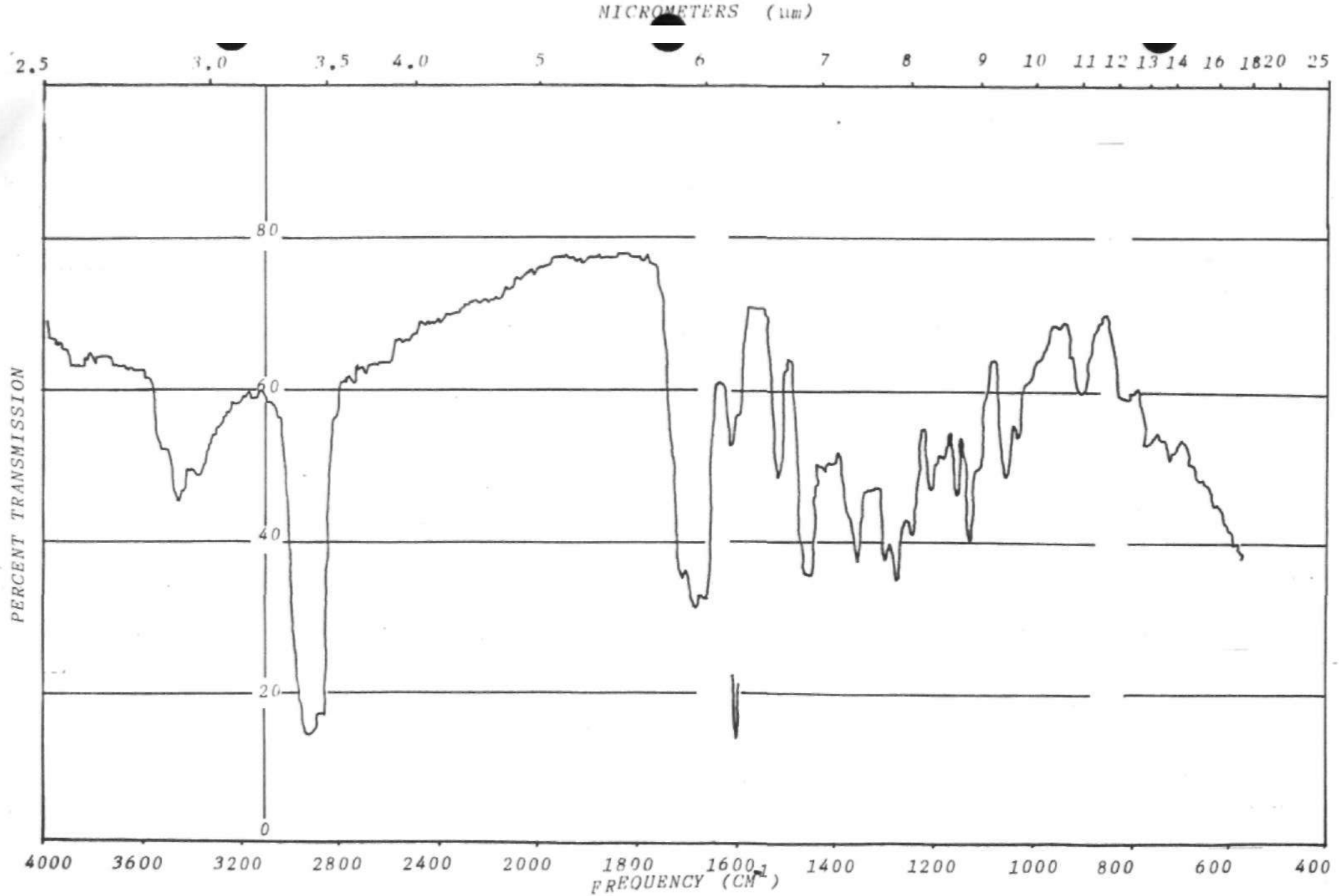


Fig. 8.3 Infrared spectrum of fraction 7/4 in phase solid + liquid paraffin with air reference.

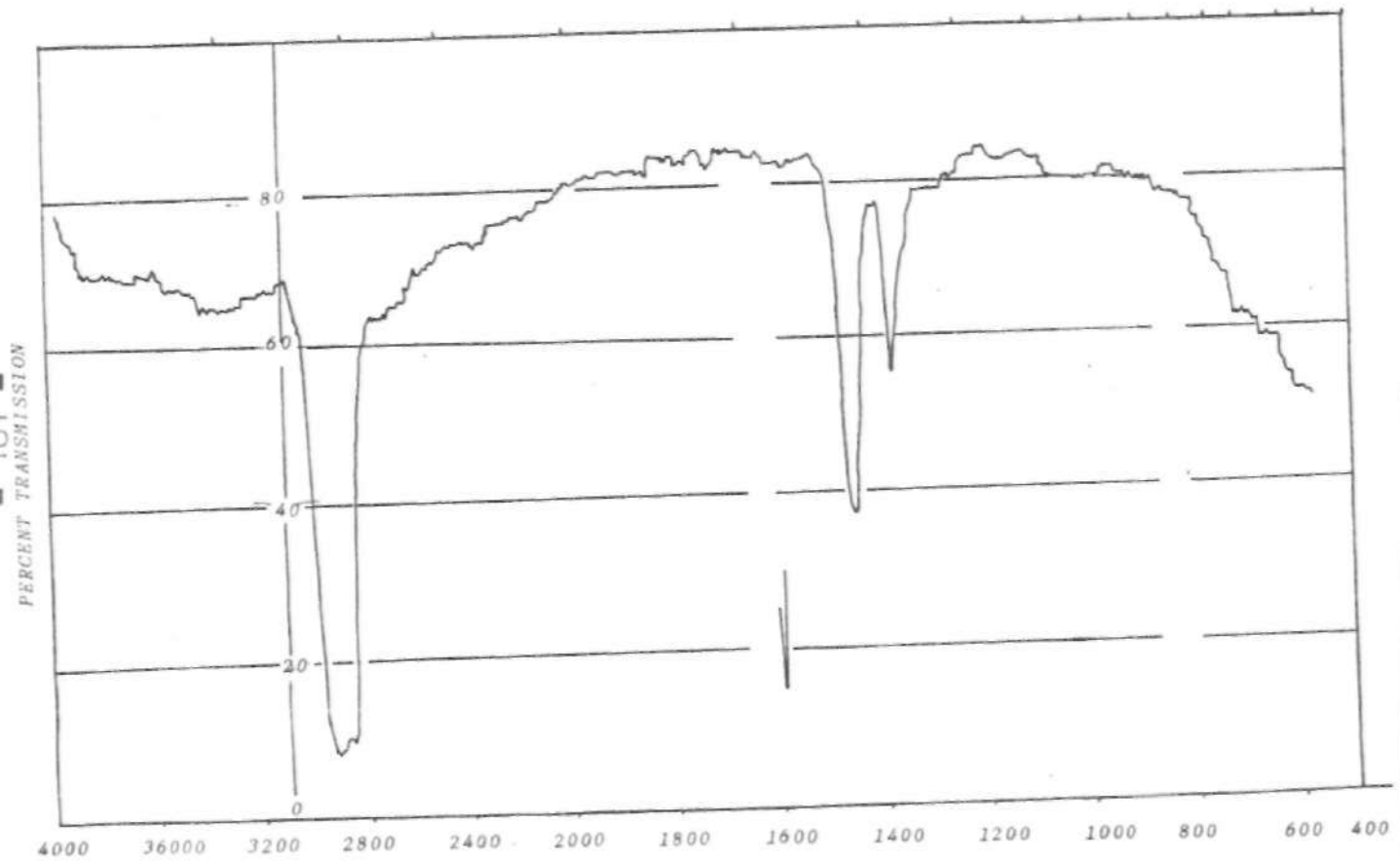


Fig. 8.4 Infrared spectrum of liquid paraffin "solvent"

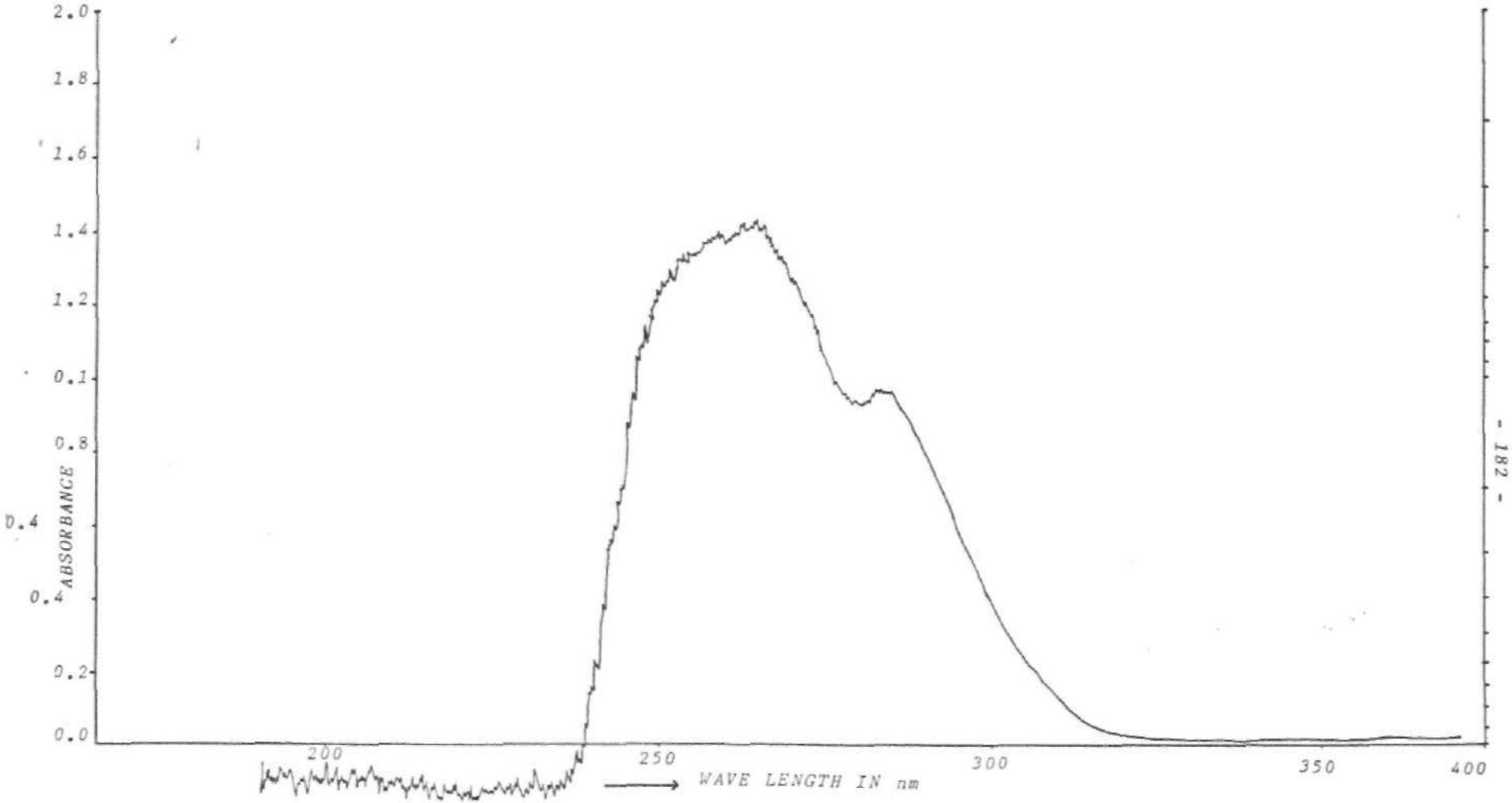


Fig. 8.5. Ultraviolet scan of fraction 7/3 dissolved in chloroform.

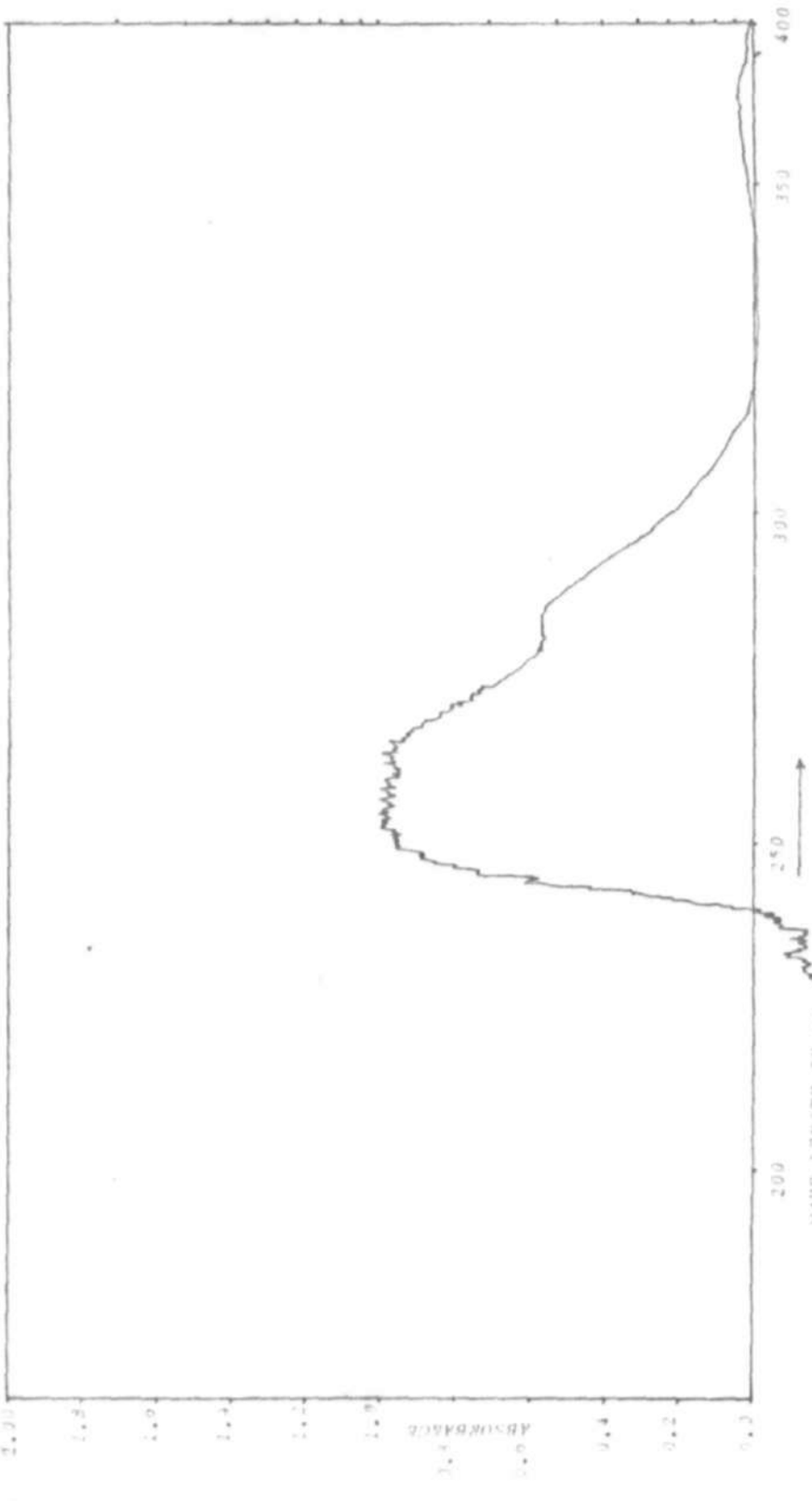
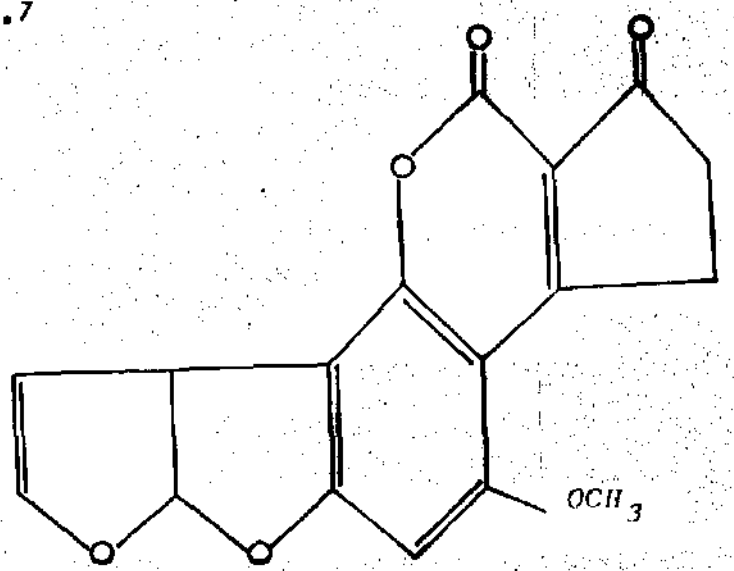
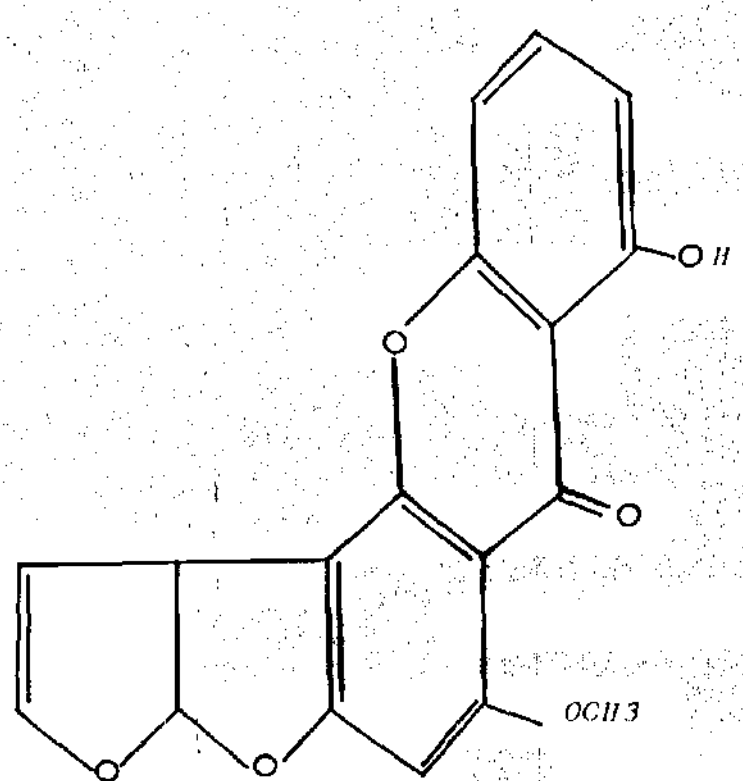


Fig. 3.6 Ultraviolet scan of fraction 7/4 dissolved in chloroform.

Fig. 8.7



AFLATOXIN B_1



STREPTOMYCATOCYSTIN

SUMMARY AND CONCLUSION

SUMMARY

Mouldy acha (Digitaria exilis,Stapf,) maize (Zea mays) and cotton seed (Gossypium spp. Linn) from different Local Government Areas of Plateau State (Barkin Ladi, Bassa, Bokkos, Jos, Lafia, Keffi, Langtang, Mangu, Pankshin and Shendam) were screened for mycoflora and mycotoxins during the dry harmattan period (November - February), the hot dry period (March-May) and the warm wet and humid period (June - October). The fungi isolated from acha were further screened for their mycotoxin potential. An in depth study was carried out on Aspergillus quadrilineatus isolated from acha and which was found to produce one of the most toxic metabolities as shown by acute and chronic toxicity studies in mice, rats, chicks and cattle. Attempts were made to isolate, purify and identify the chemical characteristics of the toxic metabolites produced by A. quadrilineatus. The following were the salient findings:

Phoma sorghina was the most common contaminant of acha during the cold dry harmattan period (November - February)(87.5% incidence) and during the humid and

wet period (June - October) (75%). This was followed by Fusarium moniliforme and Aspergillus spp. (50%). During the hot and dry period (March - May), Aspergillus spp. were the most prevalent fungi (100%) followed by Fusarium spp.(57%) and Phoma sorghina (43%) in that order. Zearalenone was the most prevalent mycotoxin in mouldy acha samples collected during the cold, dry period (44%) while aflatoxin B_1 was the most prevalent in samples collected during the hot and dry period (44%). Zearalenone was only detected in one of the samples collected during the hot, humid and wet period.

Fusarium spp. were the commonest fungi found in maize during the dry harmattan (50%) and hot and dry periods (65%) and Trichoderma spp. were the commonest fungi found during the hot, humid and wet period (84%). The highest level of aflatoxin was found in samples from Langtang (B_1 = 960, B_2 = 543 ug/kg) and the highest level of zearalenone was found in samples from Langtang area (17,500 ug/kg). The highest level of ochratoxin was found in the field samples from the Jos area (50 ug/kg).

Phoma sorghina was the only fungus isolated from one mouldy cotton seed sample collected during

the dry harmattan. Cunninghamella spp. were isolated from all three samples collected during the hot, dry period and from one of the six samples collected during the rainy season. Only aflatoxins could be detected in the mouldy cottonseed samples with the highest level (271 ug/kg) occuring in samples from Keffi.

Ninety six fungal isolates from mouldy acha were cultured on rice and extracted with methylene chloride . The extracts were then put in corn oil and screened for toxicity in mice. Fifty of the 96 isolates (52%) were found to produce toxic metabolites; 20 were mildly toxic to mice (mortality 20-40%) 13 moderately (mortality 60-80%) and seventeen were very toxic (mortality 100%) Aspergillus quadrilineatus, A. nidulans, and F. solani and Phoma sorghina produced the most toxic extracts. A. quadrilineatus produced 100% mortality at 640 mg/kg. A. nidulans and F. solani at 2560 mg/kg and Phoma sorghina produced 33% mortality at 2560 mg/kg.

Two extracts of A. quadrilineatus: the petroleum ether soluble extract (PER) and the petroleum ether defatted crude extract (PEDCR), were tested for acute and chronic toxicity in mice, and chicks. The per os LD_{50} (median lethal dose) of PER was 1148 mg/kg

and that of PEDCR was 507mg/kg in mice, and 556 mg/kg in chicks. The intraperitoneal LD_{50} of PEDCR in mice was 20.8 mg/kg. The toxic signs of PER and PEDCR in mice and chicks include tachypnea, tachycardia, anorexia, somnolence, diarrhoea, coma and death. The main post mortem findings in acute toxicity are congestion of heart, liver, kidney and lungs and sloughing of the wall of stomach and haemorrhagic entiritis. The histopathologic findings in dead animals included oedema and mild degeneration of the myocardium, necrosis of kidney tubular epithelial cells, hepatocytes and bronchioles. The only clinical observation in calf acutely dosed with culture of A. quadrilineatus on maize were transient whole body tremors which occurred an hour after dosing, tachycardia and profuse salivation. No significant histopathologic changes were found in the organs of the sacrificed calf.

Rats were alloted into five groups of five female and five males each. Group 1 received maize on which A. quadrilineatus was cultured; group 2 received normal diet substituted 60% with fungal maize culture, group 3 received 30% substituted diet, group 4, pure ground yellow maize and control group 5 received normal diet for twenty weeks.

Nine cattle were alloted into four groups. Group 1 made up of two males received normal diet substituted 20% by maize culture of A. quadrilineatus, group 2 of two males received 60% substituted diet, group 3 of two males received pure A. quadrilineatus cultured maize (100%) and group 4 control made of two males and one female received normal diet.

The animals were observed for clinical signs of intoxication. Forthnightly weight changes and various blood biochemical, mineral and haematological values were monitored in cattle over the experimental period. Post mortem and histopathologic examinations were carried out on dead or euthanatized animals.

Clinical signs observed in rats included decreased weight gain, soft faeces, lethargy, arched back, rough hair coat and death in the case of rats on 100% substituted diet. No clinical signs were observed in control rats.

No abnormal clinical signs were seen in cattle on substituted diets except that cattle on normal diet had superior weight gain as compared to those on substituted diets.

Post mortem findings in rats included hyperaemia

of the mucosa of the stomach and intestines, sloughing of the mucosa of the stomach, petechial haemorrhages on the liver and gastro-enteritis. No significant postmortem findings were observed in cattle on substituted diet except that the mucosa of the stomach and duodenum of cattle on 100% substituted diet were sloughed off.

Histopathological findings in rats included liver congestion, hepatocellular degeneration and necrosis, congestion of the venous sinuses of spleen characterized in some cases by mild to moderate lymphocytes depletion of the periarteriolar lymphatic sheath. Varying degrees of interstitial inflamation with peribronchiolitis and atelectasis and severe alveolar oedema were seen in lungs of some rats. Submucosal oedema of the stomach and granular degeneration of the tubular epithelial cells particularly of the medulla were seen. Severe meningeal congestion, haemorrhage and submeningeal degeration and perivascular cuffing in some parenchymal vessels of the brain stem, were seen.

In cattle the histopathological findings included hepatic degeneration and necrosis of the liver. Multifocal areas of haemorrhage of the liver and

extensive tubular necrosis and degeneration of glomerular tufts were seen.

Chronic feeding of A. quadrilineatus substituted diets to cattle had an increasing effect on PCV, neutrophils, serum cholesterol, triglyceride, SGOT, serum alkaline phosphatase and lactate dehydrogenase and it had a decreasing effect on lymphocytes, monocytes, Hb, and albumin when compared to control animals values. The mycotoxins produced by A. quadrilineatus were extractable from rice culture by chloroform. Column chromatographic separations in silica gel using different elution solvents and biological tests showed that the mycotoxins came off in the diethylether, chloroform and mostly in ethylacetate fractions. Use of different available mycotoxin standards on silica gel G coated chromatoplates revealed that one of the mycotoxins produced by A. quadrilineatus was sterigmatocystin. Two other more toxic mycotoxins were isolated and purified from the crude chloroform extract and their column, preparative thin layer chromatographic, infrared and UV-spectrophotometric characteristics were established. The infrared spectra of the two purified mycotoxins suggest that the carbonyl group of their structures were similar to that of aflatoxin.

CONCLUSIONS

1. The main moulds found in *acha* in Plateau State are *Phoma sorghina*, *Fusarium moniliforme* *Aspergillus* spp. while the main mycotoxins are zearalenone and aflatoxins.
2. The main moulds found in maize in Plateau State are *Neurospora* spp. *Fusarium* spp. The main mycotoxins are Aflatoxins B_1, B_2 and ochratoxin A.
3. The main moulds found in cotton seed in Plateau State of Nigeria are *Phoma sorghina*, and *Cunninghamella* spp. while the main mycotoxins are aflatoxins.
4. *Aspergillus quadrilineatus* is one of the most toxic moulds infecting *acha*. It is toxic to mice, rats, chick and cattle causing mainly hepatatoxic, nephrotic and gastrointestinal effects.
5. *Aspergillus quadrilineatus*, produces sterigmatocystin and seven other mycotoxins, two of which probably contain carbonyl groups.
6. Further work is required to fully characterize the mycotoxins of *A. quadrilineatus*.

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APPENDIX 1

CONSTITUENTS OF NORMAL AND SUBSTITUTED FEEDS FOR EXPERIMENTAL CATTLE ON CHRONIC TOXICITY STUDIES

Control Diet	100% Substitution of maize with Cultured maize	60% substitution of maize with cultured maize	20% substitution of maize with cultured maize
24kg Maize (48%)	24kg cultured maize	14.4kg cultured maize	4.8kg cultured maize
17.5kg wheat offals (35%)	17.5kg wheat offals	9.6kg clean maize	19.2kg clean maize
7.5kg Groundnut cake (15%)	7.5kg Groundnut cake	17.5kg wheat offal	17.5kg wheat offals
0.5kg Bone meal (1%)	0.5kg Bone meal	7.5kg groundnut cake	7.5kg groundnut cake
0.5kg common salt (1%)	0.5kg common salt	0.5kg bone meal	0.5 bone meal
0.005kg Vitamin Mixture	0.005kg Vitamin Mixture	0.005kg Ccmmon salt	0.5 common salt
		Vitamin mixture	0.005kg Vitamin mixture

APPENDIX 2

CUMULATIVE MEANS AND STANDARD DEVIATIONS OF HAEMATOLOGICAL VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH A. QUADRILINEATUS CULTURED MAIZE.

Haematological Parameters	Control			20% Substituted Diets		60% Substituted Diets		100% Substituted Diets
	0500(m)	2862(m)	0449(f)	0232(m)	9430(m)	0241(m)	0507(m)	0440(m)
PCV%(7)	38.0 ± 4.16	32.62 ± 3.67	38.43 ± 2.51	28.40 ± 1.14	29.87 ± 2.03	29.86 ± 2.85	35.87 ± 3.83	31.75 ± 1.18
Hbgm/dl(7)	13.08 ± 1.89	10.50 ± 1.69	13.24 ± 1.37	8.5 ± 0.5	9.82 ± 0.73	9.37 ± 1.33	10.91 ± 1.72	9.40 ± 0.88
WBC%(6)	11,833.33 ± 1,930.71	10,628 ± 1,081	9266 ± 1,689	9600 ± 9,5.41	10,064.28 ± 2,486.11	9,366.66 ± 1,011.76	9,900 ± 956.09	8,857 ± 1,629
Neutrophil(8)	29.50 ± 17.16	35.78 ± 16.47	23.37 ± 10.32	37.00 ± 14.94	34.55 ± 13.3	37.12 ± 8.90	33.67 ± 14.65	29.12 ± 11.29
Lymphocytes(8)	69.75 ± 19.37	59.67 ± 13.09	66.12 ± 12.04	53.17 ± 10.19	55.22 ± 12.84	54.87 ± 10.87	52.78 ± 12.16	54/50 ± 8.79
Monocytes(8)	1.70 ± 0.88	1.10 ± 0.92	1.87 ± 1.88	2.16 ± 2.23	3.62 ± 3.29	2.20 ± 1.30	4.00 ± 4.33	3.50 ± 0.71
Eosinophils(8)	4.60 ± 5.5	3.20 ± 4.81	8.25 ± 5.97	7.00 ± 9.16	7.50 ± 6.76	5.12 ± 6.22	9.27 ± 5.58	7.00 ± 14.57
Basophils(8)	-	0.37 ± 0.51	-	-	-	-	-	-

0500 = Code Number of Animal
(m) = Male
(f) = Female
(7) = Number of samples.

APPENDIX 3

CUMULATIVE MEANS AND STANDARD DEVIATIONS OF MINERAL VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH *A. QUADRILINEATUS* CULTURED MAIZE.

	Control			20% Substituted diets		60% Substituted diets		100% Substituted Diet
	0500(m)	2862(m)	0449(f)	0232(m)	0430(m)	0241(m)	0507(m)	0440(m)
Calcium mmol/L	2.68 ± 0.57	2.64 ± 0.66	2.70 ± 0.18	2.61 ± 0.48	2.61 ± 0.42	2.65 ± 0.60	2.95 ± 0.50	2.78 ± 0.41
Magnessium mmol/L	1.20 ± 0.25	1.60 ± 0.27	1.36 ± 0.13	1.42 ± 0.18	1.29 ± 0.17	1.35 ± 0.19	1.30 ± 0.12	1.29 ± 0.24
Potassium mmol/L	4.26 ± 0.63	3.95 ± 0.85	3.53 ± 0.82	3.30 ± 0.74	3.73 ± 0.63	2.89 ± 0.68	4.12 ± 0.67	4.23 ± 0.47
Sodium mmol/L	145.12 ± 7.02	141.16 ± 5.67	141.01 ± 9.19	136.65 ± 9.34	133.47 ± 14.59	138.96 ± 7.62	141.25 ± 9.05	148.93 ± 6.28
Copper mmol/L	17.68 ± 3.75	12.61 ± 4.82	13.45 ± 2.80	15.24 ± 2.56	16.35 ± 3.59	14.46 ± 5.41	15.38 ± 3.07	15.78 ± 4.29
Zinc mmol/L	34.78 ± 10.15	36.26 ± 9.14	42.89 ± 4.37	33.20 ± 8.83	34.68 ± 7.19	34.05 ± 9.65	34.61 ± 8.64	32.41 ± 8.84
Iron mmol/L	45.74 ± 8.73	46.54 ± 6.93	48.07 ± 5.41	42.95 ± 5.06	43.75 ± 0.08	42.93 ± 8.03	48.1 ± 6.38	40.91 ± 9.43
Inorganic Phosphate mmol/L	2.35 ± 0.26	2.66 ± 0.10	2.25 ± 0.34	2.43 ± 0.27	2.46 ± 0.21	2.29 ± 0.37	2.67 ± 0.20	2.27 ± 0.18
Chloride	102.36 ± 3.98	98.64 ± 3.14	100.00 ± 2.52	100.33 ± 2.39	102. 22 ± 3.79	101.70 ± 5.14	99.36 ± 4.25	102.18 ± 4.28

APPENDIX 4

CUMULATIVE MEANS AND STANDARD DEVIATIONS OF SOME SERUM BIOCHEMICAL VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH A!. QUADRILINEATUS CULTURED MAIZE.

	Control animals and code numbers			20% Substituted Diet		60% Substituted Diet		100%Substituted Diet
	0500(M)	2862(M)	0449(F)	0232(M)	0430(M)	2341(M)	0507(M)	0440(M)
Urea MmolL	3.81 ± 1.35	3.68 ± 1.27	3.98 ± 1.01	2.93 ± 1.53	4.68 ± 1.39	4.04 ± 1.20	5.17 ± 1.31	3.76 ± 1.15
Glucose mmol/L	4.13 ± 0.72	2.29 ± 0.15	3.87 ± 0.33	2.72 ± 0.28	3.43 ± 0.29	2.87 ± 0.18	3.88 ± 0.87	3.71 ± 0.73
Cholesterol mmol/L	2.69 ± 0.23	2.83 ± 0.23	3.07 ± 0.32	4.13 ± 0.62	3.70 ± 0.98	2.89 ± 0.43	3.03 ± 0.56	2.73 ± 0.40
Bilirubin mmol/L	0.041 ± 0.015	0.029 ± 0.018	0.05 ± 0.020	0.023 ± 0.013	0.032 ± 0.023	0.032 ± 0.014	0.042 ± 0.026	0.038 ± 0.038
Triglyceride mmol/L	0.36 ± 0.27	0.392 ± 0.10	0.41 ± 0.19	0.85 ± 0.55	0.70 ± 0.32	0.23 ± 0.11	0.52 ± 1.11	0.31 ± 0.20
Total Protein g/l	70.19 ± 6.81	68.38 ± 9.44	78.26 ± 11.08	71.04 ± 9.25	68.94 ± 11.16	73.16 ± 8.33	61.85 ± 8.61	75.83 ± 11.67
Albumin g/l	33.95 ± 6.70	33.69 ± 2.66	33.01 ± 2.67	29.82 ± 2.55	30.27 ± 2.82	29.76 ± 2.43	31.54 ± 2.43	30.92 ± 4.61

APPENDIX 4 CONTINUED

globulin g/L	36.23 ± 5.95	32.53 ± 7.13	41.62 ± 15.66	42.34 ± 8.09	39.34 ± 9.78	43.41 ± 8.44	30.32 ± 8.85	44.90 ± 12.21
Albumin/ Globulin Ratio.	0.78 ± 0.10	0.89 ± 0.04	0.67 ± 0.10	0.69 ± 0.13	0.75 ± 0.11	0.67 ± 0.14	0.86 ± 0.11	0.64 ± 0.18

APPENDIX 5

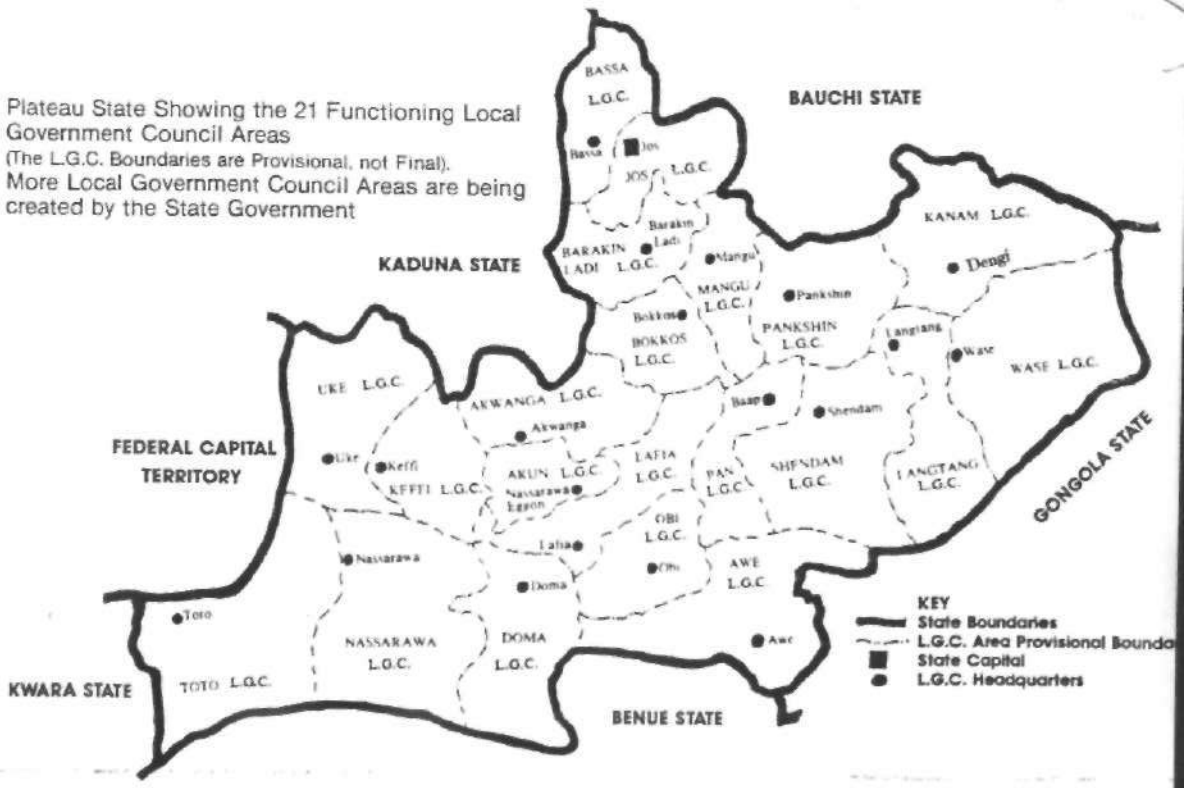
CUMULATIVE MEANS AND STANDARD DEVIATIONS OF SOME SERUM ENZYME VALUES OF CATTLE CHRONICALLY FED DIET SUBSTITUTED WITH A. QUADRILINEATUS CULTURED MAIZE.

	Control			20% substituted diet		60% substituted diet		100% Substituted diet
	0500(M)	2862(M)	0449(F)	0232(M)	0430(M)	0241(M)	0507(M)	0440(M)
Glutamate oxalacetate transaminase IU/L	21.70 ± 10.51	16.36 ± 10.36	19.50 ± 8.34	18.89 ± 9.97	21.50 ± 10.23	25.36 ± 12.85	20.54 ± 9.35	22.0 ± 12.28
Lactate Dehydrogenase IU/L	55.67 ± 23.03	28.5 ± 10.61	58.0 ± 20.22	71.00 ± 0	86.00 ± 0	66.00 ± 25.98	45.00 ± 22.87	61.67 ± 16.16
Alkaline Phosphatase IU/L	268.0 ± 93.71	209.8 ± 96.53	161.12 ± 44.19	216.7 ± 90.30	257.82 ± 105.28	155.73 ± 61.05	311.45 ± 110.24	246.83 ± 77.27



Federal Republic of Nigeria
showing the 19 states and their capitals

Plateau State Showing the 21 Functioning Local Government Council Areas
(The L.G.C. Boundaries are Provisional, not Final).
More Local Government Council Areas are being created by the State Government



APPENDIX 7

PHOTOGRAPH OF FOUR DAY CULTURE OF ASPERGILLUS QUADRILINEATUS OF POTATO DEXTROSE AGAR.

