بسم الله الرحمن الرحيم

"وَلِيَعْلَمَ الَّذِينَ أُوتُوا الْعِلْمَ أَنَّهُ الْحَقُّ مِن رَّبِّكَ فَيُؤْمِنُوا بِهِ فَتُخْبِتَ لَهُ قُلُوبُهُمْ وَإِنَّ اللَّهَ لَهَادِ الَّذِينَ آمَنُوا إِلَى صِرَاطٍ مُّسْتَقِيمٍ "

صدق الله العظيم

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إلى رمز الحنان و العطاء ..... **والدتي**

إلى أغلى ما املك ..... **زوجي**

إلى رفيق دربي ..... **أخي**

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Chapter One
Introduction

1.1 Introduction

Trichothecenes are group of closely related mycotoxins which are widely distributed in nature such as T-2 toxin, Diacetoxyscirpenol (DAS), Deoxynivalenol(DON) and HT-2 toxin.

A total of 148 trichothecenes have been isolated from fungal cultures and plants (Drove, 1988). Trichothecenes are produced in slightly low temperature and slightly different condition according to toxin type (Shepherd and Gilbert, 1986). The most common toxin producer is *Fusarium* including many species that differ in there ability to produce a toxin.

The fate of trichothecenes differs according to type of toxin and the animal receiving it. Generally muscle, liver and kidney much likely to be target organs, for example, in pigs administered with HT-2 toxin, the percentage of administered radioactivity showed that muscle, liver, bile and kidney were the most target organs (Robison *et al*., 1979).

For swan administered with labelled T-2 toxin, the greatest amount of radio activity was located in gastrointestinal tract, muscle and liver (Corley *et al*., 1986).

In chicken injected with T-2 toxin, considerable amounts of T-2 metabolites were found in the liver. Smaller amounts of HT-2 toxin, T-2 triol and other metabolite were found in lungs (Visconti & Mirocha, 1985). In cows administered with T-2 toxin, appreciable levels of metabolite remained in the bile, liver and kidney (Yoshizawa *et al*., 1981).

`T-2 toxin is considered as one of the most toxic and common toxin in trichothecenes, also HT-2 and DON (Tamm and Tori, 1984).

Trichothecenes comes after aflatoxin in toxicity and it affects both man and animal (Al Heety and Abdul Wahid, 1992).This mycotoxin affects cereal grains, farm animal which consume contaminated feed, also affects man causing diseases, such as Alimentary Toxic Aleukia and Skeletal affliction (Kashin-Back disease).

Many diseases reported after ingestion of moldy feed, these diseases characterized by feed refusal, depression, diarrhea, vomiting, and hemorrhage in intestine and muscles which lead to death of animals (Forgacs, 1965; Danko and Szerafin, 1976).

Other characters of the diseases are lesions included necrosis of the mucosa of esophagus, proventriculus and gizzard which affect many field and experimental animals with different severity according to dose intake such as mice (Siren & Feuerstein, 1986), Rat (Matsuoka *et al*., 1979), Pigs (Smalley *et al*., 1970), Birds (Speers *et al*., 1977), Lambs (Friend *et al*., 1983b), and Calves (Pier *et al*., 1976).

1.2 Aims of the study

The present work is an attempt to:-

1- Isolation and identification of *Fusarium oxysporum* from local *zea mays*.
2- Production, extraction and quantification of crude trichothecenes from isolated *F. oxysporum.*
3- Studying some of the pathological (morphological, biochemical& histopathological) effects of trichothecenes on mice.

List of Abbreviation

Code	Word
D.W.	Distilled Water
Ppb	Part per billion
TLC	Thin Layer Chromatography
U.V.L.	Ultra Violet Light
hr	hour
Min	Minutes
I.P.	Intraperitoneal
GOT	Glutamic Oxaloacitic Transaminase
GPT	Glutamic Pyruvic Transaminase
BLU	Blood Urea level
PSA	Potato Sucrose Agar
PDA	Potato Dextrose Agar
F.	Fusarium
DON	Deoxynivalenol
ATA	Alimentary Toxic Aleukia
DAS	Diacetoxyscirpenol
NIV	Nivalenol
Spp.	Species

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Chapter Two

Literature Review

2.1 History of mycotoxins:

Mycotoxins are important fungal secondary metabolites with low molecular weight, they representing potential threat to human and animal (scott &somers, 1969).

Mycotoxins present a challenge for scientists working in a wide range of disciplines such as microbiology, biochemistry, structural chemistry, toxicology, pharmacology & genetics.

Mycotoxicosis was defined as “poisoning “of the host which follows the entry of toxin substances of fungal origin into the body (Forgacs & Carll, 1962). Mycotoxicosis cause unlimited effect on plant, animal, & human.

Mycotoxins can affect many target organs and systems, notably the liver, kidney and the neurons, endocrine and immune systems. This is much concern about chronic effects brought about by low levels of exposure, and several mycotoxins have been classified by the International Agency for Research in Cancer (IARC) as human or potential human carcinogens (IARC, 1993).In some animal species, absorption within gastrointestinal tract is the first step governing the entry of mycotoxins in blood stream and furtherly tissue distribution.

In blood it has been shown that aflatoxin B1, ochratoxin A, citrinin and rubratoxin B can bind reversibly plasma protein whereas zearalenone can bind red blood cell components (Galtier, 1998).

Mycotoxins and mycotoxicosis are recognized in last two decades and received little attention. First pure mycotoxins isolated were ergot alkaloids (in 1875) and penicillinic acid (in 1913), others in 1930 and 1940; but no toxicological test done until 1960s specially when aflatoxin were discovered and defined first as poisonings of host which follow the entry of toxic substances of fungal origin in to the body (Forgacs & Carll, 1962).

Many classes of Mycotoxins such as polyketides, terpenes and nitrogen containing metabolites based on their biological origins (Steyn, 1984).

There were over 280 identified mycotoxins which arranged in 21 chemical groups (Nawar & Al-Natur, 1989).

In winter 1934-1935 in USA, over 5000 horses died because of the consumption of yellow corn contaminated with *Fusarium*, also in Egypt over 50% of horses died for the same reason (Badiali *et al*., 1968).

An outbreak occurs by consumption of contaminated cereal with *Fusarium* by farm animals that cause abortion, cervical prolapses, and pretime or posttime delivery in pigs and castles (Christensen *et al*., 1972) & (Nawar, 1975).

Other symptoms appear such as vomiting, loss of appetite of animals fed diet contaminated with *Fusarium* (Curtin & Tulte, 1966) & (Nawar, 1975).

Another dramatic outbreak caused by consumption of contaminated cereal grains that occurred in certain area in Russia between (1942-1947).

The most toxic known group is aflatoxin which produced by *Aspergillus flavus* cause what was called turkey X disease in England in 1960 which cause in few months the death of 100000 turkey and 14000 ducklings because of feeding contaminated food with *Aspergillus flavus* (Wogan, 1965).

Alimentary Toxic Aleukia (ATA) is one of the diseases caused by consuming of cereal grains that had over wintered in field (FAO, 1979).About 10% of the consumers were died with this disease (Mirocha *et al*., 1983). ATA was a result of trichothecene toxicity, and it was known that trichothecenes have severe toxic effects, that cause hemorrhage in the stomach wall and intestine and caused damage of bone marrow (Joffe, 1971).

Mycotoxin contamination is unavoidable and unpredictable which makes it a unique challenge to food safety (Park& Stoloff, 1989).

The hematoxicity of aflatoxins is characterized clinically by hematological problem due to abnormal blood cell counts &\ or dysfunction of blood cells of various lineages, based upon consideration of hematotoxicity (Lopez, 1998; Burgos 1998).

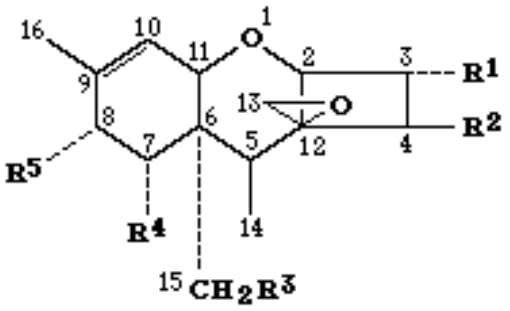
2.2 Trichothecenes (physical & chemical properties):-

The sesquiterpenoid trichothecenes possess the tetracyclic 12,13-epoxytrichothecene skeleton. More than 148 trichothecenes, 83 non-macrocyclic and 65 macrocyclic, have been isolated from fungal cultures and plants (Drove, 1988).

They can be conveniently divided into 4 categories according to similarity of functional groups (Ueno, 1977).

The first category is characterized by a functional group other than a ketone at C-8 (type A). This is the largest category containing members such as T-2 toxin and diacetoxyscirpenol (DAS). The second category of trichothecenes has a carbonyl function at C-8 (type B) typified by 4-deoxynivalenol (DON) and nivalenol (NIV). The third category is characterized by a second epoxide group at C-7, 8 or C-9, 10 (type C), and the fourth contains a macrocyclic ring system between C-4 and C-15 with two ester linkages (type D). The structures of representative trichothecenes of each category are illustrated below.

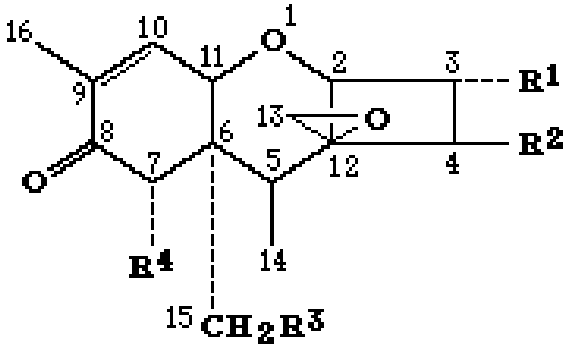
Type A Trichothecenes



Name	R_1	R_2	R_3	R_4	R_5
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
HT-2 toxin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Diacetoxyscirpenol	OH	OAc	OAc	H	H
Neosolaniol	OH	OAc	OAc	H	OH

Fig.1 Chemical Structure of type A Trichothecenes.

Type B Trichothecenes



Name	R_1	R_2	R_3	R_4
Deoxynivalenol	OH	H	OH	OH
Nivalenol	OH	OH	OH	OH
Trichothecin	H	$OCOCH=CHCH_3$	H	H
Fusarenon-X	OH	OAc	OH	OH

Fig.2. Chemical structure of type B Trichothecenes.

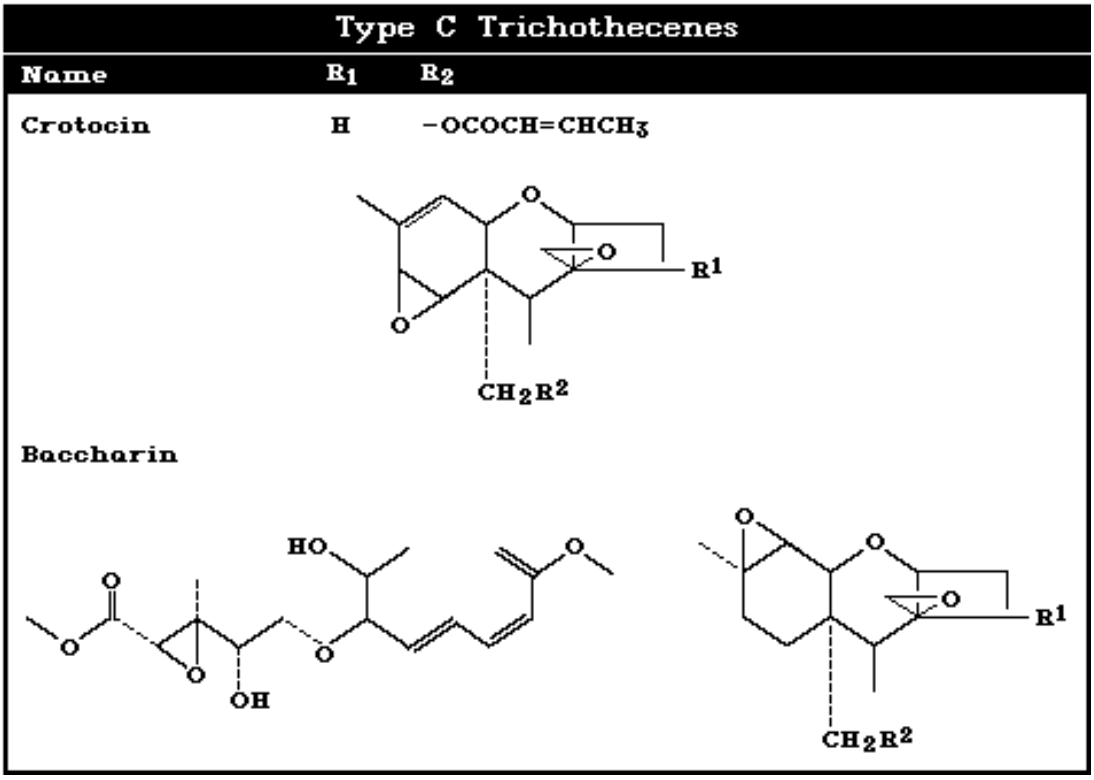


Fig.3. Chemical structure of type C Trichothecenes.

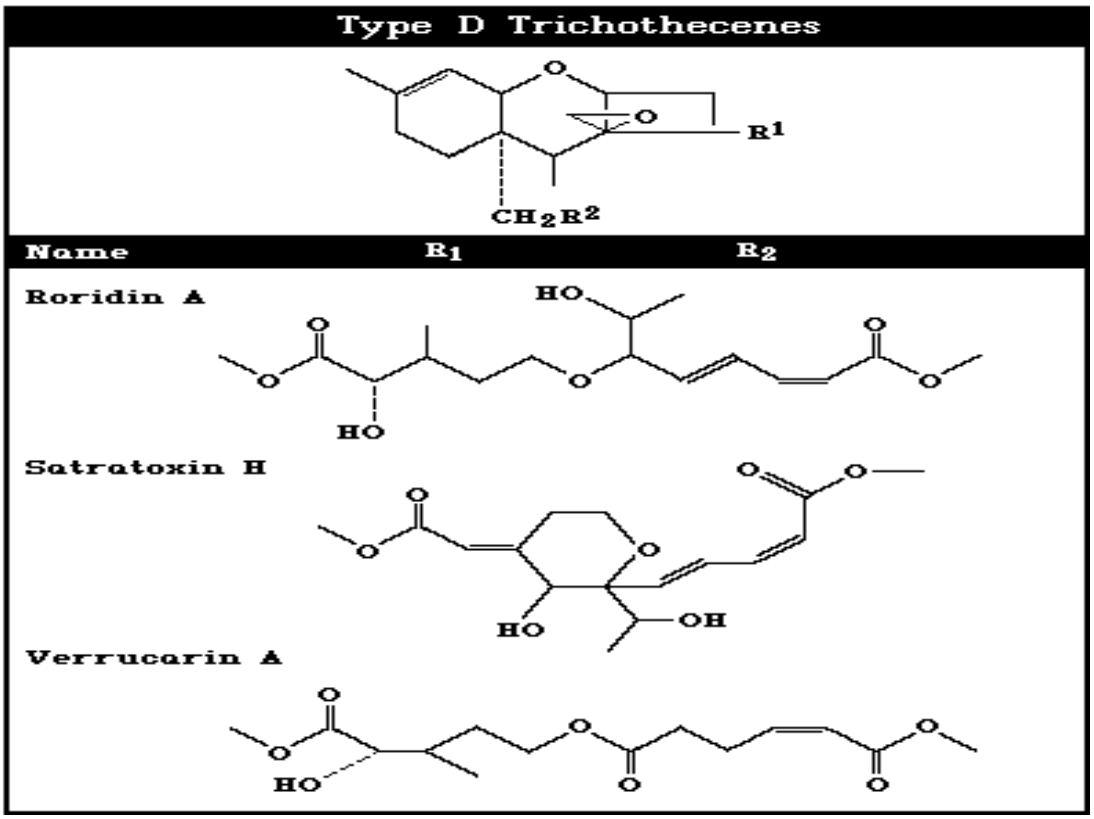


Fig.4.Chemical structure of type D Trichothecenes.

a- Physical properties

The trichothecenes are colorless, mostly crystalline solids that have been well characterized by physical and spectroscopic techniques (Cole & Cox, 1981). The type A trichothecenes are soluble in moderately polar solvents, such as chloroform, diethyl ether, ethyl acetate, and acetone, whereas the more polar type B trichothecenes require higher polarity solvents, such as aqueous methanol or aqueous acetonitrile.

Most of the trichothecenes lack conjugated insaturation in their structures with a consequent absence of absorption in the ultraviolet (UV) spectrum, except for end absorption due to insaturation at C-9. This lack of absorbance is a source of difficulty in achieving sensitive and specific detection in HPLC analysis. In contrast, the type D trichothecenes give characteristic ultraviolet spectra.

b- Chemical properties

When trichothecenes containing an ester group are treated with a base, they are hydrolyzed to their corresponding parent alcohol (Wei et al., 1971); free hydroxyl groups are readily acetylated.

The 12, 13-epoxy groups is itself extremely stable to nucleophilic attack. However, prolonged boiling under highly acidic

conditions causes an intramolecular rearrangement of the trichothecene skeleton to the apotrichothecene ring.

The trichothecenes are generally stable; for example, DON can be stored in organic solvents, such as ethyl acetate, for a long time without any significant deterioration (Shepherd & Gilbert, 1988).

They remain unaffected when refluxed with various organic solvents and also under mildly acidic conditions.

2.3 Producing organisms:-

Trichothecenes are group of metabolites produced by species of the genus *Fusarium,* in addition to other genera including *Mythecium, Trichothecium, Verticimonosporium, Cefalosporium, Trichoderma, Gibberella, & Stachybotrys.*

These fungi attack many agricultural plant products and produce toxins. In 1979 more than 45 naturally occurring derivatives of trichothecenes and more than 20 derivatives were produced by *Fusarium* species (Panther & Mirocha, 1979).

Some trichothecenes have been isolated from strains of *Fusarium* species referred as *F.episphaeria, F.latritium, F.nivali, F.tricinctum, F.sporotrichiodes, F.oxysporum, F.rigidiusculum, F.solani, F.roseum, & F.monilifome* (Ueno, 1977).

2.4 *Fusarium oxysporum*:-

Gerlach (1979), was first identifying *F.oxysporum* which is part of Elegans section of *F.* spp. Which cause toxicosis. This spp. occurred in all types of soil in all regions of the world in which farming is practiced and in many regions where there is no agriculture. Some species have extraordinarily wide distribution all over the globe.

F. oxysporum though occurring mainly in uppermost soil layers and have been found viable down to at lest 50 cm depth.

Economically this spp. considered one of the most important phytopathogenes which couse a lot of plant diseases. The average growth rate of *F.oxysporum* culture is 4.5 cm while in some strains it may reach 6.5 cm. Mycelium delicate white or peach but usually with a purple tinge, sparse to abundant then floccose, becoming felted and sometimes wrinkled in older cultures. Microconidia born on simple phialides arising laterally on hyphae or from short sparsely branched conidiophores, microconidia generally abundant, variable, oval_ellipsoid cylindrical, straight or curved.

Macroconidia, sparse in some stains, are born on more elaborately branched conidiophores or on the surface of tubercularia_like sporodochia. They are thin walled, generally 3-5 septate, fusoid_subulate and pointed at both ends; occasionally fusoid_falcate macroconidia are found with a somewhat hooked apex (Booth, 1971).

The first toxic compound isolated in pure form by *F.oxysporum* from cultures of plant is lycomarasmin, also fusaric acid, anniatins A&B, lycomarasmic acid, plytonivein and aspergillomarasmin B. It has been found that *F.oxysporum* produce trichothecenes both in nature and in culture. Ueno *et al.* (1973) *isolates* diacetoxynivalenol and fusarenon-x from *F.oxysporum*.

In addition Miroch & Christensen (1974) have reported F.oxysporum to be among the F. spp. forming zearalenone in nature.

The optimum condition for mycelium growth is at temperature 25-30 ºC, humidity between 88-91 % and pH 5.5- 6.5, also low carbon/nitrogen ratio increase fungi growth, vitamins are required for this *Fusarium* spp.

Competitive ability of this fungus as soil saprophytes is high, but nevertheless decreased by various stryptomycetes and bacteria; also by some fungi such as: *Gliocladium roseum* which is a common soil fungus of world wide distribution.

F.oxysporum considered second commonest spp. involved in superficial and deep infection. Man is also affected by *F.oxysporum*; it's associated with skin irritation and nail disease and with cornel ulcers. The only recorded of internal disease caused by F.oxysporum is that by Gutmann *et al.* (1979) describing a case of systemic granulomatous infection, in addition to alimentary toxic aleukia, and skeletal affliction (kashin-Back disease).

2.5 Natural occurrences of Trichothecenes:

It has become apparent in the past few years that whenever a Trichothecene-producing *Fusarium* species parasitizes a crop, food, or animal feed, it is highly probable that the metabolites of the trichothecene will be found as contaminants. The chance of detecting the metabolite is then clearly a function of the efficiency of the sampling procedure and the capabilities of the analytical methods used.

The trichothecenes have only been found very sporadically in natural products this may well be due to lack of extensive examination, there is little doubt that species of *Fusarium* are widely distributed geographically and associated toxin formation must be expected under specific condition.

Recent chemical and mycological date from the Transki with maize indicated a very high rate of contamination by Fusarium spp. and toxins they produced (Thiel, 1982).

As expected, samples of maize intended for beer brewing or animal feeding showed a much higher rate of contamination as well as a much higher concentration of toxins than samples from maize intended for human consumption.

Because of its toxicity, analytical procedures for T-2 toxin were developed first, and consequently early surveys for trichothecenes tended to concentrate on T-2 toxin.

Table (1): Natural occurrence of some types of trichothecenes:-

Toxin type	**commodity**	**Country**	**Level (mg/kg)**	**References**
T-2 toxin	Corn	Hungary Taiwan New Zealand	0.5-5.0 0.08-0.6 0.01-0.2	Szathmary (1983) Tseng *et al*. (1983) Hussein *et al*. (1989)
	Feed	Hungary	0.05-5.0	Szathmary (1983)
	Oats	Finland	0.01-0.05	Ylimacki *et al.* (1979)
	Peanuts	India	0.63-38.8	Bhavanishankar & Shantha (1987)
	Rice	Egypt		Abdel-Hafez *et al*. (1987)
	Sorghum	India	1.67-15.0	Bhavanishankar & Shantha (1987)
	Wheat	India	2.0-4.0	Bhat *et al*. (1989)
DON	Barley	Japan Korea Norway	t-40.4 0.004-0.5 0.006-2.1	Kamimura *et al.* (1981) Lee *et al*. (1985) Sundheim *et al.* (1988)

		U.K.	0.02-0.36	Gilbert *et al.* (1983)
	Corn	Austria	1.0-20.0	Lew *et al.* (1979)
		Canada	0.15-0.82	Scott *et al.* (1981)
		China	0.36-12.7	Qiujie *et al.* (1988)
		New Zealand	0.02-0.3	Hussein *et al.* (1989)
		Transkei	t-15.8	Theil *et al.* (1982)
		U.K.	0.1-0.3	Gilbert *et al.* (1984)
		USA	0.5-10.7	Vesonder *et al.* (1978)
	Oats	Germany	20	Bauer *et al.* (1980)
		U.K.	0.02-0.1	Gilbert *et al.* (1984)
	Rye	Korea	0.003	Lee *et al.* (1985)
	Wheat	Canada	0.01-4.3	Scott *et al.* (1981)
	Krogh	Norway	0.008-3.19	Sundheim *et al.* (1988)
		USA	0.2-9.0	Eppley *et al.* (1984)
		U.K.	0.02-0.5	Gilbert *et al.* (1984)
		Germany	t-4.7	Bauer *et al.* (1980)
NIV	Barley	Norway	0.013-1.56	Sundheim *et al.* (1988)

	Corn	China Transkei	0.054-2.67 t-1.41	Qiujie *et al.* (1988) Theil *et al.* (1982)
	Rye	Canada	0.046-0.114	Lee *et al*. (1985)
	Krogh	Germany Norway	t-7.8 0.015-0.887	Bauer *et al.* (1980) Sundheim *et al.* (1988)

2.6 Toxin production:

Mycotoxins can be carried out on grain (or any other product) at any stage of production, harvesting, transport, processing or utilization.

Mycotoxin production by a given fungus has been shown to be dependent on the following condition:-

* The actual presence of toxigenic fungus.
* A suitable substrate for the growth of fungus.
* An environment suitable for fungal growth.

Toxin production will only occur when all three conditions can be fulfilled.

Each condition will involve many inter-relating factors which together or individually can affect mycotoxin formation (Hesseltine, 1976).

The toxigenicity of the fungi differs between species, and also between the same species with different strains.

Nutrients concentration affects toxin production; carbohydrates like glucose, sucrose & galactose give high yield of crude toxin than starch, lactose, maltose, mannitol, sarbitol, xylose, & fructose. Nitrogen sources affect toxin production e.g. peptone or yeast extract with Czapek's greatly increased toxin production, while Czapek's alone or with malt reduced toxin production.

Of various metal sulfates, the Yields of crude toxin obtained with CuSO4 exceeded that of the manganese, zinc, or iron salts (Ueno *et al*., 1970), While metal ions such as MnSO4, (NH4)MoPO4, Fe2SO4, MgSO4 did not significantly increase toxin production (Ueno *et al*., 1975).

Type of cereals used also affects toxin production; white corn give highest yield of T-2 toxins as compared with wheat & rice (Burmeister, 1971). The production of trichothecenes in vivo & in vitro favoring specific condition of plant substrates, temperature, & light.

Trichothecenes are predominantly formed on over mature, senescent plant tissue: such toxin formation may then take place in field, e.g. on overwinterd grains or more commonly in the store, such as moldy corn or hay (Joffe, 1960).

Researchers (Bamburg & Strong, 1971) & (Burmeister, 1971) have reported that the production of T-2 toxin in liquid media(200ml) was optimum when incubated at 8° C. after 3 weeks

of incubation on white corn grains, T-2 was not detected at 32 ◌̊ C, while at 25,20,& 5◌̊ C were 0.67, 5.4, & 9.9 gr., respectively.

Culture on 200ml. liquid media produced 115mg. toxin at 27 ◌̊ C after 14 days compared with only 83-77 mg. at 20 or 10◌̊ C after 17 & 35 days, respectively. When grown on polished rice grain, the amount of crude toxin formed was only 0.04g. at 10◌̊ C, and 0.13g. at 20◌̊ C, but 0.38g. at 27◌̊ C. The effect of light was studied by Joffe (1974); when fungi grown on solid and liquid media in dark and light, toxicity derived from the liquid substrate was higher than that from the solid media.

Also toxicity produced in dark was higher than in light. Further studies with liquid media at 3 levels of pH carried out at 8 & 25◌̊ C, the results was highest toxin production at pH 5.6, mostly lower at pH 3.8 than at 7.2 (Joffe, 1974).

2.7 Extraction of Trichothecenes:

Solid phase extraction (SPE) method can be used for all trichothecene types, the basic principle of this method can be considered a simple chromatographic process with the sorbent being the stationary phase. It is suitable for the analysis of aqueous samples and can be performed on – or off – line. With liquid sample extraction and preconstruction can be performed at the same time.

Typical extraction method for trichothecenes is performed by shaking or blending with extraction solvents such as chloroform,

ethyl acetate, methanol, acetonitrile, aqueous methanol, and aqueous acetonitrile. Chloroform, ethyl acetate, and acetonitrile have been successfully used for the extraction of T-2 toxin, DAS, and some of their partially hydrolyzed derivatives in naturally contaminated cereals. Aqueous methanol and aqueous acetonitrile are the solvents of choice for the extraction of several trichothecenes of widely differing polarity as well as for the extraction of type B toxins alone (Trenholm *et al*.,1985).

Methods differ according to the type of solvent used, whether samples are homogenized in a blender with the solvent or agitated with a wrist action shaker, and in the length of time of the extraction process.

Spiking of samples with standards is not an adequate way of demonstrating the efficiency of extraction, and only methods validated with naturally contaminated material can be regarded as having been vigorously tested.

Extraction procedures have been assessed for DON (Trenholm *et al*.,1985) and it has been demonstrated that longer extraction times are required for naturally contaminated samples than for those that have been spiked (at least 120 min shaking). It has also been shown that aqueous acetonitrile gives a cleaner extract than aqueous methanol.

2.8 Toxic Effects of trichothecenes on animals:

2.8.1 Field observations

There are so many diseases reported after ingestion of moldy feed, as example in Hungary and other central European countries pyosepticamia has been reported in horses: This disease was characterized by hemorrhages in the intestine and muscles, severe diarrhea, and death (Forgacs, 1965; Danko & Szerafin, 1976).

An outbreak of a disease, observed in poultry (ducks, geese), horses, and pigs, was associated with mouldy barley containing T-2 toxin at approximately 25 mg/kg. Pigs fed the suspect barley exhibited signs of feed refusal, vomiting, and diarrhea. The horses became depressed and salivated excessively (Greenway & Puls, 1976).

The lesions in the geese included necrosis of the mucosa of the oesophagus, proventriculus, and gizzard. No pathological lesions were described in other animals. DON was isolated from a batch of maize that had caused vomiting in pigs (Vesonder *et al*., 1973).

2.8.2 Effects on experimental animals:

LD_{50} values for certain trichothecenes in several experimental animal species are summarized in Table 2(Ueno *et al*., 1983) and (Rye *et al.,* 1988).

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Table (2): LD_{50} values (mg/kg) of trichothecenes:

Types of the Trichothecenes	Mouse				Rat			
	I.V.	I.P.	S.C.	Oral	I.V.	I.P.	S.C.	Oral
T-2 toxin		**5.2**		**10.5**				**2.5**
HT-2 toxin		**9.0**						
Diacetoxyscirpenol (DAS)	**12.0**	**23.0**			**1.3**	**0.75**		**7.3**
Nivalenol (NIV)	**7.3**	**7.4**	**7.2**	**38.9**				**4.4**
Fusarenon-X	**3.4**	**3.4**	**4.2**	**4.5**				
Deoxynivalenol (DON)		**70.0**		**46.0**				
Trichothecin	**300**						**250**	

Studies on acute toxicity showed no marked differences observed between treated male and female animals. Newborn animals are more sensitive than adults to the toxic effects of the trichothecenes (Ueno *et al*., 1973).

The administration of trichothecenes to some animals (rats, mice, and guinea-pigs) induces diarrhea, the mycotoxin increased the absorption rate of D-xylose from the intestine *in vitro*, but the sodium level in the serum decreased(Matsuoka & Kubota, 1981).

Vomiting was one of the most significant signs of trichothecene-induced toxicosis in the cat, dog, pig, and duckling (Ueno, 1980).

For some experimental animals, T-2 toxin and related trichothecene mycotoxins at doses of 0.1-10 mg/kg induced

vomiting (Vesonder *et al*., 1973; Sato *et al*., 1975; Yoshizawa & Morooka, 1977; Ueno, 1980; Matsuoka & Kubota, 1981).

Studies on trichothecenes absorption and tissue distribution for different animals; different types of trichothecenes and different doses resulted in: pig treated with HT-2 toxin, after 18h, radioactivity showed: muscle, liver, kidney, and bile contain residue of toxin, while with T-2 toxin treatment, radioactivity of the tissue showed as follows: muscle, liver, and kidney (Robison *et al*.,1979); for guinea-pigs given T-2 toxin, after 12-24 h, radioactivity of the tissue showed that large intestine and bile, also in muscle and liver (Pace *et al.*,1985); cows administrated with HT-2 toxin orally, after 3 days, radioactivity of the tissue results were as following: bile, liver, and kidney had higher level than blood, plasma, spleen, mammary gland, ovaries, and muscles.

The cardiovascular effects of the trichothecenes have varied according to some factors such as species, dose, and duration of exposure.

Histological lesions of the animal's tissues were degeneration, necrosis and hemorrhage of the brain, thymus, spleen, intestine, lungs, heart, lymph nodes, liver, and kidneys (Croft *et al*., 1986).

Trichothecenes (T-2 toxin and Fusarinon X) used in long-term of treatment in mice, lesions were observed in the oesophageal region of the stomach of mice, the alterations included hyperplasia, hyperkeratosis, and acanthosis of the squamous epithelium. Such

changes were found 13 weeks after the start of feeding the toxins, similar gastric lesions were observed in rats fed T-2 toxin (Ohtsubo & Saito, 1977).

After 24 h of exposure to T-2 toxin, tissue of the treated mice shows lymphoid necrosis in the thymus, spleen, and lymphoid nodules of the intestinal tract, in addition to necrosis of intestinal crypt epithelial cells and necrosis of adrenal cortical cells.

In acute studies, T-2 toxin given intravenously to pigs at 1 mg/kg body weight produced a decline in blood pressure several hours after administration, the reduced blood pressure being accompanied by a decrease in heart rate (Smalley *et al*., 1970).

For poultry Chi *et al*. (1977), reported that the single oral LD_{50} dose of T-2 toxin for one-day-old broiler chicks was 5 mg/kg body weight. It was 5 and 6.3 mg/kg body weight for 8-week-old broiler chicks and laying hens, respectively.

Death of the birds occurred within 48 h of T-2 toxin administration. Within 4 h of receiving the toxin, birds developed asthenia, in appetence, diarrhea, and panting.

In a study by Wyatt *et al*. (1972), also poultry developed yellow-white lesions in the mouthparts at all dietary concentrations.

The lesions consisted of a fibrinous surface layer and a heavy infiltration of the underlying tissues by granular leukocytes. The lesions induced by fusarenon-X and NIV were similar to those

induced by T-2 toxin, but the toxins were less potent (Terao *et al*., 1978).

T-2 toxin administration reduced egg production and resulted in the production of a thinner egg shell (Wyatt *et al*., 1975). Speers et al. (1977), also observed cessation of egg production in hens fed diets containing T-2 toxin.

Coffin & Combs, (1981) observed that Dose-dependent depression of plasma-vitamin E activity and hepatic-vitamin A content were noticed.

In a study by Pier *et al*. (1976), calves received T-2 toxin orally in capsules; the high-dose calf developed a hunched stance and died on day 20, at all levels, some evidence of mild enteritis with loose faeces was obtained. Clinical signs were developed such as: bloody faeces, abomasal ulcers, ruminal ulcers, and prothrombin times and levels of serum GOT activity were increased.

Experimental lambs treated with T-2 toxin, developed focal hyperaemia and dermatitis at the mucocutaneous junction of the commissure of the lips, diarrhoea, leukopenia, lymphopenia and lymphoid depletion of the mesenteric lymph nodes and spleen (Friend *et al*., 1983).

2.8.3 Other effects:

Other pathological effect of trichothecenes can be summarized as:

*Different disturbances of central nerve system were observed on animal treated with trichothecenes, especially T-2 toxin and DON (Fitzpatrick *et al*., 1988).

*Dermal toxicity and skin necrosis have been observed by working on T-2 toxin, HT-2 toxin and DAS which were extremely potent irritants while NIV and Fusarinon-X were much less potent (Bhavanishankar *et. al*., 1988).

*Immunosuppressant observed on trichothecenes treated animals, certain trichothecenes, such as T-2 toxin and DON, have an immunosuppressive action in animals and have produced alterations in both cell-mediated and humoral immunity. Trichothecenes also induse: inhibition in antibody and inhibits allograft rejection (Rosenstein *et al.* 1979); depression in the delayed hypersensitivity response (Pestka *et al*., 1987); inhibition or suppress blastogenic response to lectins (Tomar *et al.* 1988); inhibition of the resistance to infection (increase the incidence and severity of infection in animals) (Kanai & Kondo, 1984).

*other treatment – related finding were an increased prevalent of epithelial cell hyperplasia and hyperkeratosis in the stomach of animals fed the T-2 toxin diet, this result indicated that some of the trichothecenes have carcinogenic effect (Schiefer *et al*., 1987).

2.9 Toxic effect of trichothecenes on human:-

In the period 1931-47, a human disease known as alimentary toxic aleukia (ATA) occurred in the USSR that was suggested to be related to the presence of toxic *Fusarium* species in mouldy over-wintered grain.

Two outbreaks of trichothecene-related disease have been reported, during the first incident in China in 1984/85, outbreaks of mouldy corn and scabby wheat poisoning was reported.

Out of approximately 600 persons who consumed mouldy cereals, there were 463 cases of poisoning (77% of the total).

The latency period for the onset of symptoms was 5-30 min. These included nausea, vomiting, abdominal pain, diarrhoea, dizziness, and headache. No deaths occurred. DON was detected within a range of 0.34-92.8 mg/kg and T-2 toxin and NIV were not found. (Luo, 1988).

An analogous outbreak was reported in Kashmir, India, in 1987 (Bhat *et al.*, 1987, 1989). It was ascribed to the consumption of bread made from flour that had become mouldy in storage following unseasonal rains in the wheat-harvesting season, on which *Fusarium* sp. was grown, and which was found to contain mycotoxins.

Of the 224 persons investigated on a random sample basis in a percentage, 97 were affected with symptoms including abdominal pain (100%), throat irritation (63%), diarrhea (39%), blood in stools

(5%), and vomiting (7%). Symptoms developed 15 min to one hour after consumption of locally baked bread, the following mycotoxins were found: DON (0.35-8.38 mg/kg), Ac-DON (0.64-2.49 mg/kg) (no details of estimation of this derivative were available), NIV (0.03-0.1 mg/kg) and T-2 toxin (0.55-0.8 mg/kg) (Bhat *et al*., 1987).

The clinical symptoms reported in ATA, as well as the identified occurrence of *Fusarium* in foodstuffs, suggest that it might have been associated with mycotoxins, identified years later in fungal cultures of *Fusarium* species under laboratory conditions, such as T-2 toxin (Mirocha & Pathre 1973) or wortmannin (Mirocha & Abbas 1989).

An association was established with the ingestion of grain invaded by some moulds, in particular *Fusarium poae* and *F. sporotrichioides.*

The dominant pathological changes were necrotic lesions of the oral cavity, the oesophagus, and stomach and, in particular, a pronounced leukopenia. The primary lesion was bone marrow hypoplasia and aplasia. The disease was lethal in a high proportion of cases.

When compared with the symptoms observed in experimental animals, features of both the above human diseases were similar to trichothecene toxicosis, notably symptoms caused by DON and NIV, DAS, and T-2 toxin. However, no

epidemiological studies have been reported that link ATA and scabby grain toxicosis to these chemicals.

Morphological changes following T-2 toxin administration were studied on platelets isolated from 12 healthy human volunteers. When platelets were incubated with T-2 toxin at doses of 5-500 µg/10^9 platelets for 20 min, there was a dose-related inhibition of platelet aggregation with different activators, including epinephrine, arachidonic acid, and collagen, and a release of dense bodies consisting mainly of serotonin-containing granules.

There was also a change in membrane permeability, notably suppressed aggregation, played a contradictory role in the hemorrhagic phenomena associated with these toxins in man and animals (Yarom *et al*., 1984).

There is no evidence of immunosuppressive action in man (Tomar *et al*., 1988; Kanai& Kondo, 1984).

Reported cases of human disease associated with trichothecene exposure are limited in number and information.

Symptoms of digestive disorders and throat irritation develop rapidly after ingestion of food contaminated with trichothecenes. Some scientists pointed that there is no evidence of human cancer caused by trichothecenes (W.H.O., 1990).

Chapter Three

Material & Methods

3.1 Materials

3.1.1 Equipments and Apparatus:

The following equipment and apparatus were used in this study:

Apparatus	Company
Autoclave	Gallenkamp (England)
Centrifuge	Gallenkamp (England)
Electric balance	Melter (Switzerland)
Electric oven	Gallenkamp (England)
Heamocytometer chamber	Assistant (Germany)
Incubater	Gallenkamp (England)
Light microscope	Olympus (Japan)
Micropipettes	Gelson (France)
Microtome	Gallenkamp (England)
pH-Meter	Orient research (USA)
Rotary Evaporator	Bunchi (Germany)
Scanning densitometer	Gamagii (Sweden)
Shaking incubator	Gallenkamp (England)
U. V. Light lamp	UVP (USA)
Vortex Mixer	Bunchi (Germany)

3.1.2 Chemical Materials:

The following Chemical Materials were used in this study and its company:

Material	Company
Absolute ethanol	BDH
Acetone	BDH
Acetonitrile	BDH
Agare	Oxoid
Amonium alum	BDH
Anhydrous Sodium Sulphate	BDH
Benzene	BDH
Canada Balsam	BDH
Cephalexin	S. D. I.
Chloroform	BDH
Cotton blue stain	BDH
Dextrose	Fluka
Eosin stain	BDH
Glacial acetic acid	BDH
Glycerol	Sigma chemical company
GPT kit	Bio Merieux
Haematoxylin stain	BDH
Hydrochloric acid	BDH

Lactic acid	BDH
Mayer's albumin	BDH
Mercuric oxide (red)	BDH
Paraffin wax	BDH
Phenol crystals	Fluka
Potato dextrose agar	Oxoid
Standard toxin (T-2 toxin)	Sigma chemical company
Sodium hydroxide	BDH
Sodium hypochlorite	Niktar
Sucrose	Fluka
Tween 80	Oxoid
Urea kit	Bio Merieux
Xylene	BDH

3.2. Methodes:

3.2.1. Sterilization:

A- Autoclaving: - media and other solutions are sterilized by Autoclaving under 15P/in² at 121ºC for 15 min.

B- Dry heat sterilization: - all glass apparatus are sterilized by electrical oven at 180ºC for 2 hours.

3.2.2 Preparation of solvents, media and stains:

A- Formalin 10%: prepared by mixing 10 ml of concentrated formalin to 90 ml of D.W. (Mantle *et. al.*, 1991).

B- Sodium hypochlorite solution 2%: 2 ml sodium hypochlorite was added to 100 ml D.W.

C- Antibiotic preparation: 500 ml sterilized D.W with 500 mg cephalexin, 20 ml of antibiotic for each 1L of media (Baron & Finegold, 1994).

D- PSA media (Potato Sucrose Agar):

* 500 ml Potato extract
* 15 gr. sucrose
* 20 gr. Agar
* 500 ml D.W.

Potato extract preparation: - 400 g. of mature main crop potatoes peeled and diced and suspended in 1000 ml. D.W. and boiled for 10 min., the potatoes are then discarded with slight squeeze and the liquer placed in large glass container and autoclaved at 15 P/in^2 and 121°C for 15 min., then store in refrigerator until use.

The D.W. and the potato extract have been mixed then agar and sucrose added with slow heating until the agar is dissolved; the pH is adjusted to 6.5 then the mixture autoclaved at 15P/in² and 121°C for 15 min. (Booth, 1977).

E- PDA media (Potato Dextrose Agar): -

* 500 ml Potato extract
* 15 gr. Dextrose
* 20 gr. Agar
* 500 ml D.W.

The same ways used in PSA preparation. (Booth, 1977).

F- Harris Haematoxylin stain perpetrate: -

(According to Bancroft and Steven, 1982)

* 5g Haematoxylin stain powder
* 50 ml ethanol 95%
* 100 g ammonium alum
* 2.5 g mercuric oxide (red)
* 3 ml Glacial acetic acid
* 1 L D.W.

Haematoxylin stain powder was dissolved in ethanol (95%), ammonium alum was dissolved in D.W and heat, and then both solvents were mixed and heated to boil, the solvents then

cooled and mercuric oxide (red) have been added and then reheated till the mixture gets deep violate color. The solvent cooled and filtered with 0.2 micrometer filter unite.

Before it had been used, 3 ml of Glatial acetic acid was added to each 100 ml of the prepared solvents.

G- Eosin stain: -

(According to Bancroft and Stevens, 1982)

* 1g eosin stain powder
* 99 ml ethanol 70%
* 1 ml Glacial acetic acid.

The Eosin stain powder dissolved with ethanol before used, 1 ml of Glacial acetic acid was added to each 100 ml of prepared solvents.

H- Lactophenol cotton blue stain:-

(According to McGinnis, 1980)

* 20 ml phenol crystals
* 20 ml lactic acid
* 40 ml Glycerol
* 0.05 gr cotton blue stain
* 20 ml D.W

The cotton blue stain dissolved in D.W; phenol, lactic acid and glycerol then added and mixed. Stored in 250 ml screw cap bottle.

3.2.3 Isolation of *Fusarium oxysporum*:

Fusarium has been isolated according to (Mislivec *et.al*.1975) about 30 randomly selected corn seeds (from national markets) were surface sterilized by washing with 2% sodium hypochlorite solution for 2 min and then washed with D.W for 1 min and dried by sterilized filter paper.

Then seeds were cultured on PDA, 2 seeds for each plate then incubated at 25ºC for 7 days then the grown *Fusarium* (Which was morphologically identified) purified by new subculturing (each one in single plate of PSA) this purified isolates were used for identification.

3.2.4 Identification of *Fusarium oxysporum*:

Pure isolates were identified by direct examination with light microscope on glass slide and according to taxonomic system of Snyder & Hansen (Toussoun & Nelson, 1968) & (Booth, 1977).

Slide preparation: sample has been taken from the surface of agar which fungi grown on and put on a slide contain a drop of the

lactophenol cotton blue and covered with cover slip then fixed by pass over aflame to fix the stain.

3.2.5 Spore suspension preparation:

The isolated *F. oxysporum* was used. Slants containing potato dextrose agar (PDA) medium inoculated with *F. oxysporum* were incubated at 25°C. Spore suspension were prepared according to Faraj method (Faraj, 1990), spores were harvested by adding 5 ml of sterilized distilled water containing 0.11% tween 80 to aid wetting and separation of the spores, then the fungal growth was separated by a loop.

The suspension was filtered through sterile cotton wool; the filtrate was centrifuged, and further washes with distilled water. The spore suspension was then centrifuged at 3000 r.p.m for 5 minutes. The supernatant was removed and the spores were washed twice by resuspending in sterile distilled water and further centrifuged. Then 5ml of sterile distilled water was added to the supernatant and mixed vigorously by the vortex for 1 minute. Pasteur added one drop of the suspension to hemocytometer by Pasteur pipette, spores were calculated under large power X 40 of light microscope using the following equation:

Concentration of spores = $(Z \times 4 \times 10^6)/n$ spores/ml

Where n: total No. of small squares.

Z: total No. of spores.

3.2.6 Toxin production:

According to Joffe, (1965), toxin produced as following:

1- Potato dextrose broth prepared and autoclaved.
2- Antibiotic suspension add 2 ml for each 100 ml of broth.
3- Each flask was inoculated with 1 ml of spore suspension (8.1×10^6 spore\ml).
4- Flasks were incubated at 25ºC for 1 week.
5- After that flasks were incubated at 27 ºC for 1 week.
6- And then at 8 ºC for 1 week

Note: the flasks were shaken very gently every couple days.

3.2.7 Extraction of trichothecenes:

According to Ghosal *et al*., (1978), toxin was extracted as following:

1- 250 ml of chloroform was added to each 100 ml of broth and then homogenized.
2- Then it was left for 24 hours in shaker at 200 R/m at 25ºC.
3- Filtered throw a cotton wool.
4- The mixture was placed in 500 ml separation funnel, then it was shaking for 30 sec and then it was left for separation.
5- The chloroform layer (lower layer) was taken and then filtered throw filter paper contains anhydrous sodium sulphate (repeated twice).
6- Evaporated in rotary evaporator to dryness (at 45 ºC), then the toxin was kept in small vials in dry and cold place.

3.2.8 Detection and Identification of trichothecenes:-

1. Developing system:

The developing system used for separation of trichothecenes was benzene: acetone in 3:2 ratios (Paster *et.al*., 1986).

2. Preparation of standard toxin:

Crystals of standard T-2 toxin (1mg) was dissolved in 100 ml of benzene-actonitrile solution in ratio (98:2) respectively, 1 ml of this solution was taken and diluted into 10 ml of benzene-acetonitrile (98:2). The concentration of the stock solution will be 1µg/ml. it was put in a dark vial, which sealed by parafilm and aluminum foil paper to keep it away from the light then kept in freezer.

3. Identification of trichothecenes by TLC:

TLC plates (200 x 200 x 0.25 mm) were activated in the oven at 110 ºC for one hour, a line was scored on TLC plates with 1.5 cm from each side and 2cm from top and bottom of the plates, the extract containing the crude trichothecenes was dissolved in 100 ml of benzene/acetonitrile (98:2). Samples were spotted at size of 0.1 ml and the standard of T-2 was spotted at concentration 1µg/ml, then the plate was left to air dry, and placed in a chamber containing developing solvent in exactly vertical position and in dark place. The plate was removed from the chamber, when the developing solvent reached 18 cm then allowed to dry in dark hood, and examined under U.V. light at 366-wave length with determination R**f** value, the amount of trichothecenes was

estimated in comparison with standard toxin using the scanning densitometer.

4. Confirmatory test:

Gimeno (1983) method used for the confirmatory test of trichothecenes:

The plate was sprayed with aluminum chloride solution ($AlCl_3.6H_2O$) which prepared by dissolving 20 ml of aluminum chloride in 100 ml methanol. Then the plate was placed in the oven at 115 ºC, cooled and examined under U.V. light at 366-wave length, the R**f** value was estimated in comparison with standard toxin.

3.2.9 Laboratory animals and histopathological study:

Mature Swiss mice, males and weighted (20.9-22.4) gr. were used in this study, animals were isolated in a relatively controlled environment at a temperature of about 25 ºC, in the (animal house) of Biotechnology researches center of Nahrain University. Those mice were divided into 4 groups (6 mice for each group) as following:-

Group I

Mice were treated daily (IP) with 0.1 ml of 10% chloroform for 35 days (5 weeks) and considered as control for low dose group.

Group II

Mice were treated daily (IP) with 0.1 ml of toxin (0.75mg/ml) for 35 days (5 weeks) and considered as low dose group.

Group III

Mice were treated daily (IP) with 0.3 ml of 10% chloroform for 35 days (5 weeks) and considered as a control for high dose group.

Group IV

Mice were treated daily (IP) with 0.3 ml of toxin (0.75mg/ml) for 35 days (5 weeks) and considered as high dose group.

After treatment blood sample were taken by heart puncture also liver and kidney organs were taken and stored in 10% formalin.

3.2.10 Biochemical study for liver and kidney function:

1-Kidney function (blood urea test):

By using urea-kits enables and point enzymatic determination of urea concentration (urease-medefide berthelot reaction) in urine, serum or plasma (Tietz, 1995).

$$\text{Uren} + H_2O \xrightarrow{\text{Urease}} 2\,NH3 + CO_2$$

In an alkaline media, the ammonium ions react with salicylate and hypochlorite to form green colored indophenols (2, 2- dicarboxylindophenol).

The reaction was catalyzed by sodium nitroprusside

$$NH_4 + \text{salicylate} + \text{hypochlorite} \longrightarrow \text{indophenols}$$

The color intensity is proportional to the urea concentration in sample.

	Reagent black	Standard	sample
Standard	-	10µL	-
Sample	-	-	10µL
Working solution	1 ml	1 ml	1 ml

Mixed and incubated for 5 min at 37ºC. Then alkaline solution 200 µL was added for each tube, mixed and incubated for 10 min at 37ºC, Then read in spectrophotometer at 580 nm against reagent blank, then calculated as the following:

$$\text{Sample concentration} = (A_{\text{sample}} / A_{\text{standard}}) \times n$$

n =concentration of standard.

2- Liver function (GOT& GPT test):

The glutamic transaminase enzymes, serum glutamic oxaloacetic transaminase (GOT) and serum glutamic pyruvic transaminase (GPT), catalyze the transfer of the amino group of glutamic acid to oxaloacetic acid and pyruvic acid in reversible reaction (Tietz, 1970).

$$\text{GOT: Asparatate} + \alpha\text{ Keto glutarate} \xleftrightarrow{\text{GOT}} \text{oxaloacetate} + \text{Glutamate}$$

$$\text{GPT: Alanine} + \alpha\text{ Keto glutarate} \xleftrightarrow{\text{GPT}} \text{pyruvate} + \text{Glutamate}$$

The transaminase activity is proportional to amount of oxaloacetate –pyruvate formed over a definite period of time and is

measured by a reaction with 2, 4 –dinitrophenyl hydrazine (DNPH) in an alkaline solution:-

1- 0.5 ml of substrate GPT was pre incubated for 5 min at 37ºC.

2-100 µL serum was added, then mixed and returned to W.B. at 37ºC for 30 min.

3- 0.5 ml 2, 4- DNPH was added, mixed and allow standing for 20 min at room temperature.

4- 5.0 ml of NaOH (0.4 N) mixed and let to stand for 5 min at room temperature.

5- After that the mixture was read at 505 nm against a water blank and according to calibration given with the kit the level of GPT and GOT could be known.

3.2.11 Histopathological study:

Samples (liver and kidney) were fixed in 10% formalin for 24 hr to be prepared for histopathological sectioning and according to Bancroft and Stevens, 1982: -

1. Samples were fixed in 10% formalin for 24 hr.
2. Placed in 70% ethanol over night.
3. Samples were dehydrated by 4 changes of (80%, 90%, 95%, 100%) ethanol for 2 hours for each concentration.
4. Placed in xylene for 2 hours to clear the Tissue.
5. Then embedded in melting paraffin (melting point of paraffin in 58 ºC) for 2-3 hours at 60-70 ºC in the oven.
6. Blocked in paraffin wax and sections were made by a microtome 4-5 µm in thickness.
7. Tissue sections were fixed on slides by using mayer's albumin and left to dry in oven at 37 ºC for 1-2 hrs.
8. Tissue section on slides was dewaxed by xylene.
9. Washed shortly in 3 changes of absolute alcohol. ,then with 95% alcohol and 70% alcohol.

10. Washed in water for 5 min.
11. Stained with haematoxylin for 5-10 minutes washed in water 5 min.
12. The slides were then placed in eosin for 10-15 minutes.
13. Washed in water for 2-3 min, acid alcohol for 1% dipping.
14. Sections were then dehydrated in (70,80%) alcohol, few seconds for each and 2 changes of absolute alcohol, then left to dry.
15. Put in xylene 15-30 min, and covered by cover slip with Canada balsam.

Chapter Four

Results & Discussion

4.1 Isolation and Identification of *F. Oxysporum*

Maize has been selected as a natural source for isolation of *F. oxysporum*, when maize put on surface of PDA media gave a good result for isolation of *F. oxysporum* (Booth, 1977).

The average growth rate of cultures was about 5 ± 0.5 cm, the fungus is represented by white mycelium on the surface of the dish, but with purple to brownish tinge (Fig 5). Microconidia born on simple phialides arising laterally on hyphae or from short branched conidiophores. Generally microconidia abundant, cylindrical, straight and 1-2 septate.

Macroconidia are born on more branched conidiophores, they were thin walled, 3-5 septate and pointed at both ends (Fig 6), this results obtained according to Toussoun & Nelson (1968) guide.

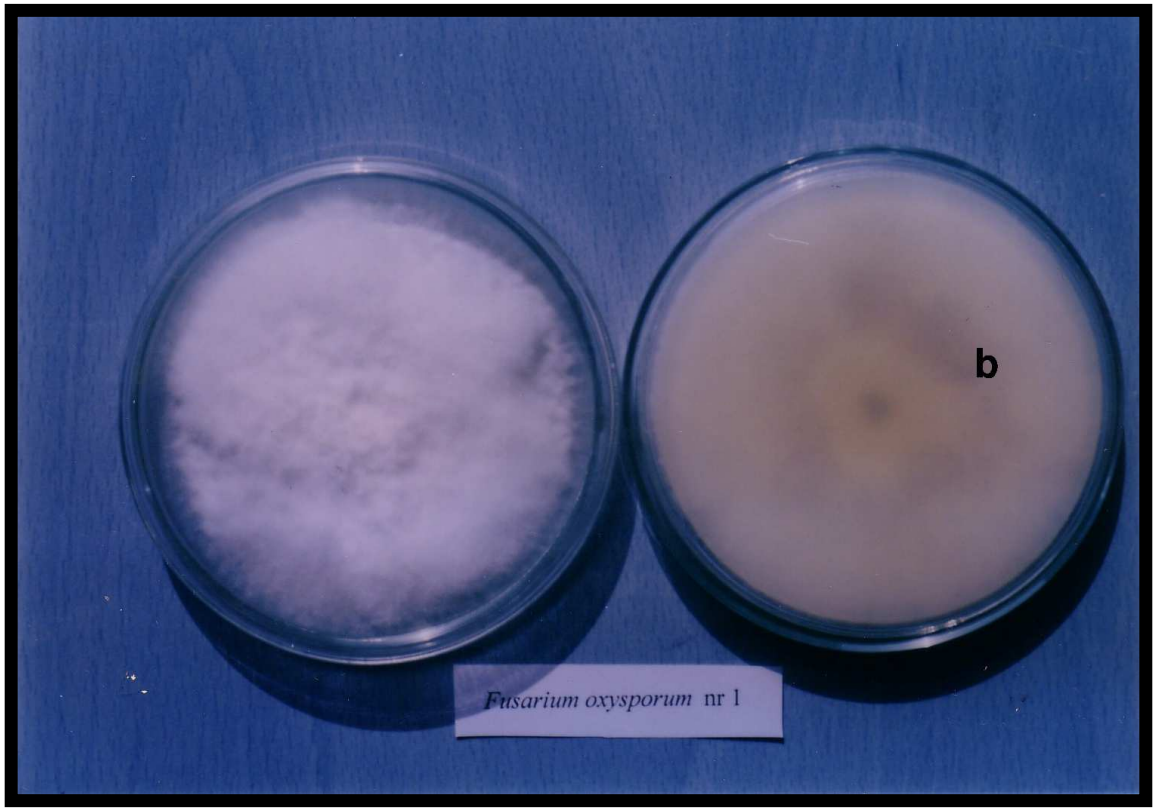

Fig.5 Culture of *F. oxysporum* on Potato dextrose agar plate.

A- *F. oxysporum* with white cotton like threads of mycelium grown on PDA (1week).

B- Inversed face of *F. oxysporum* grown on PDA with purple to brownish tinge (b).

Fig.6 Microscopic morphologi for macroconidia and microconidia of *F. oxysporum* .40X

4.2 Production and extraction of trichothecenes:-

The isolated fungi proved to be efficient producer for trichothecenes (750 ppb) in comparison with other isolates used for production of trichothecenes.

The efficiency of the fungal isolate is one of the factors that play an important role in the production of different mycotoxins (Smith *et al*., 1994).

According to our experimental work, the method used was a suitable method for recovery of the toxin from the culture, which is characterized by clear red to pink fluorescence of trichothecene derivatives under U.V. light (366 nm) with Rf value 0.2 on silica gel plate after developing the chromatogram (Betina, 1982). The solvent system is efficient in separation the components of fungal culture extract.

4.3 Toxicity of crude trichothecenes:

Marked increase in body weight was observed in treated mice when compared with the body weight of control animal, this increase sustended during the duration of treatment, these observations became slowly appeared in the last week, but in general they were dose dependent.

Body weight has increased for high dose treated mice from 21.8 ± 0.36g to 28.2 ± 0.85g, and for low dose treated animals from 22.4 ± 0.37g to 26.9 ± 0.31g and that increase is significant according to ANOVA (<o.o5) as shown in table (3).

Table(3):Body weight of mice groups:

	1 day	7 days	14 days	21 days	28 days	35 days
Group I	A 20.83 ± 0.41	A 21.46 ± 0.38	A 21.98 ± 0.35	A 22.63 ± 0.33	A 23.26 ± 0.3	A 23.95 ± 0.3
Group II	A 22.3 ± 0.3	B 23.18 ± 0.37	B 24.1 ± 0.4	B 25.15 ± 0.4	B 26.31 ± 0.37	B 27.21 ± 0.35
Group III	AB 21.25 ± 0.17	A 21.76 ± 0.19	A 22.43 ± 0.17	A 22.9 ± 0.13	A 23.56 ± 0.13	A 24.28 ± 0.12
Group IV	AB 21.85 ± 0.36	AB 22.62 ± 0.44	B 23.9 ± 0.5	B 25.9 ± 0.72	B 27.2 ± 0.73	B 28.21 ± 0.8

Group I: Control group for low dose treated animals (0.1 ml of 10% chloroform).

Group II: Low dose treated animals group (0.1 ml of crude trichothecenes).

Group III: Control group for high dose treated animals (0.3 ml of 10% chloroform).

Group IV: High dose treated animals group (0.3 ml of crude trichothecenes).

The increase in body weight of treated mice groups notably larger than in the control groups, this might be resulted from increase in water consumption and feed consumption (Munro *et al*., 1974), or might be attributed to enlargement of organs such as liver, kidney, spleen and pancreas due to mycotoxicosis (Gibson *et al*., 1989).

Ochratoxin A cause similar increase in body weight in rat fed diet contaminated with this toxin (Al-Naimi, 2001).

Other signs observed for mycotoxicosis were loss of hair, shivering, dyspnea, redness around the neck and bristling of hair. Although of the aggressive behavior of mice, loss of activity observed at the last two weeks of treatment. All signs mentioned have referred to the action of toxin resulted from mycotoxicosis, similar morphological changes were observed in chicks fed diet contaminated with citrinin (Al-Sammaraei, 1997) and in rat fed diet contaminated with aflatoxin B1 (Al-Taie, 2001) which is attributed to the mycotoxicosis.

Hemorrhage and clotting accumulation in the internal viscera are other signs of toxicosis, these signs resulted in giving abnormal dark red color for the internal viscera (Fig 7) in comparison with control (Fig 8).

Crude toxin cause exudation case for blood vesicles, oozing of blood lead to hemorrhage and oxidation of hemoglobin in the internal viscera (stomach and intestine), also formation of clots and their accumulation gave the abdomen abnormal dark red color as shown in (fig 7). This happened for most of the treated mice, but not all of them, this may be due to the difference in the animal behavior, built of body, and microanatomy between mice (Kuhn &Ghannoum, 2003).

Inflammatory cyst were observed on liver of treated mice (Fig 9) and those cysts occurred due to infection of liver or due to necrosis caused by toxicity (Cirilli, 1983 & Ryu *et al*., 1988).

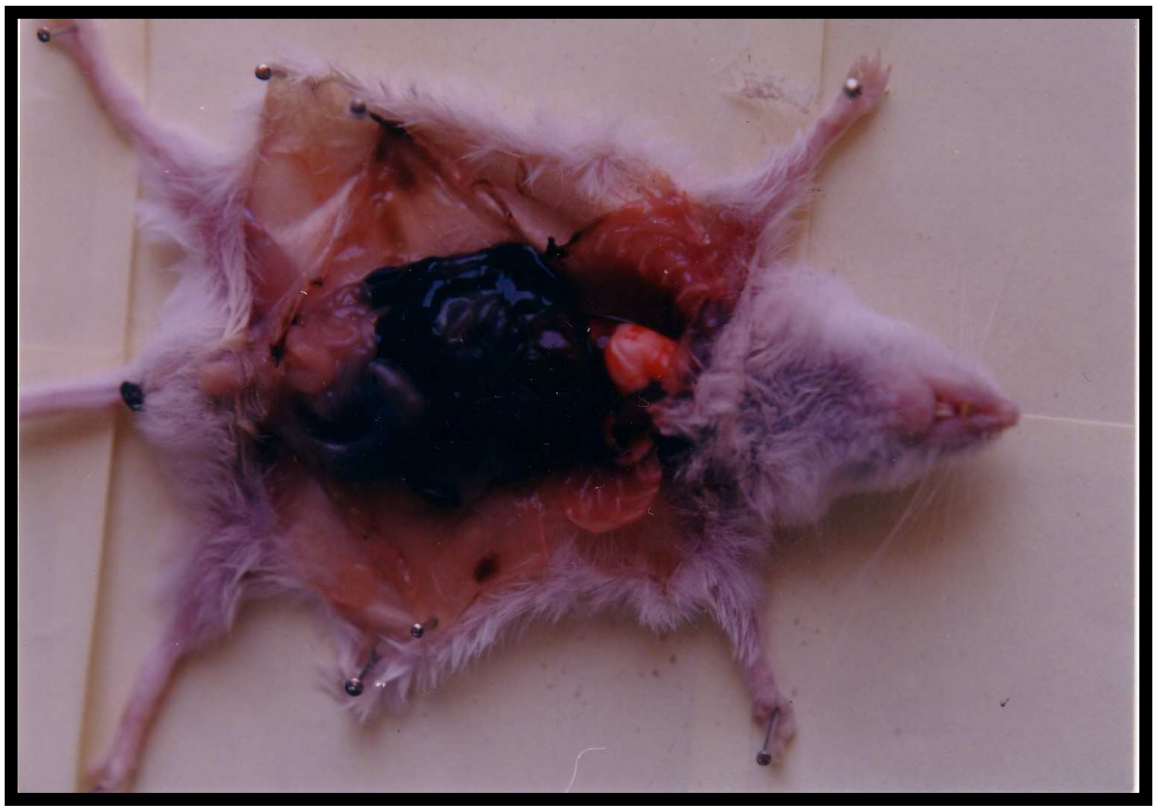

Fig.7 Mouse treated with high dose of crude trichothecenes shows hemorrhage and clot accumulation of the internal viscera (stomach and intestine).

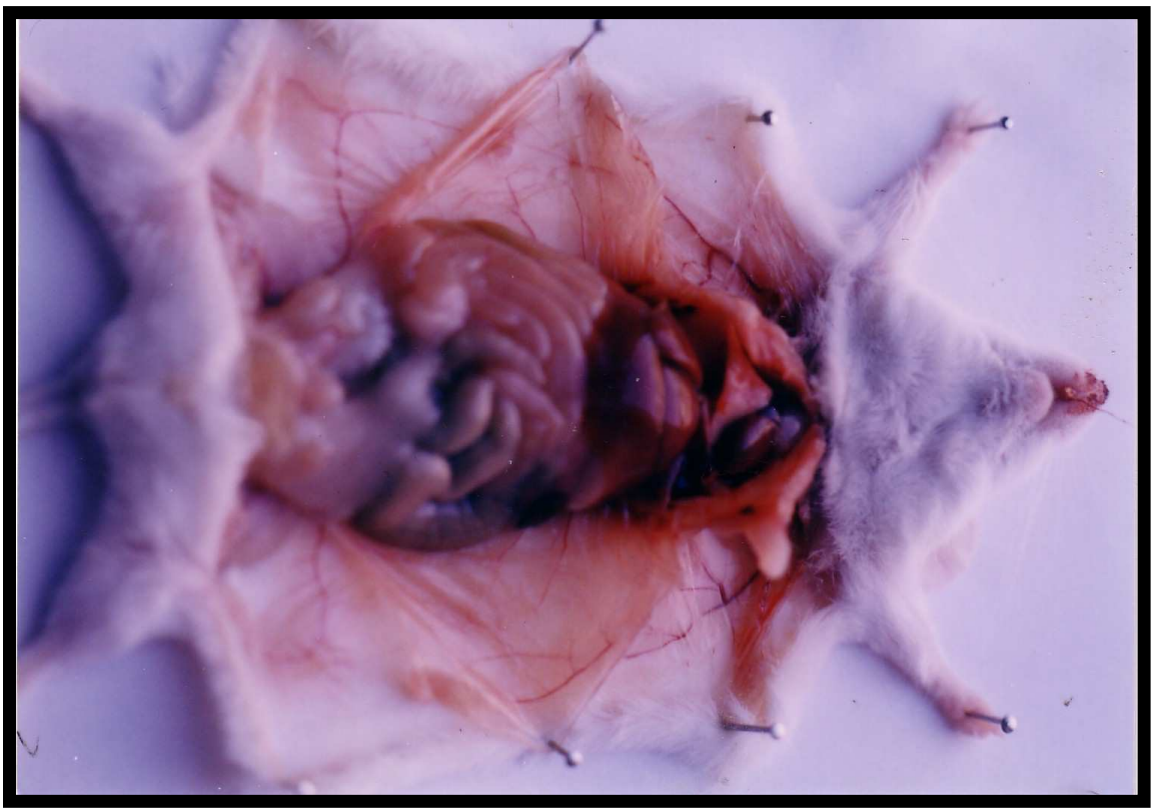

Fig.8 Mouse of the control group treated with high dose of chloroform shows normal internal viscera.

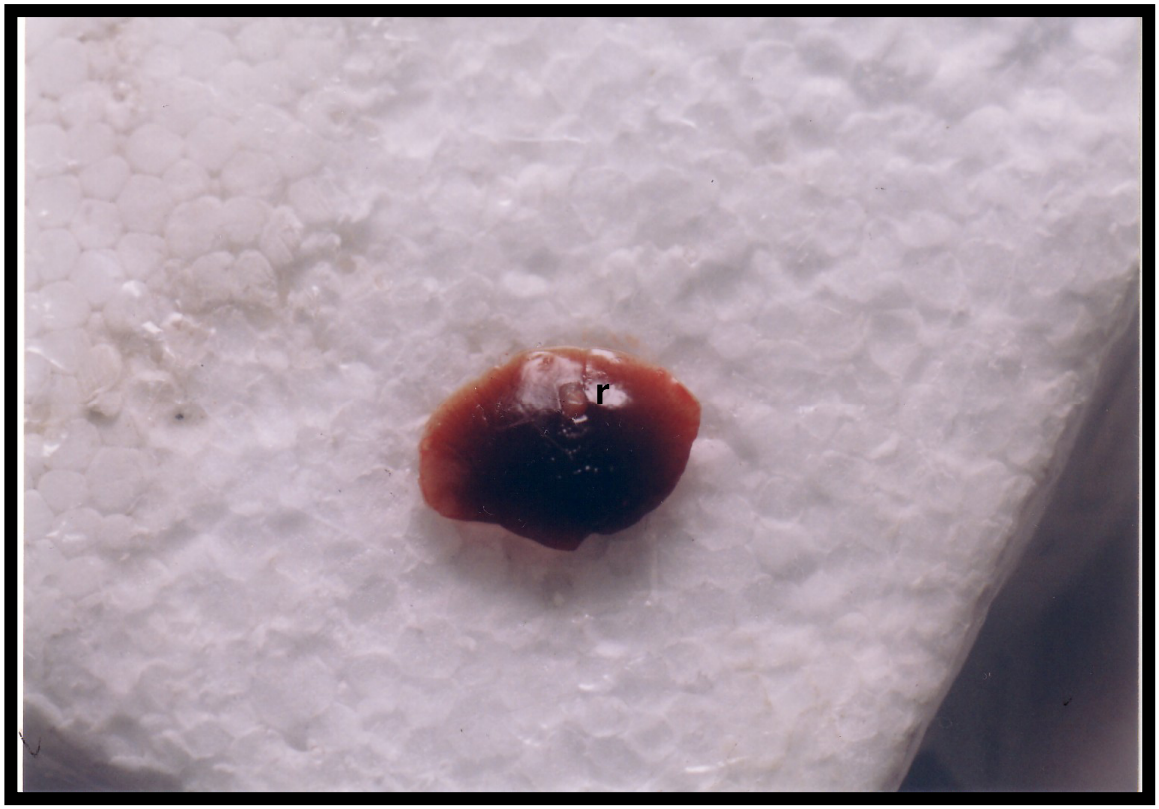

Fig.9 Liver of mouse treated with high dose of trichothecenes shows inflammatory cyst (r).

4.4 Biochemical study:

1. Blood Urea:

As shown in table 4, there is an increase in blood urea level in treated mice, this increase was related to dose concentration, and for that reason blood urea level (BUL) was more affected by the high dose than low dose of treatment in comparison with control group.

Changes in BUL seem to be results of kidney damage and renal disfunctioning obtained under the effect of mycotoxicosis.

Trichothecenes affect on kidney glomeruli and tubules which lead to increase in blood urea. Swamy *et al*., 2003 found the same effect on pigs fed grain contaminated with *F*. mycotoxins.

Table(4):Results of hematological testes:

	GPT IU/L	GOT IU/L	Urea mg/dl
Group I	A 110 ± 2.22	A 23 ± 1.76	A 44 ± 0.71
Group II	B 132 ± 6.14	B 35 ± 1.25	A 47 ± 1.08
Group III	C 195 ± 9.03	C 47 ± 1.58	B 57 ± 1.11

Group I : control animals

Group II : low dose treated animals

Group III : high dose treated animals

2. Transaminases (GOT & GPT):

Generally trichothecenes affect the liver function, this effect can be seen in the evaluation of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) enzymes level in blood of treated mice, the effect is dose dependent (see table 4). Treatment with the toxin induced hepatotoxicity by developing hepatic lesion which leads to this increase in enzymes level (Philips, 1991).

Aggab *et al*. (1997) demonstrated similar results by using fungal extracts (*C. hetronemum*) induced significant increase in GOT & GPT level resulted in treated rates causing them liver disfunctioning due to mycotoxicosis .

4.5 Histopathological study (Light microscope examination):

The cellular reaction towards any effect include cell division, damage and death of cells or enhancement of the immune system and mechanical movement to control toxic materials and stop their separation or finding other means or ways to do so.

In this study trichothecenes treated mice have suffered different kinds of damage, also in different severity according to the level of the dose been given.

For low dose treated mice, after 35 days of treatment, histopathological study pointed that kidneys have mild generative changes of glomeruli and tubules with congestion (Fig 10&11). While for high dose treatment there was moderate mononuclear cells infiltration, necrosis of tubules and slightly in glomeruli with degenerative changes in tubules (Fig 12,13 & 14).

The severity of the toxic effects on cells differs according to dose and time of exposure (Chagas *et al*. ,1994). The degenerative changes of tubules and glomeruli considered an irreversible change (a point of no return) which is a step before the cell death (Trump & Bulger, 1968).

The necrosis occurred strongly to tubules and slightly to glomeruli due to mycotoxicosis which lead to kidney damage and animal death (Robert & Mora, 1982).

Necrosis of kidney tubules and glomeruli could also be obtained by exposure to bacterial toxins (Akif ,2003).

The mononuclear cells infiltration include lymphocyte cells, macrophages and plasma cells, all were infiltrated through the vessels because of inflammatory reaction occurred because of the toxic material which act as chemo tactic factor which is responsible for attracting those cells toward the site of infection, this process called chemo taxis (Stevens & Howe, 2000).

Also congestion is another indicator of an inflammation along with inflammatory cells infiltration. This result agreed with Al-Samarraei (1997) in her study on citrinin, that mycotoxins cause inflammation which leads to mononuclear cells infiltration and congestion to kidneys.

Renal toxicity occurred in rats treated with *F. moniliforum* extract cause tubular necrosis (Voss *et al*., 1998).

The hepatic cells of animals appeared with congestion and kupffer cells hyperplasia for low dose treated mice (Fig 15) after 35 days of treatment with trichothecenes. Mild mononuclear cells infiltration in the portal area, around the bile duct was noticed in addition to congestion, sinusoidal dilation and kupffer cells hyperplasia (Fig 16, 17 and 18) upon treatment with high dose.

Death of liver cells may explain the dilatation of sinusoid (Newberne & Buttler, 1978).

The mononuclear cells infiltration around the portal area can be explained in that those cells represent a defense mechanism against any inflammation and/or any infection with some toxic materials affecting the hepatic cells (White *et al.*, 1973). Similar effects were observed in liver of rats treated with aflatoxin B1 (Al-Taie, 2001).

Kupffer cells hyperplasia means that increased numbers of macrophage cells appeared, this indicates that presence of an

inflammation in hepatic cells, the hyperplasia act as a way to limit the inflammation that occurred because of the mycotoxin. Mycotoxicosis with other mycotoxins such as ochratoxin A cause also kupffer cells hyperplasia in rat liver (Al-Naimi, 2001).

Hepatotoxicity occurred when fuminsins administrated to rats, cause kuffer cell hyperplasia and inflammatory infiltrate (Bondy *et al*., 1998).

So we can conclude from all the above results that trichothecenes like many other mycotoxins can affect significantly on liver and kidney of mice and cause to them several damages, organs disfunction, activation of the immune system and death of the animals, depending on the dose of the toxin and the time of exposure.

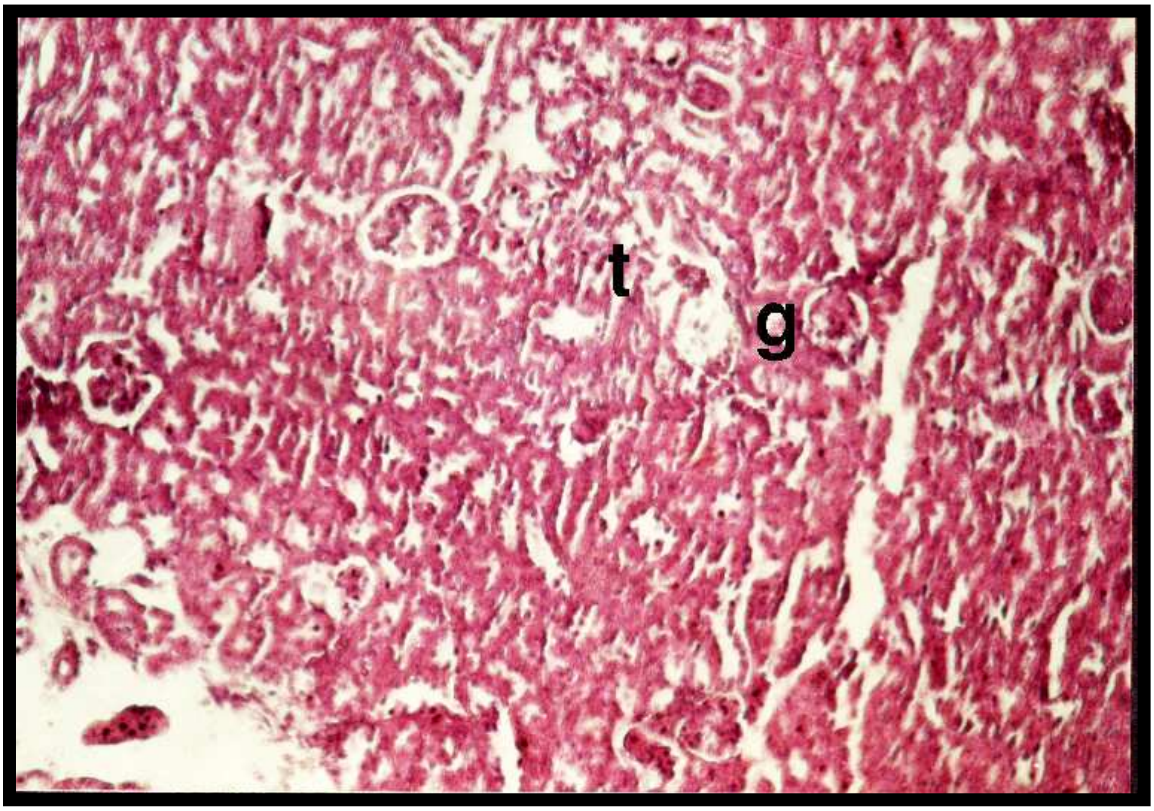

Fig.10 Kidney section of mice treated with low dose of mycotoxin showing mild degenerative changes of glomeruli (g) and tubules (t). Haematoxylin & Eosin (10X).

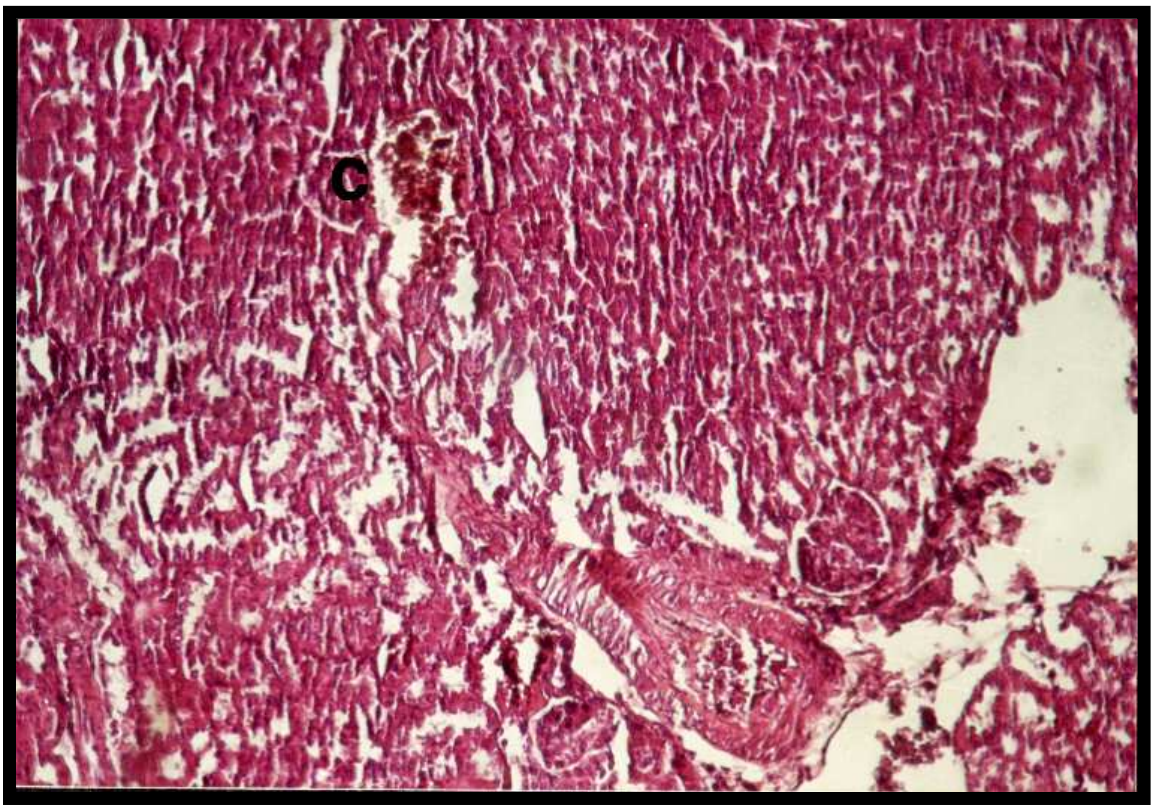

Fig.11 Kidney section of animal treated with low dose of toxin revealed congestion (c) and mild degenerative changes in tubules. Haematoxylin & Eosin (20X).

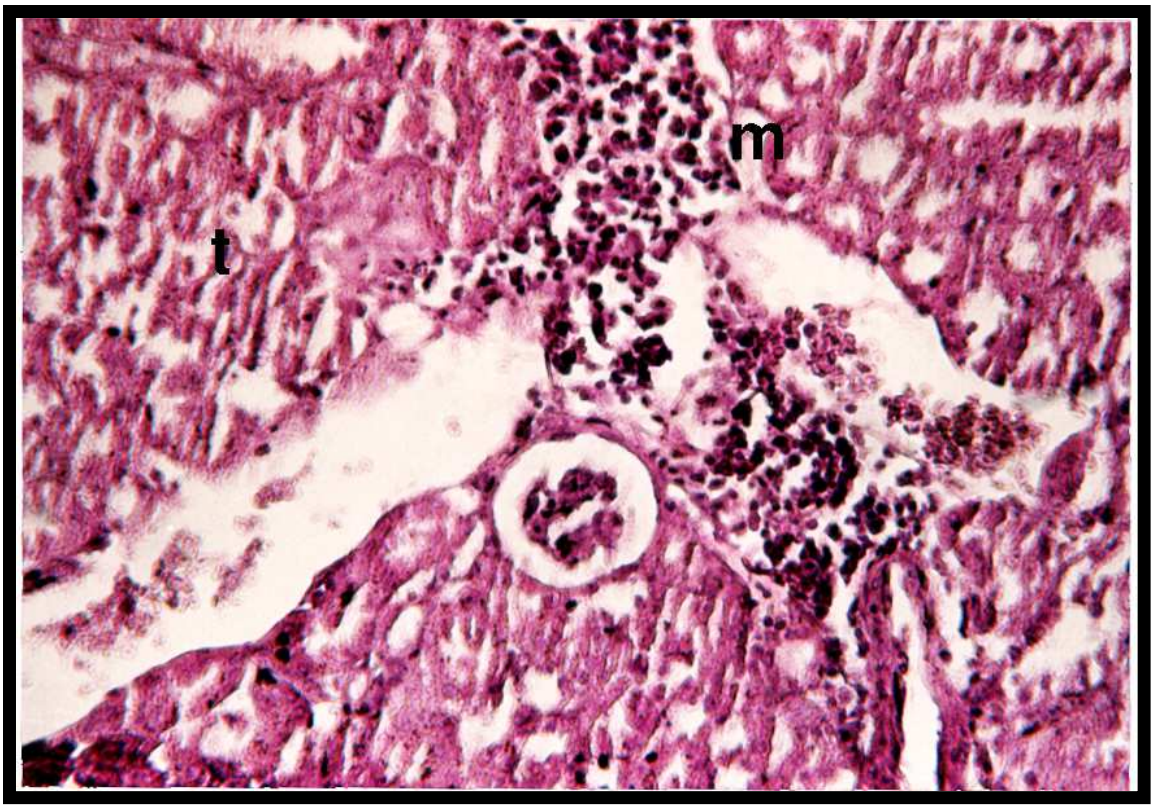

Fig.12 Kidney section of mice treated with high dose showing moderate mononuclear cells infiltration (m) with degenerative renal tubules (t).

Haematoxylin & Eosin (40X).

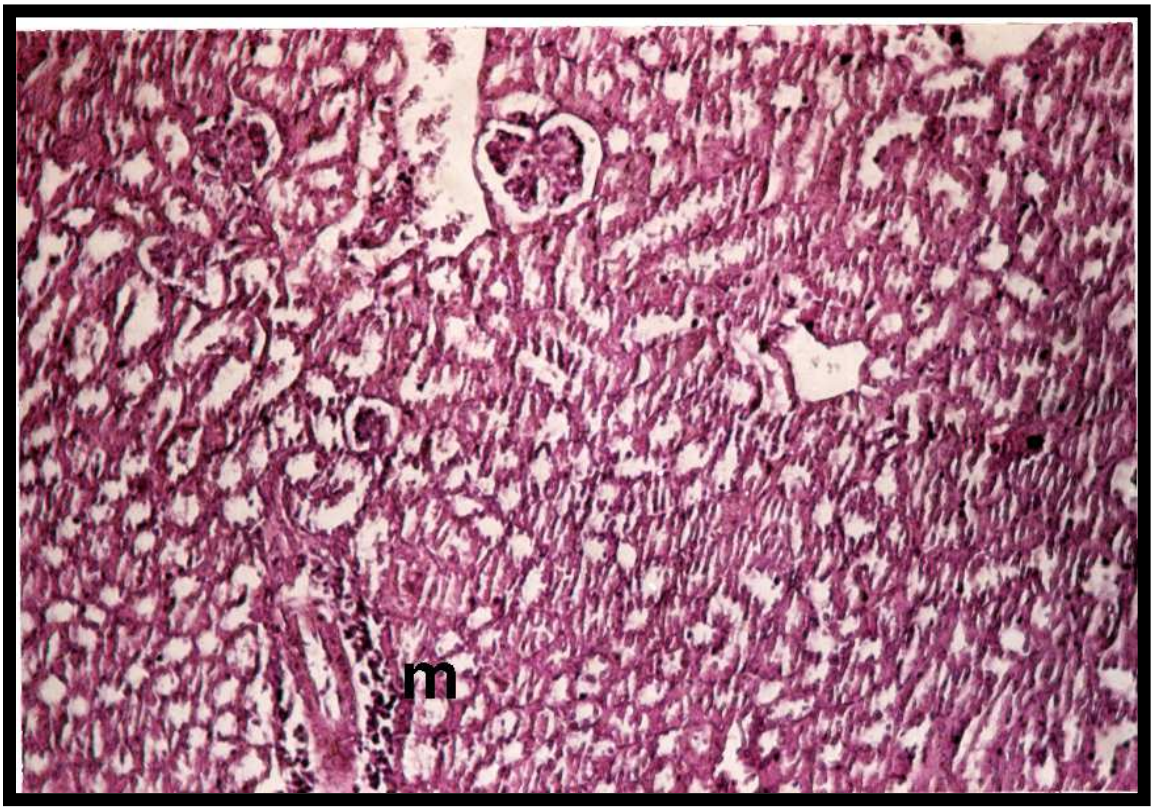

Fig.13 Kidney section of mice treated with high dose showing degeneration of tubules and moderate mononuclear cells infiltration (m).

Haematoxylin & Eosin (10X).

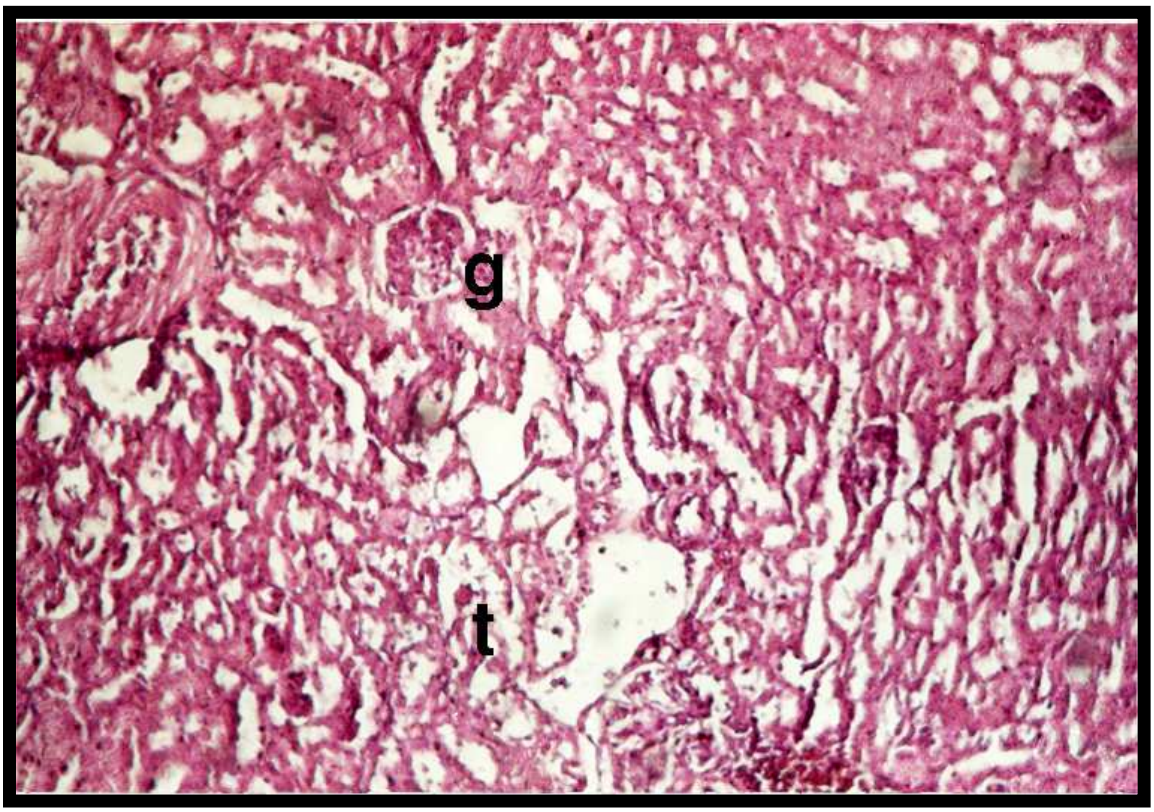

Fig.14 Kidney section of mice treated with high dose showing necrosis of tubules (t) and slightly in glomeruli (g).
Haematoxylin & Eosin (20X).

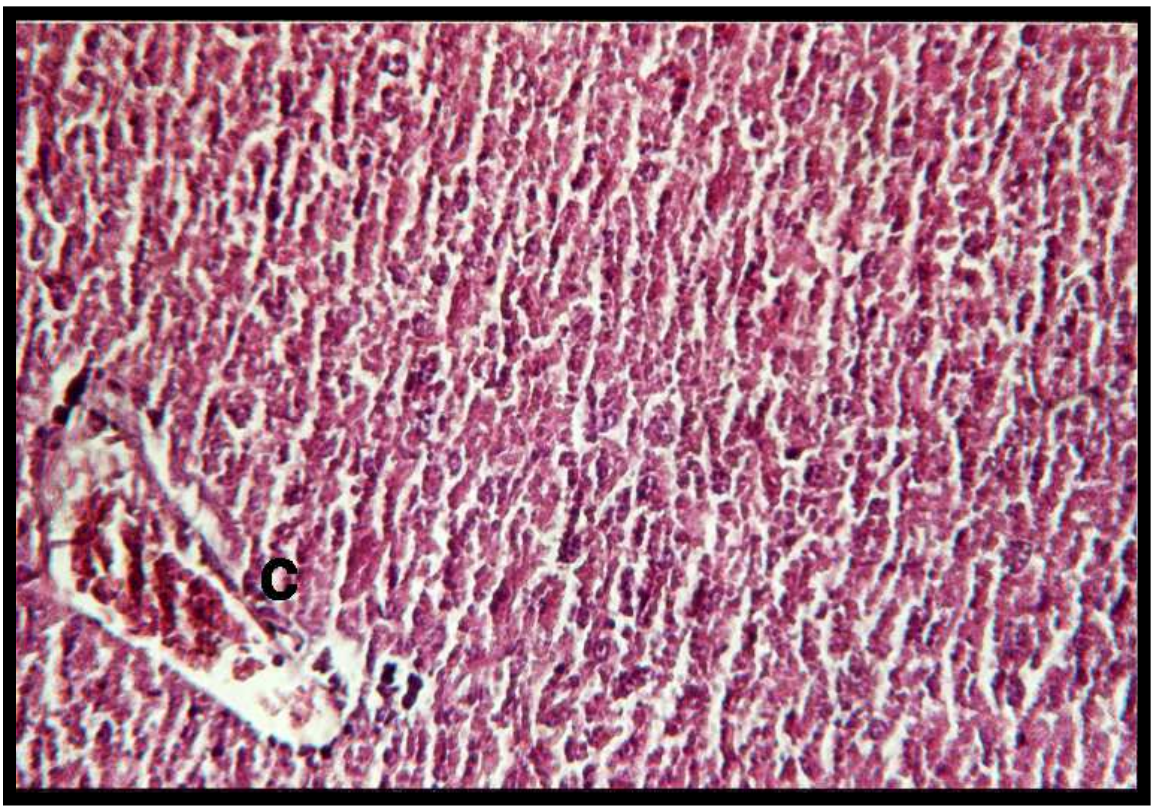

Fig.15 Liver section of animal treated with low dose of toxin showing congestion (c) and kupffur cells hyperplasia.
Haematoxylin & Eosin (20X).

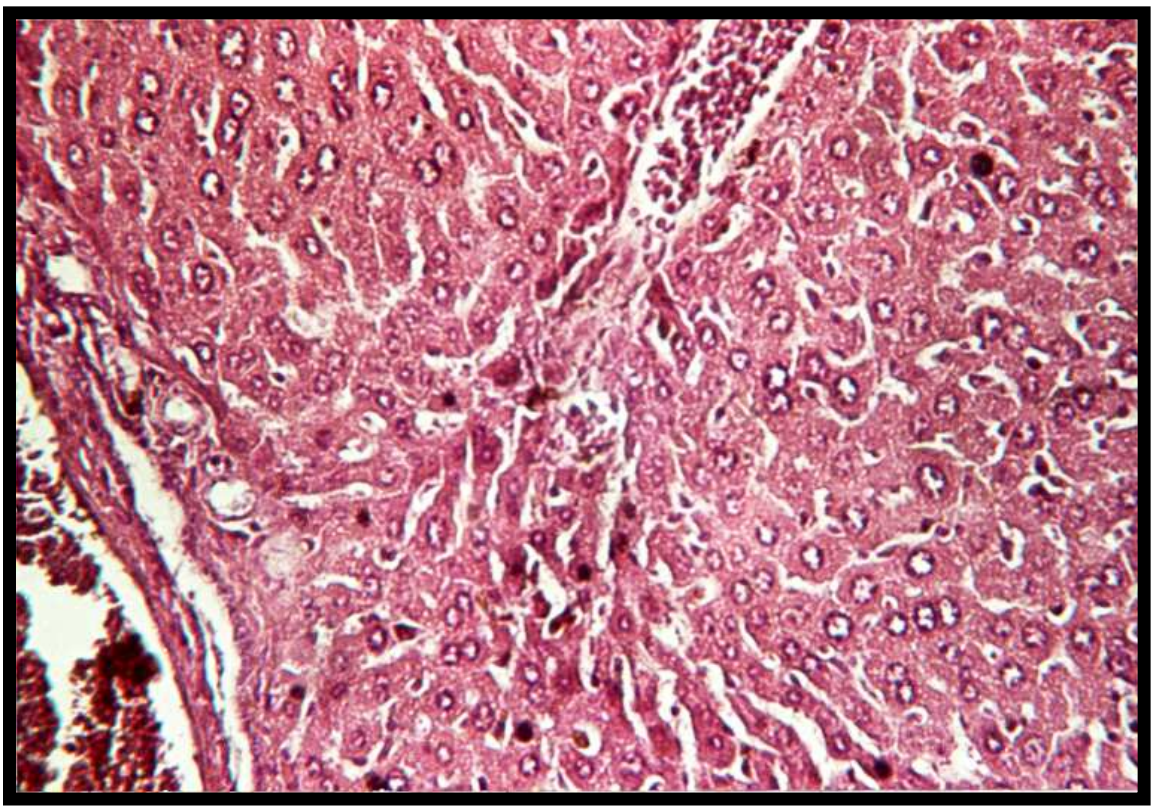

Fig.16 Liver section of animal treated with high dose of toxin showing congestion, sinusoidal dilation and kupffer cells hyperplasia. Haematoxylin & Eosin (40X).

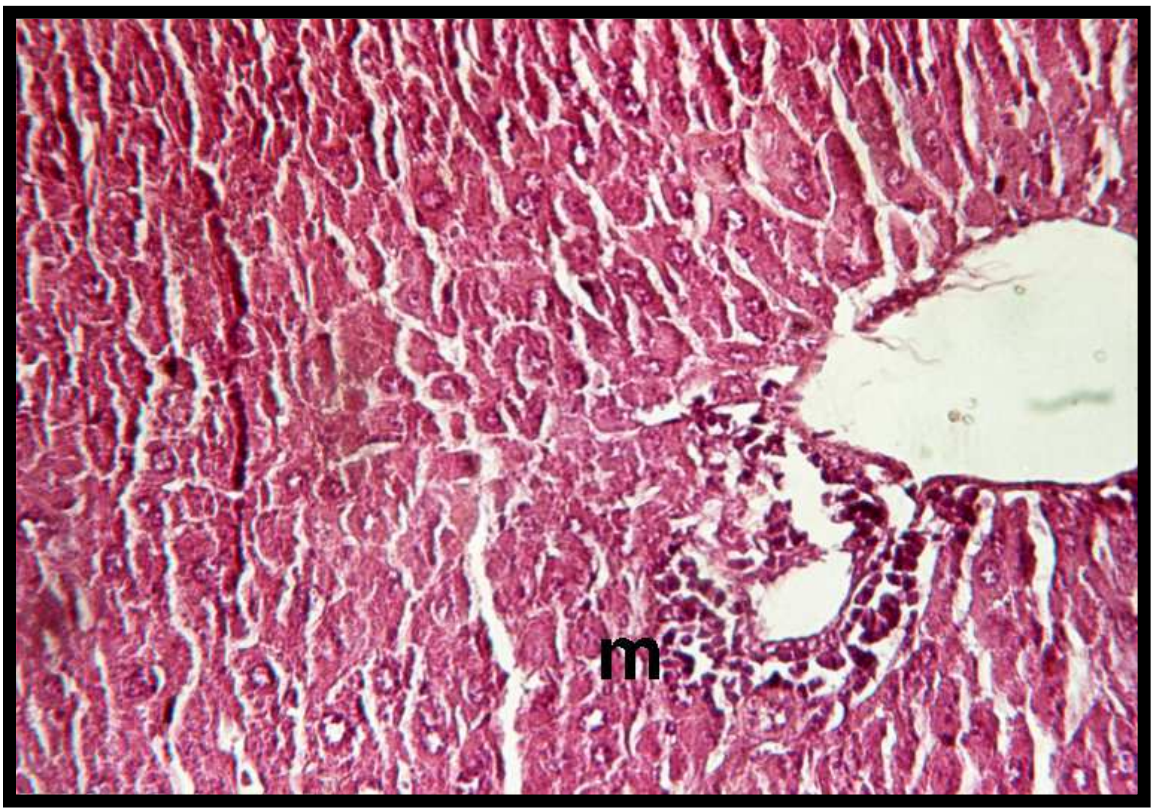

Fig.17 Liver section of mice treated with high dose of toxin showing mild infiltration of mononuclear cells in the portal area, around the bile duct (m).

Haematoxylin & Eosin (10X).

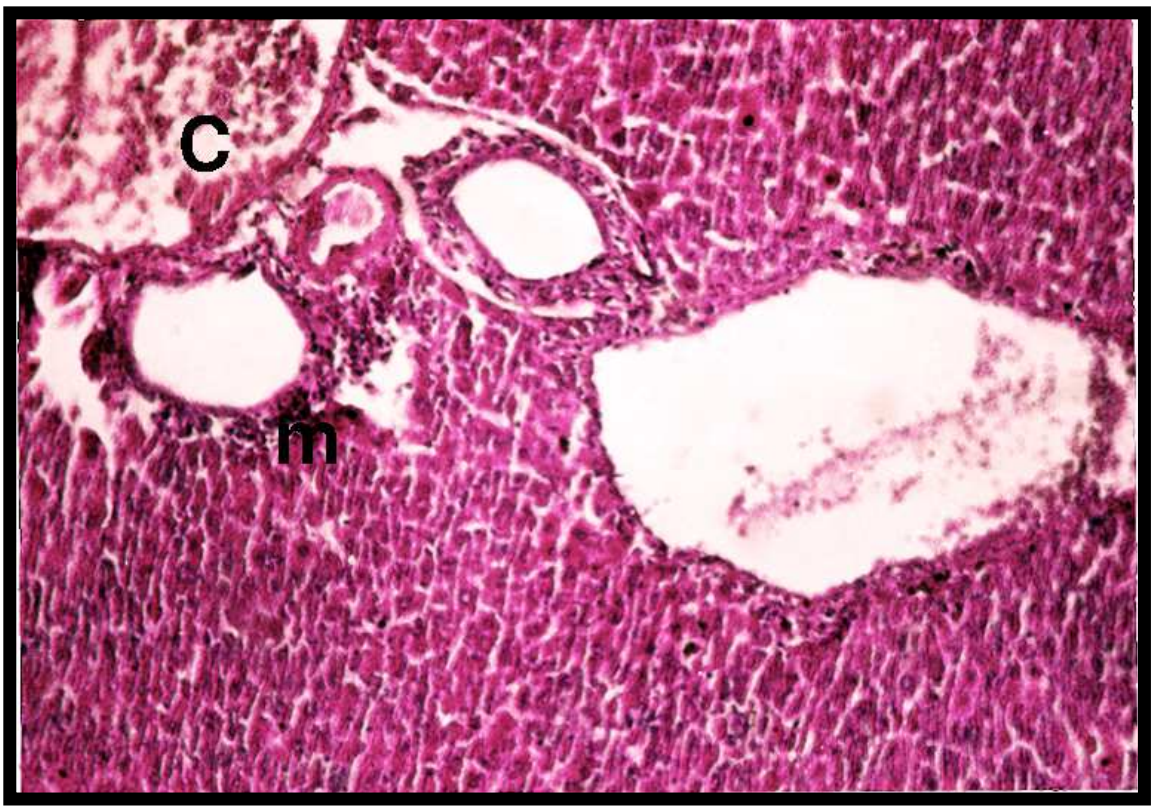

Fig.18 Liver section of animal treated with high dose revealed congestion (c) and inflammation cells infiltration in the portal area, around the bile duct (m).

Haematoxylin & Eosin (10X).

Conclusions:

From this study we conclude the followings:

1- Liver and kidney are target organs and affect there function such as increase in the level of GOT, GPT, & BUL which caused by trichothecenes.

2- Trichothecenes are considered as one of the major toxins having different types of toxicity on cell organelles even in low concentration for prolonged exposure.

3- Mouse organs function affected by Trichothecenes toxicity and this can be revealed by biochemical tests and microscopical examination.

Recommendations:

1- Studies on different pathological effects of trichothecenes on other different organs such as muscles, heart, intestine and uterus.

2- Further studies on the immunization against trichothecenes.

3- Further studies on the biochemical variation caused by trichothecenes.

4- Teratogenecity and mutagenecity of the toxin must be taken into account in further studies on mycotoxicosis.

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Supervisor certification

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We the examining committees certify that we have read this thesis and examined the student in its contents and that according to our opining is accept as a thesis for the degree of Master of Science in Biotechnology.

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SUMMARY

Trichothecenes are natural secondary metabolite produced by several species of ***Fusarium*** and some other genera in different agricultural commodities. Causing economic losses and health hazard to human and farm animals.

This investigation was planned to isolate ***F. oxysporum*** from maize, produce trichothecenes, estimate the quantity of toxin and then study some morphological, biochemical and histopathological changes induced in mice due to trichothecenes mycotoxicosis.

The results are summarized as following:

1. ***F. oxysporum*** was isolated from maize and identified according to practical guide, marked as *F. oxysporum* nr 1.
2. ***F. oxysporum*** nr.1 was proved to be trichothecenes producer on potato dextrose broth. The amount of trichothecenes produced by this isolate was 750 ppb as estimated by scanning densitometer.
3. The intraperitoneal injection of trichothecenes to mice caused pathological signs of marked increase in body weight, dyspanea, shivering, bristling up of hair, hair falling, anomalies of eyes and irritation around neck.

4. Other signs of toxicoses caused by trichothecenes are abdominal hemorrhage and clot accumulation in abdomen. In addition to inclusion (retention) cyst forms on liver.
5. The biochemical study on liver and kidney function by measuring GPT and GOT enzymes level and blood urea level, showed an increase in GPT and GOT enzymes level and blood urea level in treated animal in comparison with control animal which indicating abnormal function of liver and kidney failure.
6. The histopathological study on liver and kidney by using light microscope on treated animals with trichothecenes for 35 days of treatments revealed many alterations in liver which includes congestion, kupffer cells hyperplasia, dilated sinusoids and mononuclear cells infiltration around the portal area while in kidney includes congestion, degenerative changes of glomeruli and tubules, necrosis of tubules and slightly in glomeruli in addition to mononuclear cells infiltration.

الخلاصة

التر ايكوثيسينات نواتج ايضية ثانوية تنتج من عدة انواع من جنس ال *Fusarium* واجناس اخرى على مختلف المحاصيل الزراعية مسببة خسائر اقتصادية وتشكل خطر على صحة الانسان وحيوانات المزارع.

صممت هذه الدراسة لعزل ال *F. oxysporum* من الذرة وانتاج ال ترايكوثيسينات المنتجة منها وتحديد تركيزها ثم دراسة بعض التغيرات المظهرية والكيميائية الحياتية والنسيجية في كبد وكلى الفئران المعاملة والناتجة عن التسمم بسم الترايكوثيسينات الخام.

ويمكن ايجاز نتائج الدراسة بما يأتي :

١- عزل فطر ال *F. oxysporum* وتشخيصه بالاستعانة بدليل التشخيص العملي ورمز لهذه العزلة ب *F. oxysporum* nr.1 .

٢- انتاج سم الترايكوثيسينات من العزلة *F. oxysporum* nr.1 في وسط ال PDB ، حيث تم انتاج ٧٥٠ جزء بالبليون مقاسة بواسطة الماسح الاليكتروني.

٣- معاملة الفئران عن طريق الحقن داخل التجويف البطني ادى الى ظهور الاعراض المرضية مثل زيادة في وزن الجسم، صعوبة في التنفس، الاسهال، الارتجاف، انتصاب شعر الجسم، تهيج حول العنق، سقوط الشعر وجحوظ العين.

٤- اعراض اخرى ظهرت بسبب التسمم المايكوتوكسيني بالمايكوتوكسينات تضم نزف الامعاء وتجمع الدم وتجلطه داخل التجويف البطني، بالاضافة الى تكون اكياس تحتفظ بالسوائل في الكبد.

٥- الدراسة الكيميائية الحيوية لوظيفة الكبد والكلية قد ضمت قياس انزيم ال GOT وال GPT ومستوى اليوريا في الدم التي اظهرت زيادة في مستوياتها في دم الحيوانات المعاملة بالمقارنة مع الحيوانات الغير معاملة بالسم الفطري والذي يشير الى خلل في عمل كل من الكبد والكلية.

٦- اوضحت الدراسة النسيجية للكبد والكلية باستخدام المجهر الضوئي للفئران المعاملة بالترايكوثيسينات الخام بعد ٣٥ يوم العديد من التغيرات: مثل (الاحتقان، زيادة خلايا كفر، توسع الجيبانيات ونضوح خلايا التهابية حول المنطقة البوابية) في الكبد و(حدوث تغيرات في نسيج النبيبات والكبيبات، احتقان في النسيج، تغيرات تنكسيّة في النبيبات وبشكل اقل في الكبيبات ونضوح الخلايا الالتهابية) في الكلية.

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Pathological effect of Trichothecenes(T-2 toxin) produced by *Fusarium oxysporum* on liver and kidneys of mice

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جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة النهرين
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التأثيرات الامراضيه للترايكوثيسينات(سم T-2) المنتجه من فطر ال *Fusarium oxysporum* في كبد و كلى الفئران

رساله
مقدمه الى كلية العلوم جامعة النهرين
وهي جزء من متطلبات نيل درجة الماجستير في التقانه الاحيائيه

من قبل

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