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STUDY OF SOME CHROMOSOMAL VARIATIONS CAUSED BY OCHRATOXIN A CONTAMINATION IN MICE

A thesis submitted to the college of science
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بسم الله الرحمن الرحيم

وآيَةٌ لَهُمُ الأَرْضُ المَيْتَةُ أَحْيَيْنَاهَا وَأَخْرَجْنَا
مِنْهَا حَبّاً فَمِنْهُ يَأْكُلُونَ (٣٣) وَجَعَلْنَا فِيهَا
جَنَّاتٍ مِنْ نَخِيلٍ وَأَعْنَابٍ وَفَجَّرْنَا فِيهَا
مِنَ العُيُونِ (٣٤) لِيَأْكُلُوا مِنْ ثَمَرِهِ وَمَا
عَمِلَتْهُ أَيْدِيهِمْ أَفَلاَ يَشْكُرُونَ (٣٥) سُبْحَانَ
الَّذِي خَلَقَ الأَزوَاجَ كُلَّهَا مِمَّا تُنْبِتُ الأَرْضُ
وَمِنْ أَنْفُسِهِمْ وَمِمَّا لاَ يَعْلَمُونَ (٣٦)

صدق الله العظيم

سورة يَسِ، الآية (٣٣ – ٣٦)

الأهداء

ألى الذي يعلم تفاصيل الأمور، ودقائق الأشياء وخفايا الضمائر والنفوس .....

ربّي

ألى الحبيبة الذي ينزف دما لأجلي .... ***وطني***

ألى من غرز في نفسي العراقة .... ***أمي وأبي***

ألى من ساندتني من الألف الى الياء .... ***أمّي الثانية و خالتي د. خلود***

ألى من علّمني حرفا فملكني عبدا .... ***أساتذتي***

ألى من ساروا معي الدرب .... ***أختاي وأخي***

أهدي جهدي المتواضع

نور

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We certify that this thesis was prepared under our supervision in Al-Nahrain University, college of Science as a partial fulfillment of the requirement for the degree of Master of Science in Biotechnology.

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Chapter one
Introduction

1.1. Introduction:

Ochratoxin A (OTA) is a mycotoxin produced by a variety of species of the genera *Aspergillus* and *Penicillium*. This toxin was first described in 1965 as a toxic metabolite *of Aspergillus ochraceus,* it is also produced by a number of species of *Aspergillus* in warmer & tropical parts of the world (Scott, 1965). *Penicillium verrucosum* can produce ochratoxin in temperate or cold climates. This fungus is associated with stored cereals and is very common in Northern European countries and Canada.

Ochratoxin A has been reported as naturally occurring mycotoxin in almost all cereals including corn, barley, wheat, sorghum, rye, oats, rice & coconuts products. This toxin has been found to occur in other food commodities such as coffee, meat & meat products, and dietary products produced from animals fed on ochratoxin A contaminated diet (FAO / WHO, 2001).

There are three generally recognized ochratoxins, designated as: A, B and C. Although under experimental conditions numerous derivatives of ochratoxin have been detected, only ochratoxin A, and rarely ochratoxin B, have been found to occur naturally in molded plant products because ochratoxin A is chlorinated and it is the most toxic followed by ochratoxin B and ochratoxin C (WHO, 1979).

It was proposed by Thuvander *et. al.* that ochratoxin A is a potent nephrotoxic mycotoxin which is carcinogenic to rodents and possesses teratogenic, immunotoxic, & possibly neurotoxic properties. It has also genotoxic properties; kidney of rodents was the target organ (Thuvander *et. al.*, 1995, 1996a, 1996b; Bruinink & Sidler, 1997; Monnet-Tschudi *et. al.*, 1997). Further, it was implicated as a factor in the human disease Balkan Endemic Nephropathy (BEN) and in the development of urinary tract tumors in humans (Nikolov *et. al.*, 1996; Radic *et. al.*, 1997).

The most important animal diseases in relation to ochratoxin A is mycotoxin porcine nephropathy (PN). Toxokinetics make the kidney the primary target organ for ochratoxin A. The disease has been regularly reported in studies in Denmark for more than 60 years ago & is recognized as an endemic disease in several northern & central European countries (Krogh, 1992). So it is clear that the major target organ for ochratoxin A toxicity is the kidney in all mammalian species tested, the liver can also be affected in several animal species (Al-Naimi, 2001). In the fetus the major target is the developing central nervous system (CNS) (Kuiper, 1991).

In 1993, the International Agency for Research on Cancer (IARC) classified ochratoxin A as a possible human carcinogen, so it is inadequate evidence for human health and based on sufficient evidence for carcinogenicity in animal studies (IARC, 1993).

Ochratoxin A increases the production of abnormalities in both mitotic and meiotic chromosomes as well as in the gross morphology of the sperm head (Bose & Sinha, 1994).

Black seed (*Nigella sativa*) is related to a common garden flower that goes by several names including cinnamon flower, nutmeg flower and love-in-a-mist. In the Mediterranean and Middle East where *Nigella sativa* (*N. sativa*) seeds are traditionally used, it is also referred to as black seed and is used for a variety of ailments including upper respiratory conditions, headaches, cancer, stomachaches and jaundice. *N. sativa* seeds are traditionally eaten alone or mixed with honey (Jones, 2000).

N. sativa is found in North Africa, as well as some parts of Turkey and the Far East. The oil of black seed is so beneficial due to its content of over a hundred components such as aromatic oils, trace elements, proteins & carbohydrates (El-Zawahry, 1997). It contains 58% of essential fatty acids including omega 6 and omega 3. These fatty acids are necessary for the forming of Prostaglandin E1 which balances and strengthens the immune system giving it the power to prevent infections and allergies and control chronic illnesses. Also healthy cells are protected from viruses thus inhibiting tumors. Black seed oil also contains about 0.5 - 1.5% volatile oils including nigellone and thymoquinone which are responsible for its anti-histaminic, anti-oxidant, anti-infective and broncho-dilating effect (Randhawa & Al-Ghamdi, 2002).

1.2. Aim of the study:

This work is an attempt for studying the genotoxic effect of ochratoxin A on the somatic cells *in vivo* and trying to diminish the toxic effect of ochratoxin A by treatment with black seed (*Nigella sativa*) as a curing agent.

Chapter Two
Literature Review

2.1. History of Mycotoxin:

Mycotoxins are literally poisons that are produced by fungi. The poison that is released into the substrate by the fungus will be present even if the fungus itself is not. The term mycotoxin referred to the Greek word for fungus "mykes" & the Latin word "toxicum", it is usually refer to the toxic chemical product formed by a few fungal species that readily colonize crops in the field or after harvest & thus pause a potential threat to human and animal health through the ingestion of food products prepared from these commodities (Matossian, 1989).

Mycotoxins are recorded in history as back as 5000 years ago in China. Reports as early in 1861 indicated that a suspected mycotoxin affecting humans was reported in Russia and in Japan in 1891. It was reported on moldy rice to be potential toxic to man. The existence of mycotoxins was not documented until 1960. However, the concept that moldy food could lead to illness in people or domestic animals was long suspected before their existence was demonstrated by science (Hunter, 1989).

Mycotoxicoses are defined as "poisoning" of the host which follows the entry of toxin substances of fungal origin into the body (Forgacs & Carll, 1962).

Mycotoxins are toxins produced mostly from the genera *Aspergillus, Penicillium* and *Fusarium*. These compounds of "mycotoxins" are of interest because of their high toxicity (both acute and chronic) & the remarkable potency of some in producing tumors in laboratory animals, that with direct ingestion of mycotoxin or a toxic fungus is well known to cause illness or death (Hsieh, 1988).

Molds can be found almost anywhere, that there are numerous varieties of them ranging from these seen growing in woods & fields, paper, carpet, food and feed, to microscopic species that can invade into the growing or stored crops or can grow virtually on any organic substances, also of them many can cause diseases in plants (EPA, 2001).

Any crop that is stored for more than a few days may be considered a target for mold growth & mycotoxin formation. Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, grains, spices, oil seeds, dried peas, beans & fruits particularly apples. Mycotoxins may also found in beer & wine resulting from use of contaminated barley, other cereals & grapes in their production. Mycotoxins may be produced on products, fodder or may be present in constituents used in manufacture of meals or pelleted animal diets (Betina, 1984).

Some animal diets specially these containing grain or nuts, may contain several toxigenic species of molds, which may produce a number of mycotoxins having different toxic and / or pharmacological properties. Mycotoxins also enter the human food chain via meat or

other animal products such as eggs, milk & cheese as the result of livestock eating contaminated feed (FAO / WHO, 2001).

Most mycotoxins are chemically stable so they tend to survive storage & processing even when cooked to quite high temperatures such as those reached during baking bread or producing breakfast cereals. This makes it important to avoid the conditions that lead to mycotoxin formation as far as possible. This is difficult to achieve for the growing crop that is subject to the prevailing climate & conditions.

So drying of the cereals and grains after harvest is very important to prevent growth of mold and mycotoxins which could be formed during storage and transport. Because of these effects of mycotoxins, nowadays food crops that are commonly have to be stored for long period of time; precautionary measures have to be taken to ensure safety for people, in order to get fresh food not contaminated (Kendrick, 1992).

Mycotoxins cause a diverse range of toxic effects because of their diverse chemical structures and biosynthetic origins, their myriad biological effects, and their production by a wide number of different fungal species (Bennett & Klich, 2003).

All toxicological syndromes can be categorized as acute or chronic. Acute toxicity generally has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time period, resulting in cancers and other generally irreversible effects (James, 1985).

So the most studied mycotoxins are of the class of food-born pathogen or carcinogen because these mycotoxins generally represent the greatest harmful to human health (Hunter, 1989).

As mentioned above, mycotoxins contaminate various feed & food commodities, due to the global occurrence of toxigenic molds. The symptoms of a mycotoxicosis depend on the type of mycotoxin; the amount and duration of the exposure; the age, health, and sex of the exposed individual, and many poorly understood synergistic effects involving genetics, dietary status, and interactions with other toxic insults. Thus, the severity of mycotoxin poisoning can be compounded by factors such as vitamin deficiency, caloric deprivation, alcohol abuse, and infectious disease status. In turn, mycotoxicoses can heighten vulnerability to microbial diseases, worsen the effects of malnutrition, and interact synergistically with other toxins (Peraica *et. al.,* 1999; Bennett & Klich, 2003).

Almost certainly, the main human and veterinary health burden of mycotoxin exposure is related to chronic exposure (e.g., cancer induction, kidney toxicity, immune suppression). However, the best-known mycotoxin episodes are manifestations of acute effects (e.g., turkey X syndrome, human ergotism, stachybotryotoxicosis) (Bennett & Klich, 2003). So they are involved in the etiology of human cancers that have received particular attention. While in animals the acute intoxication causes losses in productivity, reduced weight gain & immunosuppression, those symptoms are considered as the most important feature of mycotoxicosis & genotoxic effects (Ettlin, 2003).

Different types of mycotoxins are responsible of these effects such as: aflatoxin, ochratoxin, fumonisins, trichothecenes (deoxynivalenol, nivalenol, and T-2 toxin) zearalenone, alternaria toxins, patulin & ergot (WHO, 2000).

An example of mycotoxin diseases is a disease recognized in Europe well known by the end of the first millennium; this was the result of eating rye contaminated with ergot alkaloids produced by the mold *Claviceps purpurea.* This disease was called Anthony's fire', one of the earliest recognized disease caused by mycotoxins (FAO, 1977).

Another example of mycotoxin diseases was a dramatic disease outbreak attributed to mold contaminated cereal grains occurred in certain areas of Russia in 1942 – 1947. The disease Alimentary Toxic Aleukia (ATA) was caused by eating cereal grains, particularly millet that had over wintered in the field (FAO, 1979). 10% of the populations of certain communities were reported to have died of the disease (Mirocha *et. al*. 1983).

Studies showed that some of the most common mycotoxins are carcinogenic, genotoxic, immunotoxic or teratogenic, like: Aflatoxin, for example, is a hepatotoxic, mutagenic, carcinogenic, a toxin derived from *Aspergillus*, Zearalenone is a *Fusarium* metabolite with potent estrogenic activity, & carcinogenic, while ochratoxin is nephrotoxic, immuno-supressive and genotoxic (Bennett & Klich, 2003).

Because of mycotoxins pharmacological activity, some mycotoxins or mycotoxin derivatives have found to be used as antibiotics, growth

promotants, other kinds of drugs, and still others have been implicated as chemical warfare agents.

2.2. Ochratoxin A (Physical and chemical properties):-

Ochratoxins are group of structurally–related compounds classified according to biosynthetic origin the group of polyketide-derived secondary metabolites (O`Callaghan *et. al.*, 2003).

There are three generally recognized ochratoxins designated which are A, B & C. Ochratoxin A is chlorinated and it is the most toxic, followed by ochratoxin B & ochratoxin C. Ochratoxin A molecular formula is $C_{20}H_{18}O_6NCl$ with 403 relative molecular mass & melting point $169^{\circ}C$ (using xylene as a solvent or benzene in melting point between $89-95^{\circ}C$), while ochratoxin B molecular formula is $C_{20}H_{19}NO_6$ with relative molecular mass of 369 & melting point of $221^{\circ}C$. The other kind of ochratoxins is ochratoxin alpha which is rarely designated because ochratoxin A is degraded to form it, its formula is $C_{11}H_9ClO_5$, molecular mass 256 & melting point $229^{\circ}C$ (WHO, 1979).

Ochratoxin A was discovered as a metabolite of *Aspergillus ochraceus* in 1965 during a large screen of fungal metabolites that was designed specifically to identify new mycotoxins (Van der Merwe *et. al.*, 1965), & it is a mycotoxin produced by *Penicellium verrucosum* in

temperate or cold climate & a number of *Aspergillus* in warmer & tropical parts of the world (Pitt, 1987).

Ochratoxin A is a colorless, crystalline compound, exhibiting blue fluorescence under UVL. Ochratoxin A is relatively stable to heat & is only particularly degraded under normal cooking or processing conditions. It is fat soluble, & not readily excreted, so it is accumulated in fatty tissue. The sodium salt of ochratoxin A is soluble in water, as in acid, it is moderately soluble in many polar organic solvents like chloroform & methanol (WHO, 1979).

Ochratoxin A is simply a dihydro isocoumarin, which is derived from combined pathway, the shikimic acid pathway (phenylalanine) & the polyketide pathway (Moss, 1996; 1998). Ochratoxin A is consisting of a chlorinated dihydroisocoumarin moiety linked through a 7-carboxyl group by an amide bond to one molecule of L -β- phenylalanine (Huff & Hamilton, 1979).

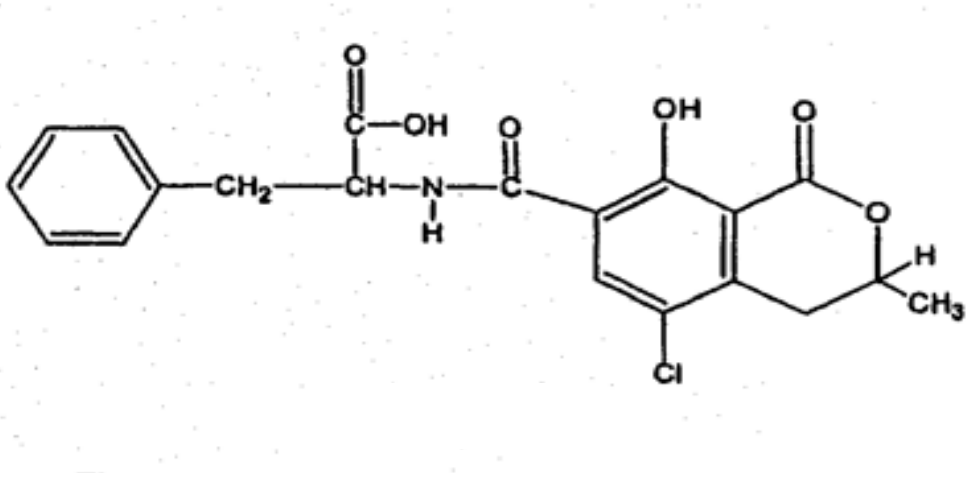

Figure (1): Chemical Structure of Ochratoxin A, designated by Hussein & Brasel, 2001.

On acid hydrolysis, ochratoxin A yields phenylalanine & an optically active lactone acid

Chu *et. al.* (1972) suggested that the free phenolic group in ochratoxin A was necessary for it is toxic effect, While Xiao *et. al*., (1996) indicated that the toxicity of ochratoxin A is related to its isocoumarin moiety.

In addition to ochratoxin A being a nephrotoxin, animal studies indicate that ochratoxin A is a liver toxin, an immune suppressant, a potent teratogen, and a carcinogen (Kwon-Chung, and Bennett, 1992; Beardall, and Miller, 1994).

2.3. Ochratoxin A Producing Microorganisms:

Ochratoxin A was first obtained from *Aspergillus ochraceous*, however, subsequent investigation have revealed that a variety of molds included in the fungal genera *Aspergillus* & *Penicillium* are able to produce ochratoxin (Frivad & Samon, 2000). Most literatures referred that ochratoxin A produced by two species of fungi they are *Aspergillus ochraceous* and *Penicellium viridicatum* (Krogh, 1976a).

Many studies suggested the other ochratoxin producing fungi of the genus *Aspergillus* including: *A. sulphureus, A. alliaceus, A. sclerotiorum, A. melleus, A. ostianus, A. petrakii, A. albertensis, A. glaucus* (Abarca *et. al*., 2001; Bayman *et. al.*, 2002; Dalcero *et. al.*, 2002) and *A. niger* (Pitt, 2000). Because *Aspergillus niger* is used widely in the production of enzymes and citric acid for human

consumption, it is important to ensure that industrial strains are nonproducers (Heenan *et. al.*, 1998).

Another finding by Varga *et. al.*, (1996) pointed that ochratoxin A could be produced by three species of *Aspergillus* in addition to the others; they are *A. albertensis, A. auricomus,* and *A. wentii* strains. Study of Abarca *et. al.*, (1997), with screening a total of 176 isolates of the genus *Aspergillus* for their ability to produce ochratoxin A on yeast extract – sucrose broth & on moistened corn, indicated that ochratoxin A was produced by one isolate of *A. fumigatus,* beside that produced by *A. ochraceus* & *A. alliaceus.*

These above different groups of species differ in there ecological niches, in the commodities affected, in the frequency of their occurrence in different regions; strain of fungus, temperature & in water activiry (Ciegler, 1972).

Further studies indicated that ochratoxin A could be produced by many *Penicillium* species such as: *P. verrucosum, P. nordicum P. purpurrescens; P. variabile; P. viridicatum; P. cyclopium;* and *P. commue* (Krogh, 1978; Pitt, 1987; Castella *et. al.*, 2002).

P. verrucosum grows only at temperature below 30°C & down to 0.80 water activity. Therefore this species is found only in cool temperature regions & is the source of ochratoxin A in cereals & cereals products (WHO, 2000).

2.4. The natural occurrence of ochratoxin A:-

Ochratoxin A is found naturally in a wide range of food stuffs (plant & animal products) but more common in dried & stored foods than elsewhere, this was reviewed by (Chu 1974a; Krogh, 1976a; 1977a; Pitt & Hocking, 1997).

However, *A. ochraceus* can affect seeds and grains more than those that are undergoing microbial deterioration in storage because it is evidently not a good competitor (Kendrick, 1992).

Naturally occurring ochratoxin A was first reported at a concentration of 110–150 μ g / kg in one sample of maize included in a survey of 283 samples from commercial markets in the USA (Shotwell *et. al.*, 1969).

It has been suggested that ochratoxin A could be found widely distributed naturally specially in soil, stored seeds, and grains undergoing microbial deterioration, cereal & cereal products. It is obvious that the presence of ochratoxin A is related to the presence of the fungi producing it, as mentioned (Cantafora *et. al*., 1983; Taniwaki *et. al.* 1999; FAO / WHO, 2001).

Investigations have revealed that strains of *P. viridicatum* are able to produce ochratoxin A at $5-10^{\circ}$ C, because the genus *Penicillium* included many species that have psychrophilic properties. So that, ochratoxin A can be produced in low incubation temperature involving the cold climates countries such as Canada & Scandinavian countries

which was observed to have a heavy ochratoxin A contamination (Harwing & Chen, 1974; Hunter, 1989).

The natural occurrence of ochratoxin A in food & feeds stuffs like wine, beer, grape juice, meat & others; are widespread, especially in temperate areas such as Canada, Denmark, Germany, Sweden & the UK, the detectable were even found in randomly collected human milk sample in Germany, Sweden & Italy (Abarca *et. al.,* 1994; Stefanaki *et. al.,* 2003).

The infection with ochratoxin A has been shown to occur pre-harvest & post-harvest, but post-harvest ochratoxin A formation is regarded as the predominant factor of ochratoxin A formation in food. Cases of mycotoxic disease porcine nephropathy (PN) have been regularly encountered in studies in Denmark since the disease was first discovered over 60 years ago. The disease is endemic in all areas of the country, although unevenly distributed (FAO / WHO, 2001).

2.5. Production of ochratoxin A:

Ochratoxin A is a mycotoxin produced by fungi occurring frequently on cereals. Production of ochratoxin, by *A. ochraceus,* was first described in South Africa by Theron, *et. al.*(1966), where it was isolated along with a number of other fungi.

Seminiuk *et. al.* (1971) investigated that the highest yield of ochratoxin A & B were obtained from strains of *A. ochraceus* 3174 (originally CSIR K-804) & NRRL 3519. Similar results were reported by Ciegler, (1972).

Fungal growth in food stuffs and subsequent mycotoxin formation is influenced by the water content of these food stuffs, and can be changed by climatic conditions such as heavy rainfalls during harvest. Thus, toxin production, are closely correlated to the degree of moisture to which they are exposed, which itself is dependent on weather conditions at harvest and techniques for drying and storage (Frank, 1991).

It is obvious that temperature and water activity of the substrate are the major factors affecting toxin production (Bacon *et. al.*, 1973; Northolt *et. al.*, 1979).

Maximum production of ochratoxin A occurred when temperature reached between $20-30^{\circ}C$ and 0.95 of water activity with only slight losses at 0.85 water activity at both $15-20^{\circ}C$ (Ramos *et. al.*, 1999).

It was known that the substrate on which the molds grow as well as the moisture level, temperature, and presence of competitive microflora interact to influence the level of toxin produced (Bennett and Klich, 2003).

Ochratoxin A was found to be produced significantly in synthetic media lower than in the natural substrate (Applegate and Chipley, 2001). In addition, the early studies used solid media such as corn or wheat (Ciegler, 1972), whereas recent studies usually used liquid media (Abarca *et. al.*, 1994). The highest levels of ochratoxin A have been reported on grain, but there is considerable variation in results among different studies testing the same isolates on grain (Hesseltine *et. al.*, 1972).

Other data indicated that small and large amount of the toxin elaborated on the Carbon and Sulfur source and on the near to the optimal quantities of Sulfur, Magnesium, and Phosphorus contained in the growth media (Lee and Magan, 1999).

It has been shown that ochratoxin A production was considerably higher in the light than in the dark. The greatest ochratoxin A production was achieved after 11 days of fermentation with light and dark grown culture at 25°C. They found that the maximum ochratoxin yield was obtained at pH 5.5 and with increasing the initial pH to near neutrality the mycotoxin yield decreased. Iron, Copper, and Zink were observed to stimulate ochratoxin A production and enhancing of growth rate of A. ochraceus (Aziz & Moussa, 1997).

2.6. Toxic Effect of ochratoxin A:

It is necessary to reviewed that ochratoxin A was recommended by the joint committee of FAO / WHO at its 37^{th} meeting, with other mycotoxins as a causer of nephropathy in pigs & human, and induction of tumors & the role of phenylalanine in antagonizing the adverse effect of ochratoxin A. It was reported as a potent teratogen, hepatotoxin and genotoxic, and it has been classified as a possible human carcinogen because it forms DNA adducts (O`Callaghan *et. al.*, 2003).

Ochratoxin A effects can be established as follows:

2.6.1. Effect on ochratoxin A laboratory and field animals:

As mentioned before, studies have indicated that ochratoxin A is harmful to a variety of laboratory animals, such as: rats, mice and ducklings (Theron *et. al.,* 1966), chicks, hens, beagle dogs, pigs, and rainbow trout (Kanisawa *et. al.*, 1977).

In Denmark, this toxin is considered to be one of the causative factors in mold nephritis of pigs & much attention has been focused on this toxin as a possible environmental hazard to humans & various domestic animals (Elling & Moller, 1973).

Several studies about ochratoxin A have been shown that it is nephrotoxic in all mammalian species tested. The etiological studies have revealed that ochratoxin A was a major disease determinant of porcine nephropathy, although other factors such as citrinin are also involved as causal determinants (Krogh, 1976a).

The main target is the renal proximal tubule, where it exerts cytotoxic & carcinogenic effects. Significant sex and species differences in sensitivity to nephrotoxicity were evident, in the order Pig > Rat > Mouse. The doses at which carcinogenicity was observed in rodents were higher than those causes nephrotoxicity (FAO / WHO, 2001).

The toxic effect of ochratoxin A has been studied exclusively in a variety of experimental animals. All the studied so far have been susceptible to orally administrated ochratoxin A, but in various degrees. The highly administration level of ochratoxin A have been found to cause changes in the kidneys & other organs & tissues

(Atroshi *et. al.*, 2000). However, only renal lesions were observed at exposure levels identical to those occurring environmentally. A study on Whole body autoradiography using intravenous injection of ^{14}C-ochratoxin A to rats resulted in the following distribution after 24 h (in decreasing order of concentration): lung, adrenal medulla, skin, liver, myocardium, kidney, salivary gland, adrenal cortex, muscle, gastric mucosa, and bone marrow (Breitholtz – Emanuelsson *et. al.*, 1992).

Ochratoxin A have shown to be slowly absorbed from gastrointestinal tract & it is distributed in a number of tissues via blood and then mainly to the kidney & lower concentrations being found in liver, muscles & fat. ochratoxin A transfer to milk has been demonstrated in rats, rabbits, & humans, but little is transferred to the milk of ruminants owing to metabolites of ochratoxin A by the rumen microflora. Ochratoxin A is a potent nephrotoxicant & induces renal tumors in rodents (Dekant *et. al.* 2001).

Ochratoxin A level of up to 29 µg / kg was found in the muscle of hens and chickens collected in one slaughterhouse (Elling *et. al.*, 1975). In another study by Krogh *et. al.*, (1976c) groups of hens were exposed for 1-2 years to dietary levels of ochratoxin A of 0.3 and 1 mg/kg. The kidneys contained the highest residues, with a mean value of 19 µg / kg tissue in the group fed ochratoxin A at 1 mg / kg, the liver and muscle contained lower levels of ochratoxin A residues.

Juszkiewicz *et. al.*, (1982) showed that ochratoxin A was found in eggs when hens were fed large amount of ochratoxin A (10 mg/kg bw). Previous study on tissue distribution of ^{14}C- ochratoxin A in laying

Japanese quail, demonstrated specific retention of unidentified radioactivity as a ring-shaped deposition in eggs, indicating that the toxin could be deposited over a short time period (Fuchs *et. al.*, 1988).

Ochratoxin A can cross the placenta. It was known that lactating rats treated orally with single doses of up to 250 µg/kg bw ochratoxin A, excreted ochratoxin A in the milk (Breitholtz – Emanuelsson *et. al.*, 1993a).

Hayes *et. al.* (1974) pointed that: Intraperitoneal injection of pregnant mice with ochratoxin A at 5 mg/kg body weight on one of gestation days 7-12 resulted in increased prenatal mortality, decreased fetal weight, and various malformations including exencephaly and anomalies of the eyes, face, digits, and tail.

An example that ochratoxin A has a neurotoxic properties was applied by Fukui *et. al.*, (1992) as follows: when mice derived from pregnant females treated intraperitoneally with 3 mg ochratoxin A / kg bw on day 10 of gestation, the somatosensory cortices of treated mice had fewer synapses per neuron compared to controls, this indicating the reduction in growth.

In another study Brown *et. al.*, (1976) showed that: treatment of rats perorally with ochratoxin A at 0.75 and 1.0 mg/kg bw on gestation days 6-15, fetuses taken on day 20 resulted in decreased weight and various anomalies (e.g., open eyes, wavy ribs, and agenesis of vertebrae).

It was found that male rats are more sensitive than female while mice are 100 fold less sensitive than rats to tumorginicity of ochratoxin A (Dekant *et. al.*, 2001).

2.6.2. Effect of ochratoxin A on Human:

Ochratoxin A has been found in human blood samples, most notably in a number of countries in the cool temperate climatic areas of the Northern Hemisphere; however, no cases of acute intoxication in humans have been reported. Ochratoxin A was found more frequently and at higher concentrations in blood samples obtained from people living in regions where a fatal human kidney disease occurs and is associated with an increased incidence of tumors of the upper urinary tract (FAO / WHO, 2001).

Ochratoxin A was suggested to be possible determinant of endemic nephropathy (EN) & further observations also suggested that individuals with urinary tract tumors had evaluated levels of ochratoxin A in the blood & urine (Radovanovic *et. al.*, 1991). Considerable effects have been made to determine a correlation between human exposure to this toxin and the incidence of the disease.

Human endemic nephropathy (HEN) is a fatal human renal disease, recognized as a specific entity and affecting predominantly rural populations in limited areas of the central Balkan Peninsula. So far, the disease has been reported in Bosnia and Herzegovina, Bulgaria, Croatia, Romania, and Yugoslavia (Serbia). The disease was first

recognized in the 1950s (Tancev *et. al.,* 1956), but there is evidence that it occurred even earlier (Belicza *et. al.,* 1979).

The disease starts without an acute episode. Onset is common between the ages of 30 and 50, although there have been reports of patients aged 10 – 19 (Stoyanov *et. al.*, 1978). Its progress is very slow, and after development of nonspecific signs and symptoms there is atypical manifestation of renal impairment (Radonic *et. al.,* 1966). The effect on the primary tubules is characterized by a decrease in tubular transport and becomes evident through proteinuria. The ultrasonic appearance of the kidney is normal at the early stage of the disease, but it becomes smaller as the disease progresses (Borso, 1996).

Since there are neither characteristic clinical data nor pathogenomic laboratory indicators, the early diagnosis of HEN is difficult & relies on repeated findings of proteinuria, anemia, & a family history of the disease. As this disease cause a renal failure & atrophy of the kidney with multiple clinical features & it occurs in Balkan it is called Balkan Endemic Nephropathy (BEN) & it is linked to the appearance of ochratoxin A. The disease affects women more than men, and women die more frequently from BEN (Ceovic *et. al.*, 1992).

So far, studies on the etiological factors for BEN have not disclosed a single environmental causative agent of this puzzling disease. The data reject the possibility of a purely environmental causation of BEN. The pattern of BEN transmission in families at risk is not typical for single gene disorder. Extensive epidemiological & genetic studies have discovered some characteristics of the multifactorial (polygenic)

inheritance of BEN. There is evidence of a "familial tendency" variation of the risk-for BEN, depending on the number of sock parents & the degree of relatedness. The development of BEN in individuals is according to at-risk families who were born in non-endemic areas on another feature. Thus in general, genetic factors must play an important causal role (Ettlin, 2003; Wijnands & van Leusden, 2000).

The impact of additional environment and/or toxicological triggers on individuals genetically predisposed to BEN is supported by the followings: (1)Cytogenetic results of the increased frequency of floated sensitive Fra sites, (2)Spontaneous & radiation-induced chromosomal aberrations in BEN patients, (3)Data from detailed analysis of chromosomal breaks in BEN patients & control, (4)The occurrence of BEN in migrants. Hence, genetic & epidemiological approaches to the etiology of BEN have been proposed. Important factors are the transforming growth factor-beta (TGF-β), the genetic heterogenecity of xenobiotic-metabolising enzymes, and defects in the host's immune system. The predisposing genes for BEN patients with urinary tract tumors could be germ-line mutations in tumor suppressor genes of acquired somatic mutations in oncogenes (Ettlin, 2003).

2.6.3. Effect on Immunity:

Ochratoxin A causes immunosuppression following prenatal, postnatal and adult-life exposures. These effects include reduced phagocytosis and lymphocyte markers, increased susceptibility to bacterial infections and delayed response to immunization in piglets. In

adult mice natural killer cells (NK) activity is suppressed by ochratoxin A (Muller *et. al.*, 1999; Wijnands, & van Leusden, 2000).

Purified human lymphocyte populations and subpopulations are adversely affected by ochratoxin A *in vitro* (Lea *et. al.*, 1989). Further, B cells do not respond to polyclonal activators following a brief exposure to ochratoxin A. The authors suggested that the toxin causes immunosuppression through interference with essential processes of cell metabolism irrespective of lymphocyte population or subpopulation.

Ochratoxin A has known to have immunosuppressive effects in a number of species. Prenatal administration of ochratoxin A to rats caused immunosuppression, but prenatal administration stimulated certain aspects of the immune response in rats. The mode of action of ochratoxin A in the immune system is proposed through inhibiting the proliferation of B- and T-lymphocytes & affecting the late stage of T-lymphocyte activation *in vitro*. However, both the immuneological & teratogenic effects have been observed only at doses much higher than those causes nephrotoxicity (FAO / WHO, 2001).

Functional changes to splenocytes were not observed, which lead to the conclusion that low level exposure to ochratoxin A alters the relative & absolute numbers of lymphocyte organs but does not affect the immune functions (Thuvander *et. al.*, 1996a).

Muller *et. al.* (1995) found that in a mouse model ochratoxin A has a non-selective suppressive effect on various immune & defence system. Not only do weight depression, lymphopenia, neutrophilia, & eosiniphilia occur, but also antibody production & phagocytosis become suppressed.

In a previous study, the size of the mouse thymus was reduced to 33% that of controls after four intraperitoneal injections of ochratoxin A at 20 mg/kg bw on alternate days, a dose which caused minimal nephrotoxicity. Bone marrow depression was shown as dose-related, significantly decreased marrow cellularity, including a reduction in bone marrow macrophage–granulocyte progenitors, a decreased in the number of haematopoietic stem cells and a significant decrease in erythropoiesis as measured by ^{59}Fe uptake; decreased phagocytosis by macrophages was also observed (Boorman *et. al.*, 1984).

In another study applied by Singh *et. al.*, (1990) observing an immuno-suppression in chicken fed diets containing 0.5 or 2 mg ochratoxin A / kg of feed for 21 days. Compared to controls, treated animals had reduced total serum protein, lymphocyte counts, and reduce weights of thymus, bursa of Fabricius and spleen.

2.7. Chromosomal Studies:

Living organisms may expose to different kinds of effects which may cause chromosomal abnormalities (or aberrations) (CA). CA considered as a genetic damage of chromosomal level observed as an

alteration either in chromosome number or in chromosome structure (Al-Obaidy, 2003).

Numerical CA includes trisomy, monosomy, triploidy, & tetraploidy. A part from tirpoidy & tetraploidy involving sex chromosomes like the mosaic 45, X cell line, or it could involve autosomes that are usually presumed to be resulted from a germinal mutation. Ninety percent of numerical anomalies in recognized conceptuses terminated as fetal deaths; thus, a study restricted on live-births showed that there is a miss in a major proportion of chromosome as detectable abnormalities (WHO, 1985).

Studies in the resolution of chromosome substructure (high resolution banding) have significantly reduced the size of detectable lesions and such advances appear likely to continue. Thus, it is difficult to specify precisely the proportion of recognized conceptuses with a structural abnormality. With currently available technique, these are much less frequent than those with numerical abnormalities. Although numerical changes represent a significant proportion of human heritable genetic diseases, their sequences in somatic cells are less well characterized (WHO, 1985). Among live-births, the ratio of detected structural to numerical abnormalities is about 1:4 to 1:5 (Hook & Hamerton, 1977). Among human fetal deaths, the ratio is much lower, about 1:30 (Warburton *et. al.*, 1980).

Alterations in chromosome structure are more accurately assessed in somatic cells and are observed cytologically as CA or sister chromatid exchanges (SCE). Unlike numerical abnormalities, a significant

fraction of detected structural abnormalities are known to be inherited. Chromosomal aberrations can be studied in any cycling cell population, or in any non-cycling cell population that can be stimulated by a mitogenic agent to enter the cell cycle (WHO, 1985).

CA occur at various stages of mitosis (i.e. G1, S, G2, or M) and during meiosis. Auerbach, (1976) and Therman, (1980) provided comprehensive reviews of this subject. If breaks occur during the G1 stage, only one chromatid is affected. Both chromatids are involved in breakage take place during the S phase. When two breaks occur in the same chromosome, centric rings or acentric fragments are produced. There are certain autosomal diseases that show a high frequency of spontaneous CA or a high susceptibility to induce damage.

These effects could be resulted from a natural source such as physical effects like Ultraviolet light- ray (UVL), cosmetic ray, X-ray and heat; other chromosomal effects like: the effect of contaminated diet with chemical compounds or toxins of a chemical origin or may be by microbial toxins such as aflatoxins, ochratoxins, fusarium toxins & others. These compounds have the ability to defect the chromosomes (Brach and Hart, 1978).

It was reported that chemically induced aberrations present low sensitivity when compared to the frequency of other causes of CA. It is more appropriate to consider that the probability of producing aberrations in G_1 or G_2 cells following chemical treatments is low, but is considerably increased when treated cells are in, or pass through, the S-phase. Chemically – induced DNA damage in non-cycling

lymphocytes will not generally be converted into aberrations until the cells are stimulated to re-enter the cell cycle *in vitro,* and undergo DNA replication (Evans & Vijayalaxmi, 1980; Preston & Gooch, 1981).

The amount of DNA damage & CA caused by chemical agents depends on several factors including: (a) the dose received; (b) the value that can vary with the agent. (c) the time between exposure and sampling. (d) the amount of repair in cell cycle (WHO, 1985).

Generally, one of the most important CA is the Sister Chromatid Exchange (SCE) which is known to be manifestation of damage to the chromosome (Latt *et. al.,* 1981).

SCE results from the breakage and rejoining of DNA at apparently homologous sites on the 2 chromatids of a single chromosome. Other study showed that SCE and CA could be resulted from the effect of DNA damaging agents that cause DNA strand breaks in the treated animal leading to SCE and CA (Shubber and Al- Shaikhly, 1988; Shubber and Salih, 1988).

It is well documented that SCEs are produced during DNA replication (Wolff *et. al.,* 1974) and that the polarity of DNA is maintained in the process of exchange (Wolff & Perry, 1975).

The baseline of SCE may vary by a factor of two or more. Several potential sources of variation have been identified & they generally fall into 2 categories: (a) culture factor associated with the *in vitro* growth of the lymphocytes; (b) the biological factors associated with the genotype, lifestyle, or general health of the individual. Other factors those are unique to the individual being sampled include finding

variables as sex, age, diet, genotype, medication, & smoking. Each of these factors potentially plays a role in the induction or expression of SCE (WHO, 1985).

Baseline SCE frequencies are increased in some human diseases such as Bloom's syndrome (Chaganti *et. al.*, 1974) and multiple sclerosis (Sutherland *et. al.*, 1980; Vijayalaxmi *et. al.*, 1983a). Cells from patients with the inherited disease Xeroderma pigmentosum are hypersensitive to the induction of SCEs by UVL radiation and alkylating agents (Wolff, 1977).

There are many cytogenetic changes that may lead to cancer development including deletions, translocations and inversions. Deletions often resulting in loss of tumor suppressor gene, while translocations and inversions can be divided into those consistently found in certain tumor types (specific) and those observed only in the tumor from one patient (idiopathic) (Rabbitts, 1994).

Others have reported consistent defects in dozens of different cancers. The defects include translocations, associated mostly with leukemia and lymphomas, deletion or missing pieces, which occur in solid tumors such as lung and kidney cancer, and extra-chromosomes, which are linked to various other tumors (Gold, 1983).

Chromosomal mutations are operationally defined as changes in either chromosome number or structure observable with standard karyotypic techniques. More minute changes in DNA structure are classified as gene mutations, often referred to as "point mutations". It has to be noted that the covalent binding of chemicals or either reactive

metabolites to DNA is generally believed to be a key step in the initiation of carcinogenesis by genotoxic agents (SCF, 1998).

2.6.4. Effect of Ochratoxin A on Chromosomes:

The genotoxic effect may be explained by an indirect mechanism involving impaired protein synthesis. It has been shown that the covalent binding of chemicals or their reactive metabolites to DNA is generally believed to be a key step in the initiation of carcinogenesis by genotoxic agents (SCF, 1998).

Several studies have investigated on the carcinogenic effect of ochratoxin A in lab animals, especially mice. However, the exposure of mice to a single dose of ochratoxin A (about 2.5 mg / kg) through gastric intubations led to modification in DNA kidney, liver, and spleen after administration about 24 hours. Although ochratoxin A genotoxicity in all organs modifications were found, as shown in table (1), the level of changes differed in a tissue dependent manner with the kidney and liver being the most sensitive organs (Pfohl-Leszkowicz *et al.,* 1995).

The genotoxic effects, inhibition of DNA synthesis and mitosis, as well as histopathological effects on the nuclei of ochratoxin A-treated cells may be explained by ochratoxin A-inflicted DNA damage, which include DNA adduct formation and DNA single strand breaks (Wei & Sulik, 1993).

Table (1): Assay for Genotoxicity of Ochratoxin A.

Effect	Affected Cell	Concentration	Refrences
Gene mutation	Lymphoma cells Mammary cells	0.1-13 ppb 5-10 ppb	Bendele *et. al*., (1985b) Umeda *et. al*., (1977)
UDS	Hepatocytes	4-40 ppb	Mori *et. al*., (1984)
DNA damage	Spleen cells Kidney cells Liver cells	2500 ppb 600 ppb 1200 ppb	Pfohl-Leszkowicz *et. al*., (1991)
CA	Blood cells	1 ppb	Bose & Sinha, (1994)
Single-strand breaks	Spleen cells Kidney cells Liver cells	2500 ppb	Creppy *et. al*., (1985)
DNA adducts	Kidney cells Liver cells	400 – 500 ppb	Pfohl-Leszkowicz *et. al*., (1993a)

In vitro studies on cells from laboratory animals indicated that ochratoxin A cause inhibition of macromolecules biosynthesis (protein, RNA, & DNA). Macromolecules biosynthesis studies revealed that the lowest concentration of ochratoxin A that causes inhibition in DNA biosynthesis was 0.001mM (milli Molar), while protein synthesis was

inhibited at concentration of 0.01mM, and finally about 60 % of RNA synthesis was inhibited in concentration 1mM (Braunberg *et. al.*, 1992).

However, using different test conditions and/or different endpoints, ochratoxin A is reported to be able to cause DNA-strand breaks *in vitro* and *in vivo* studies (Dorrenhaus & Follmann, 1997), in addition to gene mutations in bacterial cells (modified Ames test) (Henning *et. al.*, 1991).

Maaroufi *et. al.*, (1994) proved that in renal biopsies taken from patients suffering from CIN, ochratoxin A binds to DNA (more specific to Guanine in DNA), thus, rendering the DNA less active and thus affecting the renal function.

Ochratoxin A causes DNA single-stranded breaks and DNA adducts in the DNA of spleen, liver and kidney in treated male mouse & in male and female of rats in both *in vivo* and *in vitro* studies and it gives rise to liver tumor in both sexes (Creppy *et. al.* 1985; Kuiper-Goodman & Scott, 1989; Pfohl-Leszkowics *et. al.*, 1991; 1993).

Several investigations studying the mode of action of ochratoxin A, suggesting inhibition of protein synthesis, formation of DNA adducts, and provocation of DNA single stranded breaks as a result of oxidative stress due to ochratoxin A effects (Luhe *et. al.*, 2003).

The highest DNA adduct levels were found in the target organs (kidney and bladder), being most persistent in the kidney (Pfohl-Leszkowics *et. al.*, 1993a; Obrecht-Pflumio *et. al.*, 1996). In addition ochratoxin A has also been showed to induce DNA-adduct in monkey

kidney cells & human bacterial cells in vitro (Grosse *et. al.,* 1995; 1997a).

An unusually large number of DNA adducts (up to 30 individual adducts) was formed from ochratoxin A in low yields in various experimental systems. Experimental observations support this hypothesis: Castegnaro *et. al.,* (1998); Pfohl-Leszkowicz *et. al.,* (1998).

Treatment of mice with oral doses of 0.6, 1.2, or 2.5 mg / kg bw ochratoxin A caused formation of DNA-adducts in the kidney & to a less extent in the liver & spleen. These adducts were measured after 24, 48, and 72 hours by using of modified ^{32}P-postlabelling method (Pfohl-Leszkowicz *et. al.,* 1991). but none of these adducts has been demonstrated to contain a fragment of ochratoxin A. it was, therefore; uncertain whether ochratoxin A interacts directly with DNA or whether it acts by generating reactive oxygen species (ROS) (WHO, 1985).

Thus, it was found that, at least some of those adducts might have been due to ochratoxin A-induced cytotoxic effects that generate reactive oxygen species (FAO / WHO, 2001).

Formation of DNA adducts has also been postulated as an important event in the tumorigenicity of ochratoxin A. It is obvious that ochratoxin A causes renal tumors by covalent binding of reactive intermediates to DNA, thus; this DNA damage considered due to oxidative stress represent an alternative explanation for the description data & is more consistent with observations (FAO / WHO, 2001).

The mechanisms of tumor induction in rodent kidney by ochratoxin A have been addressed in many studies, including investigations of the role of biotransformation and bioactivation, and the formation of ochratoxin A-derived nucleic acid derivatives in target and non-target organs for toxicity. The results diverge, as do those of the studies on mutagenicity (FAO/ WHO, 2001).

Ochratoxin A is negative in conventional mutagenicity tests carried out according to standard protocols, i.e. Ames test and tests for gene mutations and CA in mammalian cell cultures (SCF, 1998).

Gene mutation was induced in bacteria and mammalian cells in a few studies, but not in most, ochratoxin A did, however, induce DNA damage, DNA repair & CA in mammalian cells *in vitro*, and damage & CA in mice treated *in vivo* (FAO / WHO, 2001).

DNA single-stranded breaks were also observed *in vivo* in spleen, liver, or kidney cells of mice after intraperitoneal (IP) injection of ochratoxin A. DNA repair, manifested as UDS, was observed in most studies with primary cultures of rat & mouse hepatocytes, porcine epithelial cells from bladder & human urothelial cells (WHO, 1985).

In more details ochratoxin A induces (UDS) in rat hepatocytes in a very narrow concentration range. At 750 nM (nanoMole) a weak and at 1μM (microMole) a marked induction was observed. Concentrations less than 750 nM had no effect, while concentrations more than 1μM were cytotoxic. Similar experiments were performed using porcine urinary bladder epithelial cells, and similar results were found at slightly different concentrations. Between 350 and 1000 nM dose

dependent USD was observed, while concentrations less than 250 nM had no effect & concentrations above 1 nM were cytotoxic (Dorrenhaus & Follmann, 1997).

Ochratoxin A is thus, genotoxic *in vitro* & *in vivo*, but the mechanism of genotoxicity is unclear, and there is no evidence that it is mediated by direct interaction with DNA. The doses used in studies of genetic toxicity were in the same range as those at which the incidence of renal tumor was increased in mice. In rats the incidence of nephrotoxicity and renal tumors were increased at much lower doses (FAO / WHO, 2001).

Methotrexate:

One of the important genotoxic compounds is methotrexate (MTX). MTX is a folate antimetabolite. It is an analog of aminopterin, which is also derived from folic acid. The molecular structure of MTX differs from folic acid in that it has a hydroxyl group in place of the 4-amino group on the pteridine ring and there is no methyl group at the N position. Its administration leads to low white blood cells counts and it can decrease the number of the platelets ( ). This will lead to Anemia, hair loss & irritate the liver that causes a type of hepatitis. MTX could decrease the fertility and should be avoided in pregnancy and breast feeding because teratogenesis is a serious concern if MTX is administered during pregnancy. MTX crosses the placenta to the fetus and cause fetal abortion, congenital anomalies, & CA. MTX may influence cell proliferation including the synthesis of thymidylic acid, DNA nucleotide precursors, and inosinic acid, a purine precursor

required for DNA and RNA synthesis. Also MTX can also affect the CNS and cause chemical meningitis (Lederle and Mylan, 2002).

2.9. *Nigella sativa* (The black seed):

Nigella sativa (*N. sativa*) (black seed) is traditionally known in Middle Eastern countries as "Habbat al Barakah" - 'The Blessed Seed', due to its powerful healing qualities for different ailments. Black seed is believed to be indigenous to the Mediterranean region but has been cultivated into other parts of the world including Saudi Arabia, northern Africa & parts of Asia. Black seed has been used as a natural remedy, frequently used in folk medicine in the Middle East, some Asian countries, and Africa for more than 3000 years ago, and it is now well known in the USA and Europe for its promotion of good health and treatment of many ailments (El-kadi & Kandil, 1986).

Coequal names of its seed in Arab countries are Al-Habbah Al-Sawda (means black seed), Habbet el-baraka, Kamoun Aswad, and etc… In Pakistan, India and Sri Lanka it is called as Kalvanji, and in English language is known as black seed, black cumin and black caraway (Randhawa & Al-Ghamdi, 2002).

Clinical trials have validated the efficiency of black seed in promoting health & wellness. It is obvious that increasing the effectiveness in treating specific conditions, the healing principles of black seed in its pure, natural form should also be taken into account (Nooruddin, 2003).

The most pertinent point to be made about black seed is that it should be regarded as part of an overall holistic approach to health & ideally should be incorporated once every day lifestyle. In this way, the many nutritional & healing properties contained in the body's immune system over time, supplying it with the optimum resources, it needs to help prevent & fight illness. Black seeds are traditionally eaten alone or ground with honey (Jones, 2000).

2.10. Plant Description:

Black seeds (*N. sativa*) belong to the botanical family Ranunculaceae and commonly grow in Europe, Middle East, and Western Asia (Randhawa & Al-Ghamdi, 2002).

The black seed is an herbaceous plant, 15 - 60 cm high, the leaves are 2.5 - 5 cm long, and pale bluish purple or white flowers. The seeds are ovate, tiny & hairy, being no more 3 mm in length, and those seeds are originated from common fennel flower plant. The plant has divided foliage; the flowers grow terminally on its branches, while the leaves grow opposite each other in pairs on the either side of the stem. Its lower leaves are long, while the upper leaves are small of about 6 – 10 cm (Mukerji, 1953; Townseed, 1980).

Black seed reproduces with itself and forms a fruit capsule which consists of many white trigonal seeds. Once the fruit capsule has matured, it opens up and the seeds contained within are exposed to air becoming black in color.

2.11. Active Ingredient of black seeds (*N. sativa*):

Different methods were applied to identify the active component of black seed. It contains over 100 valuable nutrients including the following active ingredients: Fixed oils (32 – 40 %) involving saturated and unsaturated. Black seed contains a high rate of essential fatty acids such as linoleic, linolenic & oleic. These are important for healthness, because they are not produced by the body but eaten in food. These are just as vital to our body as a vitamin and mineral intake, as well as for hormone production (Gad *et. al.,* 1963; Babayan *et. al.,* 1978).

Also it was reported that linoleic and oleic are play a major role in protection against mutagens by blocking metabolic activation with enzyme and / or in trapping mutagen molecules (Giorgio, 1994).

Nowadays because our food is more and more denaturalized, our bodies are full of free radicals which produce cancer. The essential fatty acids in black seed bind the free radicals and eliminate them. Since our bodies are not able to synthesize thereby making these fatty acids, black seed an important addition to our diet (Tierra, 2004).

It contains carbohydrates (33.9 %), fibers (5.5 %); it was mentioned that black seed contains a non-starch polysaccharide component which is considered as a useful source of dietary fiber, and finally 6% water (Randhawa & Al-Ghamdi, 2002).

A literature search by the University of Potchefstroom (1989) revealed that black seed's capacity to increase the milk flow of nursing

mothers could be attributed to a combination of lipid portion and hormonal structures found in the black seed (Tierra, 2004).

The volatile oils is about (0.4 – 0.45 %) in the black seed showed anti-bacterial properties.

Volatile oil like: nigellone, thymoquinone and dithymoquinone (Badary *et. al.*, 2000) were detected in black seeds. Crystalline nigellone was first isolated & identified as providing many health benefits. Nigellone is known to be an important factor in the treatment of asthma and it is also causes inhibition of histamine release. Studies have shown that black seed protected against histamine induced bronchospasm in guinea pigs. Nigellone has a suppressive effect that inhibits protein kinase C, a substance known to trigger the release of histamine. Nigellone also, decreased the uptake of calcium in mast cells which also inhibits histamine release. These above studies are mentioned by Nooruddin, (2003).

The other active compound of black seed is thymoquinone which is considered as anti-inflammatory agent (Nooruddin, 2003); and also it was reported to be anti-oxidant agent (Nagi *et. al.*, 1999).

One of the main constituents in black seed is carotene, which is converted by the liver into vitamin A, is well known for its anti-cancer and anti-oxidant activity (Al-Azzawi, 1999). β- carotene, is considered to be a potent anti-oxidant, it is fat soluble protects fat stores and lipid membranes against oxidation (Giorgio, 1994).

The other ingredients involving: Proteins (16 – 19.9 %) that including 15 amino acid like arginine, leucine, lusine…etc. it was

reported that arginine is essential for infant growth (Babayan *et. al.*, 1978), and alkaloids like nigellicine & nigellidine. The main alkaloids of black seeds are diterpene alkaloids called nigellamines. In pharmacology experiments, the isolated alkaloids have been shown to lower cholesterol and triglycerides, coumarins which is known to stimulate the immune system (Kumara & Huat, 2001).

Also there is saponins involving triterpenes & steroids (Ansari *et. al.,* 1988), and minerals (1.79 – 3.74 %) like Calcium, Phosphorous, potassium, sodium & iron (Kumara & Huat, 2001) they are required only in small amounts by the body, these elements' main function is to act as essential cofactors in various enzymes functions. The elements known to be toxic to animals such as arsenate, uranium, and vanadium are well below 0.5 ppm level. Thus black seed is considered as safe food ingredient.

2.12. Uses of the Black seed:

Black seed (*N. sativa*) has been used historically for more than 3000 years ago, that it can play an important role in several kinds of treatment. Black seeds, in its complete, pure, natural form; act on the principle of assisting the body's own natural healing process in overcoming illness or maintaining health. It works on the part or system of the body affected without disturbing its natural balance elsewhere. The effect of black seed's natural combined, nutritional & medical value not only helps to relieve the current condition at hand,

but also helps the body build further resistance against future ailments or diseases (Randhawa & Al-Ghamdi, 2002).

The Greek physician Dioskorides used black seed to treat headaches, nasal congestion, toothache and intestinal parasites. Hypocrates, the grandfather of today scientific medicine regarded black seed as a valuable remedy in hepatic and digestive disorders. Black seed was also to be found in the tombs of Pharoahs, including that of Tutenkamun, believing that they could use it to fight sicknesses in the afterlife. It is known to have been used by Cleopatra for her health & beauty giving qualities (Nooruddin, 2003).

For nearly three millenniums, Arabs have proved to be adept pharmacists, blending and mixing herbs to enhance their therapeutic value. Islamic medical and scientific studies show that they also used the black seed oil to treat colds, head and toothaches, as well as for general well-being, to boost the immune system, and against inflammations and skin disorders by improving skin elasticity. Our Islamic profit Mohammad wrote in ancient script how black seed prevents and indeed heals many illnesses, all except death itself (Abu-Abdulla, 1984).

Ibn Sina (980-1037) in describing the black seed as that which “stimulates the body’s energy & helps recovery from fatigue or dispiritedness”, still holds true to “Tibb” (the Islamic Medicine) health practitioner today. The rich nutritional value contained in black seed as

outlined by scientific analysis of black seed, also points to it as a great source of energy.

As mentioned before black seed has a powerful healing quality for many ailments. Examples of these ailments & diseases are: Acne, cold & flue, lethargy, nervous tension, healthy complexes, high blood pressure, diarrhea, hair loss, headache, earache, sinusitis, and also hormone imbalances which can occur during menopause for instance can be treated (Randhawa & Al-Ghamdi, 2002).

Black seed when taken regularly aids a variety of sicknesses including flatulence, stomach and bowel discomfort, keeping the stomach and bowel system free from infection and bacteria. It is also used for scorpion and spider stings and bites of snake, cat and dog. Black seed provides nutritional support for the body's defense system. In addition, it is used as a flavoring additive to bread and prickles (El-kadi & Kandil, 1986).

The multiple uses of black seed *(N. sativa)* in the folk medicine encouraged many investigators to isolate the possible active components and to conduct *in vivo* and *in vitro* studies on laboratory animals and human beings in order to understand its pharmacological actions. These include immune stimulation in human and animal that administration of 1gm *N. sativa* seed twice daily in human volunteers enhanced immune functions (El-kadi & Kandil, 1986), anti-inflammatory & anti-allergic (Gilani, 2001); a group of scientists decided to test the effectiveness of the fixed oil of black seed and its

derivative, thymoquinine, as an anti-inflammatory agent, and in the traditional medicine to treat a wide range of diseases including diarrhea and asthma. Their study found that the oil inhibited eicosanoid generation and demonstrated anti-oxidant activity in cells. The inhibition of eicasanoid generation, however, was higher than could be expected from thymoquinone alone (Houghton *et. al.*, 1995).

Black seed has anti-parasitic properties (Akhtar & Riffat, 1991) it was found that black seeds can prevented liver damage induced by *Schistosoma mansoni* infection in mice (Mahmoud *et. al.*, 2002), and anti-microbial (El-Fatatry, 1975).

The activity of the black seed was compared with five antibiotics: ampicillin, tetracycline, cotrimoxazole, gentmicin, and nalidixic acid. The oil proved to be more effective against many strains of bacteria including: *V. cholera, E. coli,* and all strains of *Shigella* species except *S. dysenteriae.* These results suggest that of black seeds would probably be a good therapeutic agent in the treatment of diarrhea (Nooruddin, 2003).

The anti-cancer (Salomi *et. al.* 1991) and anti-oxidant (Nair *et. al.* 1991), black seeds and some of its active principles have been shown to possess protective effect against haematological, hepatic, renal and other toxicities induced by anti-cancer drugs and some toxins.

Also black seeds have the ability to decrease the blood sugar of healthy human volunteers treated with 1 gm of *N. sativa* seed capsules

twice daily, i.e. black seeds is essential for treatment of diabetes (hypoglycemic effects) (Bamosa *et. al.*, 1997), etc.

In one particular study in Munich, 600 allergy patients were given 500 mg of black seed oil twice a day for 3 months. A clear improvement was observed in 85% of the patients. The oil can be mixed into any cold liquid or yogurt. However black seed may be used as a therapeutic aid together with the exclusive sickness that require more immediate action as a treatment in serious medical complaint (Tierra, 2004).

The efficacy on black seed as alternative to vaccination and antibiotics in poultry as well as to see the growth performance of growing turkeys supplement with black seed was evaluated. Twenty five percent of the birds that received 0 % black seed diet developed signs of infection (green and watery faeces and refused feed) after the 3rd week. This condition was replaced with one containing 5 % black seed for 8 weeks. This study suggested that black seed meal was potential as an alternative to antibiotics and vaccination as well as an ingredient in poultry diet (Al-Azzawi, 1999).

2.12.1. Effect on the immune system:

In recognizance of its substantial nutritional components, as well as its specific medical properties, the body's ability to maintain health & promote healing of a lasting nature is best increased through regular use of black seed.

As a natural remedy people take black seed which can play an important role to enhance human immunity, particularly in immunocompromise proteins (El-kadi & Kandil, 1986).

Black seed could be also suitable for diseases of the immune system itself, e.g. allergies; tuberculosis (TB); cancer; AIDS, etc… The immune system is strengthened with regular doses, and as scientists at the Immuno Biology Laboratories in South Carolina have reported, regular doses of this black seed extract actually stimulate bone marrow and immune cells. Cell damaging viruses are reduced as the black seed increases the number of anti-bodies which produce the B-cells in the body. All these functions make black seed an ideal supplement to human everyday in order of well-being (Randhawa & Al-Ghamdi, 2002).

Haq *et. al.* (1999) revealed that black seed can enhance the production of interleukin-3 (IL-3) by human lymphocytes when cultured with pooled allogenic cells or without any added stimulator and has an effect on macrophages as well. When cells treated with black seed proteins produced greater amounts of cytokines, specifically interleukin-1-beta (IL-1-β) and tumor necrosis factor alpha (α).

However, Stimulant effect was observed with fractionated black seed proteins (P1 and P2) with a maximum effect at $10\mu g / ml$. The stimulatory effect of whole *N. sativa* and its fractionated proteins was noticed on the production of TNF-α in both non-activated and mitogen activated cells (Haq *et. al.*, 1999; Randhawa & Al-Ghamdi, 2002).

It was also established by El-kadi & Kandil, (1986) that four weeks after administration of black seed to volunteers the majority of complete lymphocyte count displayed a 72% increase in T-helper cells to T-suppress cells ratio, as well as an increase in natural killer cells (NK) functional activity. They reported: “These findings may be of great practical significance since a natural immune enhancer like the black seed could play an important role in treatment of cancer, AIDS, & other disease conditions associated with immune deficiency state”.

2.11.2. Effect on tumor calls:

The anticancer activity of black seed was first noticed by El-Kadi and Kandil, (1986) who observed enhancement of natural killer (NK) activity ranging from 200-300 % in advanced cancer patients receiving multimodality immunotherapy program in which *N. sativa* was one of the components. Later on, the anti-cancer effect of black seed was investigated both *in vitro* using cancer cell lines and *in vivo* using animal models (Randhawa & Al-Ghamdi, 2002).

Black seed is considered as a biological response modifiers because studies show that extracts from the seeds are toxic to cancer cells and, in mice, prevent blood cell toxicity caused by the anti-cancer drug cisplatin (Nair *et al.*, 1991).

The active components of black seeds, thymoquinone and dithymoquinone, both of which inhibit tumor cells in laboratory experiments; even tumor cells which are resistant to anti-cancer drugs (Worthern *et. al.*, 1998). A cell study conducted at the International

Immuno-Biology Research Laboratory in South Carolina showed that on incubation with black seed, cancer cells were unable to produce fibroblast growth factor and the protein collagenase, both necessary for blood-vessel growth into the tumor. Without a blood supply, a tumor cannot grow (Medenica, *et. al.*, 1997).

Thymoquinone and dithymoquinone, active principles of black seed that had cytotoxic effect against parental and multi-drug resistant human tumor cell lines which were over 10-fold more resistant to doxorubicin and etoposide (Worthern *et. al.*, 1998). Similarly, thymoquinone reduced the incidence and multiplicity of benzo-a-pyrene induced forestomach tumour in female Swiss albino mice by 70 % and 67 %, respectively (Badry & Gamal El-din, 2001).

Another experiment indicated that thymoquinone may also prevent some toxic side effects of cancer treatments. Scientists from King Saud University in Saudi Arabia have found that mice pretreated with thymoquinone were protected from carbon tetrachloride-induced liver toxicity which may be how it protects the liver. Carbon tetrachloride is a toxic compound that in small amounts can kill the animal by causing the liver and kidney to atrophy. Thymoquinone also demonstrated antioxidant activity (Nagi *et. al.*, 1999).

Topical application of black seed extracts inhibited two-stage initiation/promotion of skin carcinogenesis in mice by delaying the onset of papilloma formation and reducing the number of papillomas per mouse (Salomi *et. al.* 1991).

Moreover, using an active principle of fatty acids derived from black seed demonstrated, *in vitro,* in Swiss albino mice showed that this active principle could completely inhibit the development of a common type of cancer cells called Ehrlich ascites carcinoma (EAC) (Randhawa & Al-Ghamdi, 2002). Mice which had received the EAC cells & black seed remained normal without any tumor formation, illustrating that the active principle was 100% effective in preventing EAC tumor development (Salomi *et. al.* 1992).

A second common type of cancer cells called Dalton's lymphoma ascites (DLA) cells, *in vivo,* were also used to inhibit its development in mice. Results in mice, received DLA cells & black seed showed that the active principle had inhibited tumor development by 50% less compared to mice not given the active principle (Salomi *et. al.* 1991).

The study of Salomi *et. al.*, (1991) & (1992) concluded that:

"It is evident that the active principle isolated from *N. sativa* seed is a potent anti-tumor agent, & the constituent long chain fatty acid may be the main active component".

Chapter Three
Materials & Methods

3.1. Materials:

3.1.1. Equipment and Apparatus:

The following equipment & apparatus were used in this study:

Apparatus	Company
Autoclave	Gallenkamp (England)
Centrifuge	Gallenkamp (England)
Water bath	Gallenkamp (England)
Cold incubator	Gallenkamp (England)
Electric balance	Metler (Switzerland)
Electric oven	Gallenkamp (England)
Hot plate	Gallenkamp (England)
Micropipette	Gelson (France)
Microscope	Olympus (Japan)
PH-Meter	Orien Research (USA)
TLC plates	Merk
U.V. light lamp	CAMAGll, 1987
Vortex Mixer	Bunchi (Germany)
Scanning densitometer	Bunchi (Germany)
Laminar air flow	Metalab (France)

Magnetic stirrer	Retsch (Germany)
Rotary Evaporator	Merk
Electric shaker	Merk

3.1.2. Chemical Materials:

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

Material	**Company**
Chloroform	BDH
Celite	BDH
Anhydrous Sodium Sulphate	BDH
Methanol	BDH
Hexan	BDH
Ethanol	BDH
Glacial acetic acid	Fluka
Czapek's dox agar	Oxoid
Hydrochloric acid (HCl)	BDH
Potassium chloride (KCl)	Fluka
Tween 80	Oxoid
MTX	Sigma chemical company
Heparin	Denemarca
RPMI 1640 (1X)	Flow laboratories-V.K.

Glucose	Sigma chemical company
Hepes	Sigma chemical company
Calcium Chloride ($CaCl_2$)	Sigma chemical company
Sodium pyruvate	Sigma chemical company
Sodium bicarbonate (Na_2HCO_3)	Sigma chemical company
Potassium phosphate (KH_2PO_4)	BDH
Ochratoxin A standard	Sigma chemical company
Antibiotics (Penicilline, Streptomycin & Amphotricin)	Samara drug factory
Gimsa stain	Fisher
Acetonitrile	BDH
Benzene	BDH
aluminum chloride ($AlCl_3$)	BDH
methanolic sulphuric acid	Fluka
Colchicine	AL-Hikma
Sodium sulphate (Na_2SO_4)	Sigma chemical company
Silica gel	Merk

3.2. Methods:

3.2.1. Spore suspension

The isolate of *Aspergillus ochraceus* which used in this study was received from Mycology Laboratory of Biotechnology Department / Collage of Science / University of Al-Nahrain).

Slants containing Czpek's dox agar medium were inoculated with the isolate of *Aspergillus ochraceus* the slants were incubated at $25^{\circ}C$ for 7 days and kept at $5^{\circ}C$ in the refrigerator until use.

Spore suspension were prepared according to Faraj method (Faraj, 1990), spores were harvested by adding 5ml of sterilized distilled water (D.W.) containing 0.1% tween 80 to aid wetting & 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 tubes, further washed with D.W. The spore suspension was then centrifuged at 3000 r.p.m for 5 minutes. The supernatant was removed & the spores were washed twice by resuspending in sterile D.W. and further centrifuged. Then 5ml of sterile D.W. was added to the supernatant & mixed vigorously by the vortex for 1 minute. One drop of the suspension was added to haemocytometer by Pasteur pipette, spores were calculated using light microscope under large power X 40 of and the following equation (Faraj, 1990):

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

Where:

n: total number of squares.

Z: total number of spores.

3.2.2. Production of ochratoxin A on natural substrate

The ochratoxin A producing fungi were tested for their ability to produce ochratoxin A according to Faraj, (1990) using corn (or barley) as a substrate.

1. 50 grams of corn grains were weighted & grind then placed in 250 ml conical flask.
2. 11 ml of D.W. was added to the weighted corn.

The initial moisture content was 13%, the required moisture calculated according to 0.98 water activity (98% Rh) was 28%.

$$X = w (y – a) / 100 – y$$ (Al-Samarraie, 1997)

Where **X:** water to be added (ml).

y: required distilled water.

a: initial distilled water.

w: weight of the sample (50 gm).

$X = 50 (28-13) / 100 - 28 = 11$

3. The flasks were allowed to stand for 2 hours with frequent mixing & then sterilized for 20 minutes at $121^{\circ}C$ & under pressure 15 pounds / in^2.
4. Each flask were inoculated with 1ml of spore suspension of fungal isolate, and inoculated for 7-14 days at $27^{\circ}C$.
5. The flask were shaken every day by hand to loosen any clamps of mould growth and to redistribute the moist grains from the bottom.

6. After incubation time the flasks were dried in oven at $60^{\circ}C$ for 24hours then the ochratoxin A was extracted as mentioned below.

3.2.3. Extraction of ochratoxin A

According to the method of Hald & Krogh, (1973), the extraction involved the following steps:

1. 20 ml of D.W. was added to 50 gm of the sample (grounded corn) and mixed by using the magnetic stirrer.
2. Hydrochloric acid (HCl) of 6N was added to make the PH of the sample about (2-3).
3. 100 ml of chloroform was taken with 5 gm of celite with continuous shaking by the electric shaker for 30 minutes.
4. The mixture is allowed to pass through Anhydrous Sodium Sulphate (Na_2SO_4) by a filter paper.
5. The resulting solution collected & evaporated by the rotary evaporator.
6. In a clean flask, a mixture of 50 ml methanol & 75 ml hexane was added to the remaining component.
7. The mixture was added to the separatory funnel with continuous shaking, and then the funnel was left to be separated into two layers.

8. The last step is repeated for three times & in each time the methanol layer was taken & discarded the hexan layer which contains the unwanted impurities.
9. Again the resulting extract was passed through the (Na_2SO_4) on the filter paper.
10. The extract was evaporated to dryness.

3.2.4. Preparation of standard toxin

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

3.2.5. Quantification of ochratoxin A by thin layer chromatography (TLC)

Silica gel TLC plates F366 ($200\times200\times0.25$ mm) were activated in the oven at 110°C for one hour; a line was scored on TLC plates with 1.5cm from each side and 2cm from top & bottom of the plate, the extract containing the ochratoxin A was dissolved in 100 ml of benzene/acetonitrile (98:2). Samples were spotted at size of 0.1 ml with 1.5 cm intervals & the standard of ochratoxin A was spotted at

concentration 1μ/ml, then the plate was left to air dry, & inserted in to a chamber containing developing solvent in vertical position & dark place. The plate was removed from the chamber, when the developing solvent reached 18 cm then allowed to dry in dark hood, & examined under UVL with determination of R**f** value, the amount of ochratoxin A was estimated in comparison with standard ochratoxin A using the scanning densitometer.

3.2.6. Confirmatory test

Two chemical methods were used for the confirmatory test of ochratoxin A:

a. Gimeno method (1983):

The plate was sprayed with aluminum chloride solution ($AlCl_3.6H_2O$) which prepared by dissolving 20 gm of aluminum chloride in 100 ml methanol. Then the plate was placed in the oven at 151°C, cooled & examined under UVL at 366 - wave length, the R**f** value was estimated in comparison with standard ochratoxin A.

b. Abdelhamid method (1995):

The plate was sprayed with solution of methanol sulphuric acid: methanol (20:100), & placed in an oven at 105°C for 2 min, then cooled & examined under UVL, R**f** value was estimated in comparison with standard ochratoxin A.

3.3. Laboratory animals:

Thirty white Swiss mice belong to the species of *Mus muscless* which obtained from Biotechnology Research Center / Al-Nahrain University with weight 25-30 grams were used. They were divided into tow separated groups. The first one was given the ochratoxin A orally for 20 days, and then divided into 7 subgroups according to the ochratoxin A concentration to be tested. The second group was given the ochratoxin A in feed as contaminated diet, which also divided into 5 subgroups according to the type of feeding. Each group of the above includes 3 mice that were put in a separated plastic cage, and those cages were kept at normal conditions: $23-25^{\circ}C$ room temperature, day sun light and normal humidity. In addition to that, those animals of the ochratoxin A oral administration were fed with a suitable quantity and quality of complete diet & water which include the locally mixed feeding from the following materials:

Product	Percentage (%)
Crushed barley	24.50
Crushed wheat	30.00
Crushed yellow corn	22.50
Soya bean	15.20
NaCl	0.45
Calce stone	0.20
Animal protein	7.15

3.4. Exposure of mice to ochratoxin A

A. Ochratoxin A oral administration

The ochratoxin A was diluted with 50% ethanol to make tested doses. In order to achieve the appropriate dose that affect the animal without killing it, the following procedure was administered to limit the acute dose:

5 ppb / 1 ml	animals killed directly after administration
2.5 ppb / 0.5 ml	animals dead after 2 days
2 ppb / 0.4 ml	animals dead after 7 days

1.5 ppb / mouse were the acute dose, which is the final dose that affects the animal extensively without killing it. The results were found after experimenting on 5 mice for this purpose.

Groups of mice which were orally administered ochratoxin A were as the followings:

Group I: Normal mice (control).

GroupII: Animals treated with 0.1ml PBS which is considered as a (-ve) control.

Group III: Animals treated with 2.5 mg / 0.1 ml MTX which is considered as a (+ve) control.

Group IV: Animals treated with 0.2 ppb/mouse ochratoxin A.

Group V: Animals treated with 0.5 ppb/mouse ochratoxin A.

Group VI: Animals treated with 1 ppb/ mouse ochratoxin A.

Group VII: Animals treated with 1.5ppb/mouse ochratoxin A.

The doses were given orally for 20 days (daily treatment), then blood was collected by heart puncture & spleen was taken after anatomy & cut into small pieces, to get white blood cells, were taken for the cytogenetic study.

B. Mice fed ochratoxin A contaminated diet

This treatment involved 5 subgroups. Each mouse was fed a complete diet as followings:

Group I: Normal mice that fed normal feeding & considered as a control.

Group II: Animals fed normal diet containing black seed in ratio 1:1.

Group III: Animals fed diet contaminated with the culture of the fungus that produced ochratoxin A, (*Aspergillus ochraceus*), it equals to about 50 ppb as measured before by using scanning densitometer after extraction.

Group IV: Animals fed diet contaminated with the culture of the fungus that produced ochratoxin A, (*Aspergillus ochraceus*); and the black seed at the ratio 1:1.

Group V: Animals fed a diet containing black seed for two weeks, after that the animals fed diet contaminated

with the fungus *Aspergillus ochraceus* for another two weeks.

The protection ratio of the black seed against ochratoxin A was estimated by using the following equation according to Rawat *et. al.*, (1997):

$$\textbf{Protection ratio} = \frac{\text{Ochratoxin A} - \text{Ochratoxin A with Black seed}}{\text{Black seed with Ochratoxin A}} \text{ X } 100$$

$$\text{Ochratoxin A} - \text{Control}$$

3.5. Chemical Preparations:

1. Colchicine: This chemical was purchased from Al-Hikma pharmaceutical products, and was prepared by dissolving one tablet (0.6 mg) of colchicine in 0.6 ml of D.W. It was obtained as sterile solution & stored at 5° C.

2. Phosphate Buffer Saline (PBS): the solution was prepared by dissolving of the following chemicals in 1000 ml of distilled water the PH was adjusted to 7.2

Sodium chloride (NaCl)	8gm
Potassium chloride (KCl)	0.2gm
Sodium phosphate hydrate (Na_2HPO_4)	1.15 gm
Potassium phosphate dihydrat (KH_2PO_4)	1.15gm

3. Potassium chloride (KCl): the hypotonic solution was prepared by dissolving 5.587 gm of KCl in one liter D.W. to get 0.075M concentration of KCl, the solution was kept in 4° C until use.

4. Fixatine Solution: The fixative used for cytogenetic studies was a freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid, and then kept in 4˚C until use.

5. Phytohaemaglutinin (PHA): PHA was obtained as sterile solution from Biotechnology Research Center, and then stored at 20° C.

6. Giemsa Stain: This stain was prepared by dissolving 2 mg of Giemsa in 100 ml of methanol with stirring on a hot plate at 37° C for 5 min and left it at room temperature with stirring for 2 hours. The stain solution was then filtered through filler paper and stored at room temperature. The stain was diluted in ratio of 1:4 with PBS or distilled water immediately before use (Yaseen, 1990).

7. RPMI 1640 medium: This medium contained the following components in one litter of distilled water (Freshney, 1994):

RPMI 1640 medium base	10.4 gm/litter
Newborn bovine serum	15 %
Glucose	2.5 gm
Hepes	5.986 gm
$CaCl_2$	0.1 gm
Sodium pyrouvate	0.1 gm
Sodium bicarbonate	2 gm

Penicillin	0.006 gm
Streptomycin	0.013 gm
Amphottricin	3 ml

The volume was completed to 1 litter by D.W.

3.6. The Genetic Experiments:

3.6.1. Chromosomal preparations from blood & spleen cells of the mouse:

The experiment was done according to (Verma and Babu, 1989) as follows:

1. The animal was anesthized by chloroform.
2. The animal was fixed on his ventral side on the anatomy plate and the abdominal side of the animal and its thigh region was swabbed with 70% ethanol.
3. The blood was collected by heart puncture using sterile heparinized syringe by cervical dislocation
4. About 0.3ml or 6-7 drops of heparinized whole blood was inoculated in RPMI 1640 culture medium supplemented with(0.3 – 0.5)ml PHA in sterilized conditions.
5. The spleen was cut from the abdominal cavity of the mouse and put in test tubes with PBS solution.
6. The spleen was cut into small pieces and dispersed with a Pasture pipette.

7. The solution was taken from these test tubes and transferred into another sterile test tube containing RPMI 1640 medium adding to it (0.3 – 0.5)ml PHA leaving the remaining of the spleen organ.
8. The culture medium was incubated in CO_2 incubator at $37^{\circ}C$ for 71 hours with frequent shaking every 24 hours.
9. After the 71 hours of incubation 0.1ml of colcemide was added to each tube with mild shaking and transferred back to incubator.
10. After one hour of incubation the culture was centrifuged at 1500 r.p.m for 10 minutes.
11. The supernatant was discarded by pipetting of media, leaving as little medium as possible over the cell pellet.
12. Then the pellet was resuspended in 5ml of KCl as a hypotonic solution (0.075M) drop by drop with continues gently shaking (Hypotonic solution must be prewarmed to $37^{\circ}C$ before use), and then the test tubes get back to the incubator for 30 minutes at $37^{\circ}C$ and the tubes were shacked from time to time.
13. After the 30 minutes the tubes were centrifuged at 1500 r.p.m for 10 min. The supernatant was removed leaving little volume as possible over the cell pellet.
14. The fixative solution was added as drops on the inside wall of the test tube with the continuous shaking, and then, it was fixed to 5ml and the content was shaked well. In this step, culture tubes can be kept in refrigerator ($4^{\circ}C$) until use.

15. The culture tubes where centrifuged at 1500 r.p.m for 5 minutes.
16. The last step was repeated for three times at lest until the suspension become clear.
17. After the final centrifugation, the cells were suspended in a small volume of fixative (approximately in the range of 1–3 ml), depending on the size of the cell pellets to give a slightly opaque suspension.

The final suspension in fresh fixative may be used immediately to make slides or may be stored for several days before the slides are made, storage should be at 4° C or -20° C.

3.6.2. Slide preparation (Yassen, 1990):

The slides used must be cleaned from any blot or impurity to get perfect results in the slide examination. By a Pasture pipette few drops from the tube were dropped vertically on to wet, chilled, grease free slide from at least a height of 30 cm at a rate of (4-5) drops to give the chance for the chromosomes to spread well. Later, the slides were dried on a hot plate at 50˚C for 1 minute. After that staining method were applied. Three slides for each animal were prepared for cytogenitic assays.

3.6.3. Staining:

Slides were stained with freshly made Giemsa stain (1 part Giemsa stain to 4 parts PBS or D.W.) for 20 min, and then washed with distilled water at room temperature, and then the slides were examined under the microscope to observe the chromosomes at metaphase.

3.7. Cytogenetic Analysis Test:

3.7.1. Mitotic Index (MI) Assay:

The slides were examined under high power (40X) of compound light microscope and (1000) of divided and non divided cells were counted and the percentage rate was calculated for only the divided ones according to the following equation (Shubber & Al-Ala'ak, 1986):-

$$\textbf{Mitotic index (MI)} = \frac{no\ of\ the\ devided\ cells}{total\ no.of\ the\ cells\ (1000)} \times 100$$

3.7.2. Chromosomal Aberration (CA) Assay:-

In this procedure, the prepared slides were examined by light microscope using the oil immersion lens, scanning of every 100 divided cells per each animal or blood lymphocytes culture, and the cells should be at the metaphase stage of the mitotic division where the

chromosomal aberrations are clear and the percentage of these aberrations like (chromosome breaks, ring chromosome, acentric chromosome, etc…) could be estimated.

3.8. The Statistical Analysis:-

Date was analyzed statistically using the general linear model procedure of SAS system (SAS, 1992). Whenever there was a significant effect, Duncan's Multiple Range Test (Duncan, 1955) was used for comparison of means.

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Chapter Four
Results & Discussion

4.1. Production & Extraction of Ochratoxin A:

Ochratoxin A gave a blue fluorescence under UVL. The results by scanning densitometer indicated that the isolate of *A. ochraceus* is efficient for production of ochratoxin A in concentration of 50 ppb. The result was identical with that of the standard toxin used on TLC; while the concentration of ochratoxin A produced by this isolate is low in comparison with that of Al-Naimy, (2001) which produced ochratoxin A of 300 ppb.

In a study applied by Bayman *et. al.*, (2002); revealed that the concentration by the isolate of *A. ochraceus* was 10 ppb. The relatively low ochratoxin A production observed may reflect the decline of ochratoxin A production during long time of maintenance in culture.

4.2. Toxicity of ochratoxin A:

The results revealed that mice have response to both, ochratoxin A oral administration & ochratoxin A contaminated diet. In ochratoxin A oral administration experiments, the highest dose 5 ppb/mouse resulting in killing the animals (table 2); otherwise the lethal effect was delayed with decreasing the toxin dose until reach to 1.5 ppb/mouse in

a period of treatment for 20 days. These results ensure that ochratoxin A has toxic & lethal effects on mice.

A study mentioned by FAO / WHO, (2001); showed that ochratoxin A has toxic & lethal effects in mice treated orally in the concentration of 1 ppb.

Table (2): Responding of mouse to oral administration with ochratoxin A.

Giving Dose of OTA	Observations
5 ppb	Killed directly after administratio
2.5 ppb	Death occurred after 2 days
2 ppb	Death occurred after 7 days

During the period of treatment with ochratoxin A orally & with contaminated diet, pathological symptoms were observed on the treated animals. The first symptoms to be observed is the increase in mice body weight (table 2); this may be attributed to swelling and hemorrhage of liver, kidney, spleen & pancreas due to ochratoxicosis. These results agreed with previous study published by Al-Naimy, (2001) who studied the effect of ochratoxin A on male rats liver.

In another study, treating of chicks on diet containing 300 ppb showed increase in a relative weight of several organs (liver, kidney, spleen, and pancreas) (Elissalde *et. al.*, 1994).

Table (3): Increase in body weight per gram (bw/gm) of mice treated orally and with diet contaminated with ochratoxin A.

Days / Groups	1	6	12	18	24	30
Group I (control)	30.25	30.3	30.5	30.5	30.8	31.06
Group II (BS)	29.3	30.03	30.1	30.5	31.5	32.08
Group III (OTA)	30.4	31.78	33.34	34.5	35.35	36.04
Group IV (OTA + BS)	27.45	30.55	32.7	34.6	35.25	36.19
Group V (BS +OTA)	28.06	30.7	33.46	34.7	34.95	35.5

BS: Black Seed.

OTA: Ochratoxinn A

OTA + **BS:** Ochratoxinn A treated with Black Seed.

BS + **OTA:** Pretreatment with Black Seed then Ochratoxinn A contaminated diet.

Other symptoms observed were: hair loss, diarrhea, shivering, dyspnea, emaciation, irritation around the neck & anomalies of the eyes, in addition to the aggressive behavior of the mouse. All signs mentioned have referred to the action of toxin resulted from mycotoxicosis.

4.3. Effect of ochratoxin A on mouse blood & spleen cells:

Different pathological effects were seen involving the above mentioned pathological symptoms. Also there are different genotoxic effects seen under the effect of ochratoxin A including decrease in mitogenic activity (effect of stimulating cell division) and increase in level of different chromosomal aberrations. The degree of these aberrations varies depending on the type of treatment or administration as follows:

A. Ochratoxin A oral administration:

Depending on our results, it is clear that ochratoxin A concentrations of oral administration have a significant effect ($P \leq 0.01$) in the mitogenic activity of blood & spleen cells, as the ochratoxin A concentration increased particularly when compared with the normal & the (-ve) control. In addition to that, it is obvious from tables (4, 5, 6 & 7) that the four concentrations have no significant differences with each others but they are significant in comparison with methotrexate (MTX) treatment.

The results of the CA were shown with highly significant ($p \leq 0.01$) clastogenic effects. Those CA were observed in cells of mice treated with ochratoxin A at different concentrations like structural chromosomal changes including: chromosome break (figure 2),

chromatid breaks (figure 9), dicentric chromosome (figure 5), acentric chromosome (figure 3, 7, 8), ring chromosome (figure 6), & end to end chromosome (figure 2, 5, 6, 8) etc...

Also ochratoxin A has the ability to cause numerical chromosomal changes including aneuploidy and polyploidy (figure 4).

Toxin concentration of 1.5 ppb gave the maximum level of CA, which are similar to those caused by MTX. According to these results, it is obvious that the incidence of aberrant cells & number of aberration per cell were dose dependent. Ochratoxin A oral administration also causes a decrease in MI.

This was also mentioned by Bose & Sinha, (1994); who reported that oral administration of ochratoxin A to mice at concentration of 1 ppb causes increase in the production of abnormalities in both mitotic & meiotic chromosomes in the treated animal.

Table (4): Structural chromosomal aberrations in mouse blood cells caused by ochratoxin A oral administration.

Parameter / Doses	MI	Chromatid Break /100 cells	Dicentric /100 cells	Acentric /100 cells	Ring Chromosome /100cells	Total
Normal	6.62 ± 0.40 a	1.33 ± 0.33 d	0.0 ± 0.0 d	0.0 ± 0.0 c	0.0 ± 0.0 c	1.33 ± 0.33 e
0.1ml PBS (-ve control)	6.45 ± 0.26 a	1.67 ± 0.67 d	0.0 ± 0.0 d	0.0 ± 0.0 c	0.0 ± 0.0 c	1.67 ± 0.67 e
2.5 mg MTX/mouse (+ve control)	4.71 ± 0.68 b	14.67 ± 3.76 a	7.67 ± 0.33 a	14.67 ± 3.76 a	6.67 ± 1.2 a	43.64 ± 1.45 a
0.2ppb OCA / mouse	2.97 ± 0.36 c	4.0 ± 0.58 cd	2.67 ± 0.58 c	4.0 ± 0.58 b	2.67 ± 0.67 b	13.24 ± 0.89 d
0.5 ppb OCA / mouse	3.54 ± 0.33 c	4.67 ± 0.89 cd	3.33 ± 0.58 c	5.0 ± 0.58 b	2.0 ± 0.58 b	15.0 ± 1.53 d
1 ppb OCA / mouse	3.24 ± 0.44 c	7.67 ± 0.89 bc	5.0 ± 1.0 b	6.67 ± 0.33 b	3.67 ± 0.88 b	23.01 ± 0.58 c
1.5 ppb OCA/ mouse	2.76 ± 0.31 c	8.67 ± 0.88 b	8.0 ± 1.0 a	7.33 ± 0.33 b	6.67 ± 0.88 a	30.67 ± 0.33 b

Table (5): Structural chromosomal aberrations in mouse spleen cells caused by ochratoxin A oral administration.

Parameter / Doses	MI	Chromatid Break /100 cells	Dicentric /100 cells	Acentric /100 cells	Ring Chromosome /100cells	Total
Normal	6.62 ± 0.40 a	1.67 ± 0.66 c	0.0 ± 0.0 d	0.0 ± 0.0 e	0.0 ± 0.0 c	1.67 ± 0.67 d
0.1ml PBS (-ve control)	6.45 ± 0.26 ab	1.67 ± 0.33 c	0.0 ± 0.0 d	0.0 ± 0.0 e	0.0 ± 0.0 c	1.67 ± 0.33 d
2.5 mg MTX/mouse (+ve control)	4.05 ± 0.37 c	14.33 ± 4.05 a	13.33 ± 4.91 a	20.0 ± 4.04 a	8.67 ± 1.76 a	56.33 ± 5.61 a
0.2ppb OCA / mouse	5.29 ± 0.56 bc	3.0 ± 0.58 c	1.67 ± 0.67 d	2.67 ± 0.88 de	2.0 ± 0.58 bc	9.24 ± 2.19 c
0.5 ppb OCA / mouse	5.12 ± 0.38 c	3.33 ± 0.88 c	2.67 ± 0.67 cd	4.33 ± 0.88 cd	2.67 ± 0.66 bc	13.0 ± 0.58 c
1 ppb OCA / mouse	4.38 ± 0.23 c	6.33 ± 0.33 b	4.67 ± 0.88 c	7.33 ± 1.20 bc	4.0 ± 1.53 b	22.33 ± 1/20 b
1.5 ppb OCA / mouse	4.31 ± 0.53 c	11.33 ± 2.03 a	7.0 ± 0.58 b	9.67 ± 1.76 b	4.67 ± 0.66 b	32.67 ± 0.33 b

Table (6): Numerical chromosomal abnormalities in mouse blood cells caused by ochratoxin A oral administration.

Parameter / Doses	Aneuploidy	Polyploidy
Normal	0.0 ± 0.0 d	0.0 ± 0.0 d
0.1ml PBS (-ve control)	0.0 ± 0.0 d	0.0 ± 0.0 d
2.5 mg MTX/mouse (+ve control)	8.0 ± 0.58 a	8.0 ± 1.53 a
0.2ppb OCA / mouse	3.33 ± 0.33 c	3.0 ± 0.58 c
0.5 ppb OCA / mouse	4.0 ± 0.58 c	3.33 ± 0.88 c
1 ppb OCA / mouse	6.0 ± 0.58 b	5.0 ± 1.15 bc
1.5 ppb OCA / mouse	7.33 ± 1.20 ab	6.33 ±0.88 ab

Table (7): Numerical chromosomal abnormalities in mouse spleen cells caused by ochratoxin A oral administration.

Parameter / Doses	Aneuploidy	Polyploidy
Normal	0.0 ± 0.0 d	0.0 ± 0.0 e
0.1ml PBS (-ve control)	0.0 ± 0.0 d	0.0 ±0.0 e
2.5 mg MTX/mouse (+ve control)	12.0 ± 3.21 a	9.33 ± 2.03 a
0.2ppb OCA / mouse	2.67 ± 0.67 d	2.67 ± 0.33 de
0.5 ppb OCA / mouse	4.0 ± 0.58 cd	3.67 ± 0.88 cd
1 ppb OCA / mouse	5.67 ± 0.88 c	5.33 ± 0.88 bc
1.5 ppb OCA / mouse	8.0 ± 0.58 b	6.67 ± 0.88 b

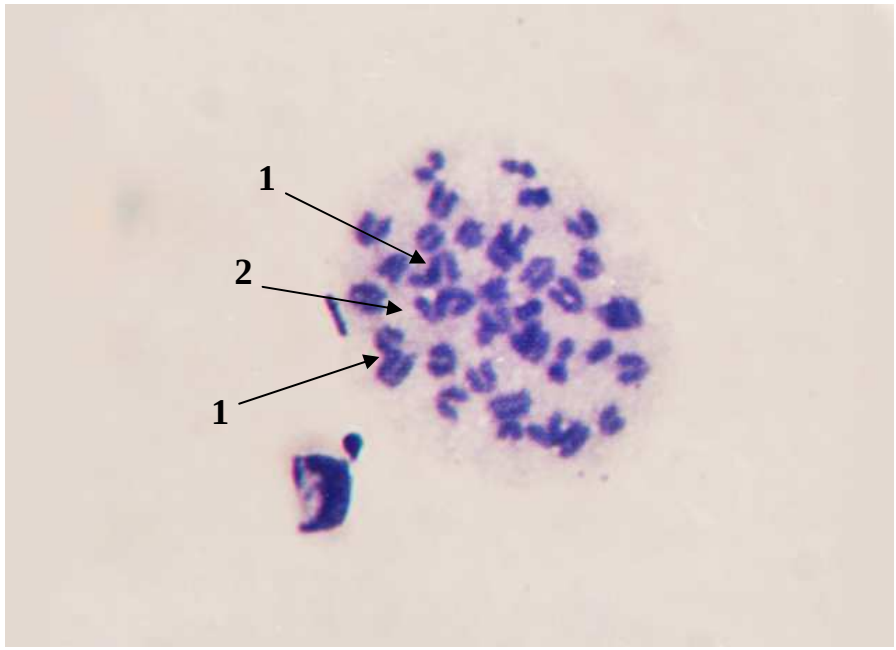

Figure (2): Metaphase of blood cells from animals treated with 0.2 ppb ochratoxin A oral administration showing the following effects:

1. End to end chromosome adhesion.
2. Chromosome break.

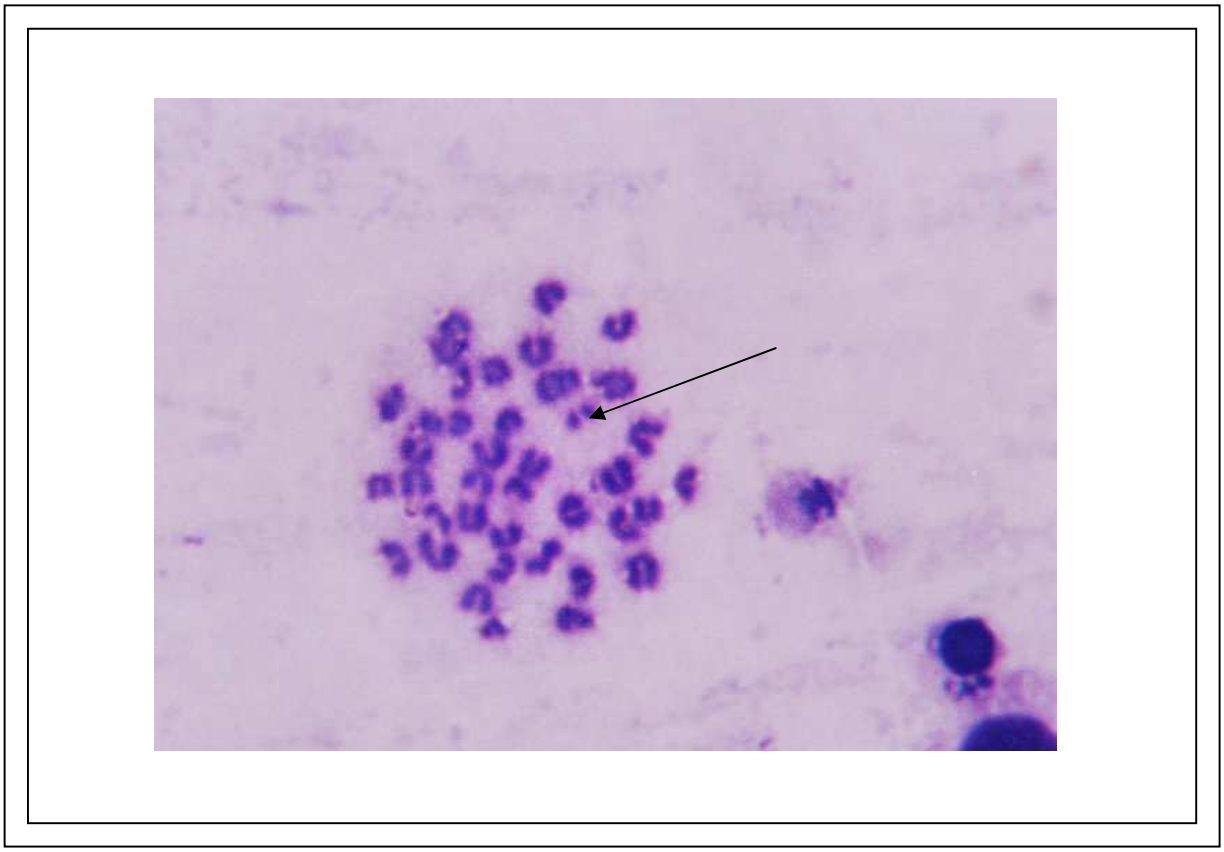

Figure (3): Metaphase of spleen cells from animals treated with 1.5 ppb ochratoxin A oral administration showing the acentric chromosome.

Figure (4): Metaphase of spleen cells from animals treated with 1 ppb ochratoxin A oral administration showing the polyploidy cell.

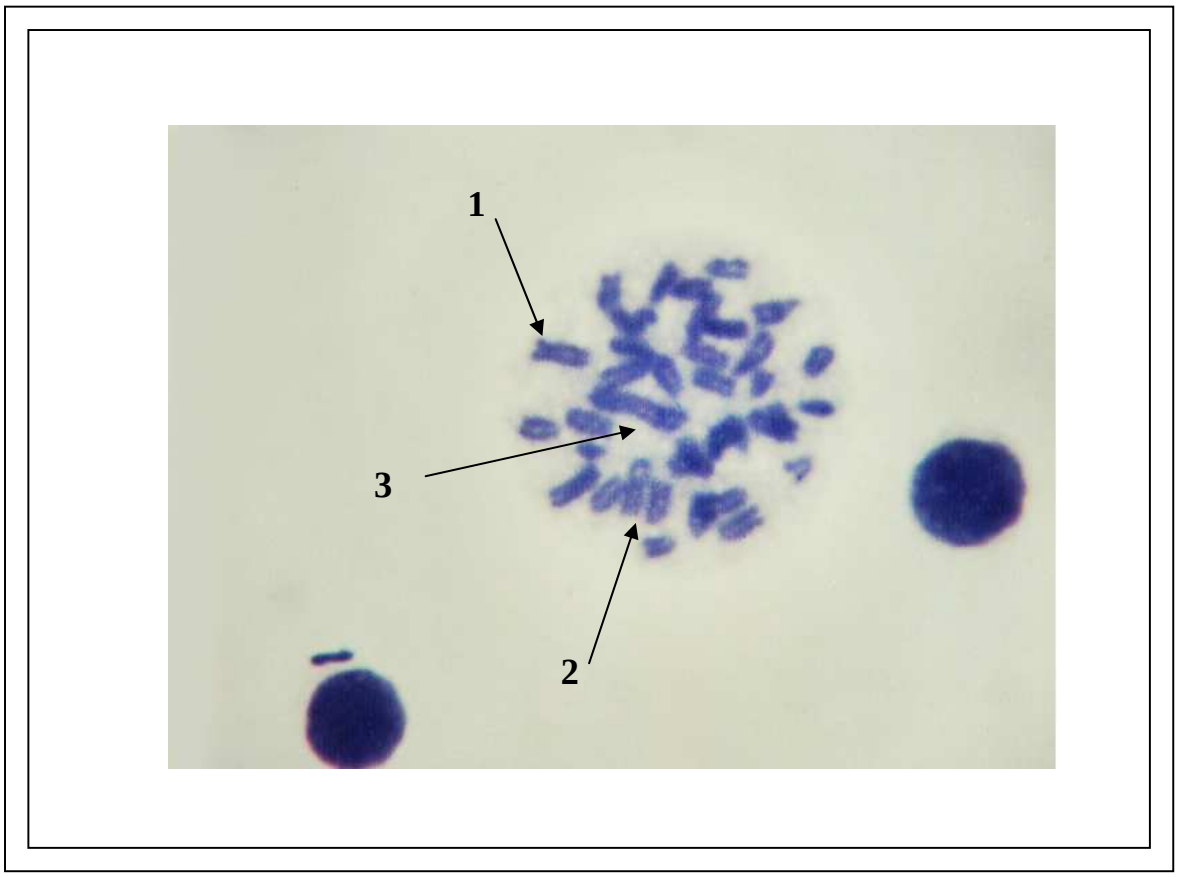

Figure (5): Metaphase of spleen cells from animals treated with MTX showing the following effects:

1. Dicentric chromosome.
2. End to end chromosome adhesion.
3. Sticky – ends overlaping

Depending on the above results, each kind of CA which occurred in the cell due to specific cause; the existence of polyploidy in the cell might be explained on the basis of errors in the final phases of mitosis or may be attributed to endoreplication (Dazzi & Goldman, 1998), while the aneuploidy aberrant may results from non-disjunction or chromosome lag, that a chromosome may lag at anaphase & be excluded from the new nucleus.

Chromatid break resulted in apparently random visible lesion in metaphase chromosome, which can lead to other structural changes such as deletion, translocation & acentric chromosome. Finally the Ring chromosome is a clinical feature dependent on the break points at the **p** end & **q** end of the chromosome & these points will linked together according to basis of sticky ends (Friedman *et. al.,* 1999).

B. Ochratoxin A contaminated diet:

As mentioned before, the results of mitogenic activity of blood & spleen cells showed a highly significant ($P \leq 0.01$) reduction when compared with the control. This includes group III of the contaminated diet in which it has a highly significant inhibition in the mitotic index (tables 7 & 8) when compared with group I (control) & with group II (feeding black seed alone).

In the same group (group III) there is a high significant variation in the CA between and group I (control) (tables 8, 9, 10 & 11) which

could be due to the geno-cytotoxic effect caused by ochratoxin A toxicity. A suggestion by McLean & Dutton, (1995) that ochratoxin A is a very potent mycotoxin because it passes through the food chain.

In the group IV which (treatment with black seeds beside the ochratoxin A contaminated diet), the results was significant in both mitogenic activity (tables 8 & 9) and CA (tables 8, 9, 10 & 11) when compared to control animals, while it is non-significant to the ochratoxin A contaminated diet (figures 6 & 7), but it is possible to notice that there is a numerical differences between the treatment with ochratoxin A alone & treatment with ochratoxin A beside black seed, that the number of defects decreased after treatment with black seed.

There is a significant decrease in CA and increase in the mitogenic activity of group V (pretreatment with black seed for 2 weeks) as a comparison with the previous two groups (tables 8, 9, 10 & 11) (figure 9).

Table (8): Structural chromosomal aberrations in mouse blood cells caused by ochratoxin A contaminated diet.

Parameter / Doses	MI	Chromatid Break /100 cells	Dicentric /100 cells	Acentric /100 cells	Ring Chromosome /100cells	Total
Group I (control)	7.12 ± 0.17 a	1.67 ± 0.33 b	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	1.67 ± 0.33 d
Group II (BS)	5.82 ± 0.22 ab	0.67 ± 0.33 b	2.0 ± 0.47 bc	0.0 ± 0.0 c	0.0 ± 0.0 c	2.67 ± 0.87 d
Group III (OTA)	4.41 ± 0.5 bc	13.0 ± 3.78 a	8.67 ± 1.2 a	15.33 ± 3.48 a	8.33 ± 0.88 a	45.33 ± 1.20 a
Group IV (OTA+BS)	3.97 ± 0.50 c	5.67 ± 0.88 b	7.0 ± 0.58 a	10.67 ± 1.76 a	7.0 ± 1.15 ab	30.0 ± 2.06 b
Group V (BS+OTA)	3.75 ± 0.69 c	3.0 ± 0.58 b	3.0 ± 0.58 b	4.0 ± 0.58 b	3.67 ± 1.2 b	13.67 ± 1.77 c

Table (9): Structural chromosomal aberrations in mouse spleen cells caused by ochratoxin A contaminated diet.

Parameter / Doses	MI	Chromatid Break /100 cells	Dicentric /100 cells	Acentric /100 cells	Ring Chromosome /100cells	Total
Group I (control)	7.12 ± 0.17 a	1.67 ± 0.33 b	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 b	1.67 ± 0.67 d
Group II (BS)	6.26 ± 0.41 a	1.67 ± 0.33 b	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 b	1.67 ± 0.33 d
Group III (OTA)	4.41 ± 0.47 b	13.67 ± 4.41 a	8.67 ± 1.2 a	14.67 ± 4.1 a	8.0 ± 2.52 a	45.01 ± 2.91 a
Group IV (OTA+BS)	3.45 ± 0.47 b	6.67 ± 0.33 b	7.0 ± 0.58 a	10.67 ± 1.76 a	7.0 ± 1.15 a	31.34 ± 0.88 b
Group V (BS+OTA)	3.97 ± 0.50 b	3.0 ± 0.58 b	3.0 ± 0.58 b	4.0 ± 0.58 b	3.67 ± 1.45 ab	13.67 ± 2.08 c

Table (10): Numerical chromosomal abnormalities in mouse blood cells caused by ochratoxin A contaminated diet.

Parameter / Doses	Aneuploidy	Polyploidy
Group I (control)	0.0 ± 0.0 c	0.0 ± 0.0 b
Group II (BS)	2.33 ± 0.33 b	3.0 ± 0.58 b
Group III (OTA)	9.33 ± 1.86 a	10.0 ± 2.52 a
Group IV (OTA+BS)	6.67 ± 0.88 a	9.0 ± 1.15 a
Group V (BS+OTA)	3.0 ± 0.58 b	3.33 ± 0.88 b

Table (11): Numerical chromosomal abnormalities in mouse spleen cells caused by ochratoxin A contaminated diet.

Parameter / Doses	Aneuploidy	Polyploidy
Group I (control)	0.0 ± 0.0 d	0.0 ± 0.0 c
Group II (BS)	3.0 ± 0.47 cd	3.0 ± 0.72 b
Group III (OTA)	9.33 ± 1.86 a	9.0 ± 1.15 a
Group IV (OTA+BS)	7.33 ± 0.33 ab	6.67 ± 0.88 a
Group V (BS+OTA)	4.33 ± 0.88 bc	3.33 ± 0.88 b

Table (12): The Protection Ratio of black seeds against ochratoxin A in mice blood cells.

	Chromatid Break	Dicentric	Acentric	Ring Chromosome	Aneuploidy	Polyploidy
OTA+BS	**64**	**19**	**30**	**15.9**	**28.5**	**10**
BS+OTA	**88**	**65.3**	**73**	**55.9**	**67.8**	**66.7**

Table (13): The Protection Ratio of black seeds against ochratoxin A in mice spleen cells.

	Chromatid Break	Dicentric	Acentric	Ring Chromosome	Aneuploidy	Polyploidy
OTA+BS	**58.3**	**19.2**	**27.2**	**12.5**	**21.4**	**25.8**
BS+OTA	**88.9**	**65.3**	**72.7**	**54.1**	**53.5**	**63**

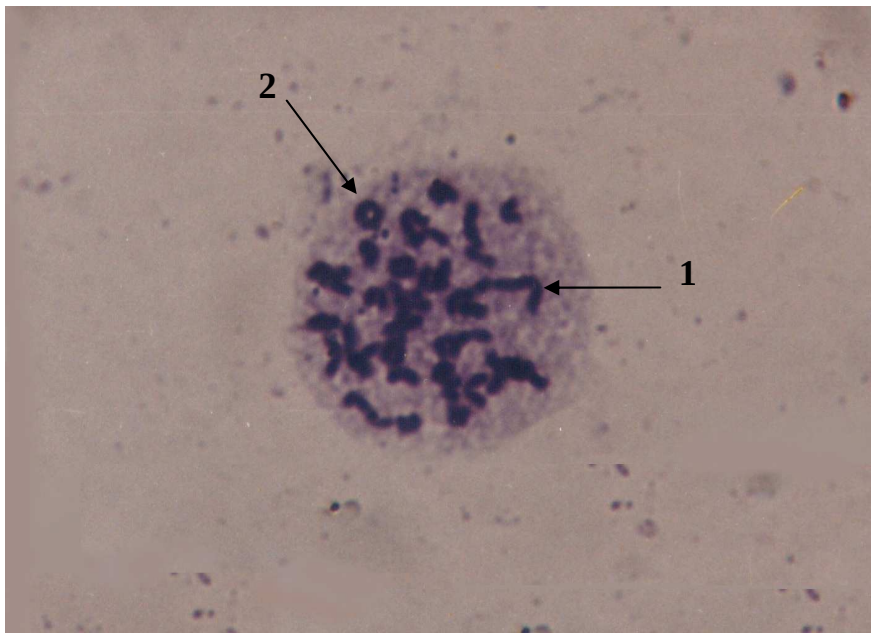

Figure (6): Metaphase of blood cells from animals treated with ochratoxin A contaminated diet (group III) showing the following effects:

1. End to end chromosome adhesion.
2. Ring chromosome.

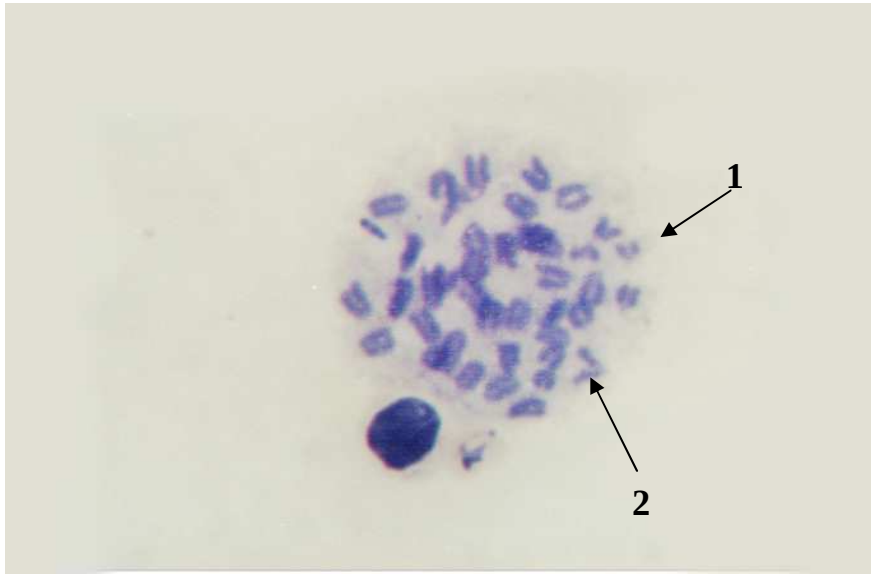

Figure (7): Metaphase of spleen cells from animals treated with ochratoxin A contaminated diet (group III) showing the following effects:

1. Acentric chromosome.
2. Chromatid break.

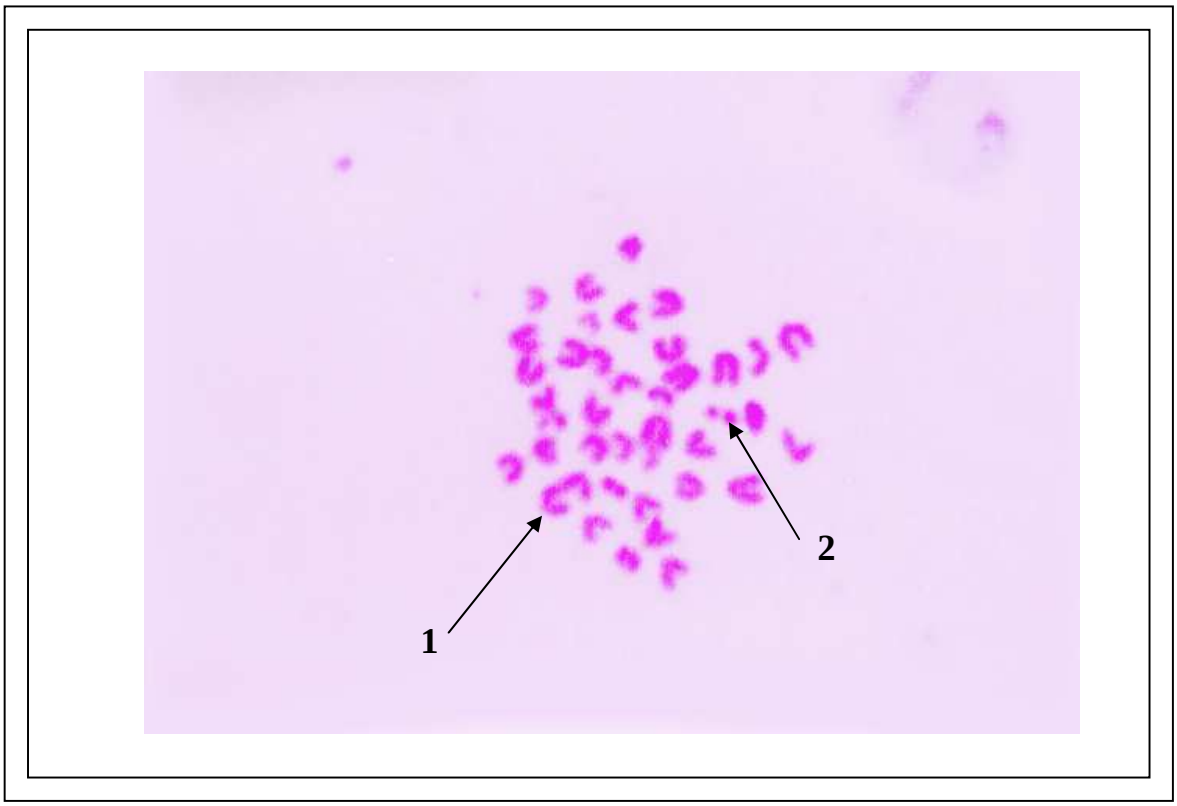

Figure (8): Metaphase of blood cells from animals treated with ochratoxin A contaminated diet & black seed (*Nigella sativa*) (group IV) showing the following effects:

1. End to end chromosome adhesion.
2. Acentric chromosome.

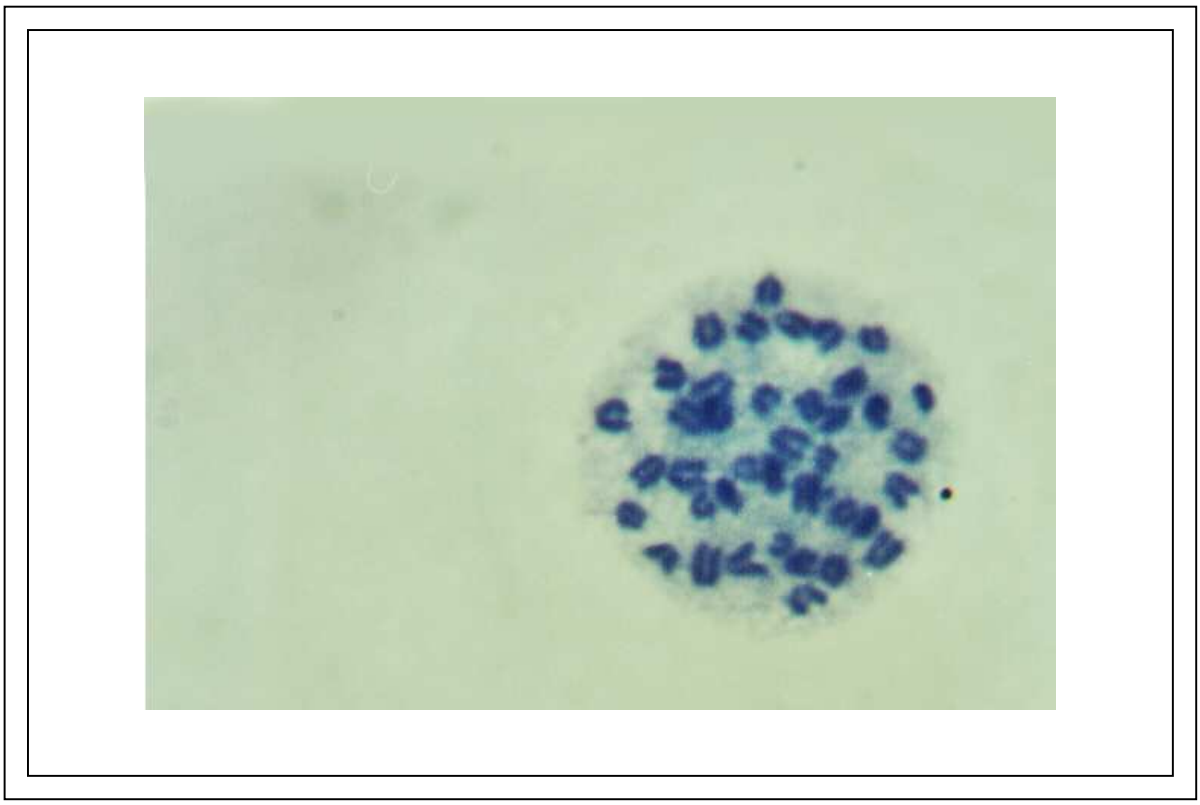

Figure (9): Metaphase of spleen cells from animals treated with black seed (*Nigella sativa*) first and then with ochratoxin A contaminated diet & (group V) showing the detoxification.

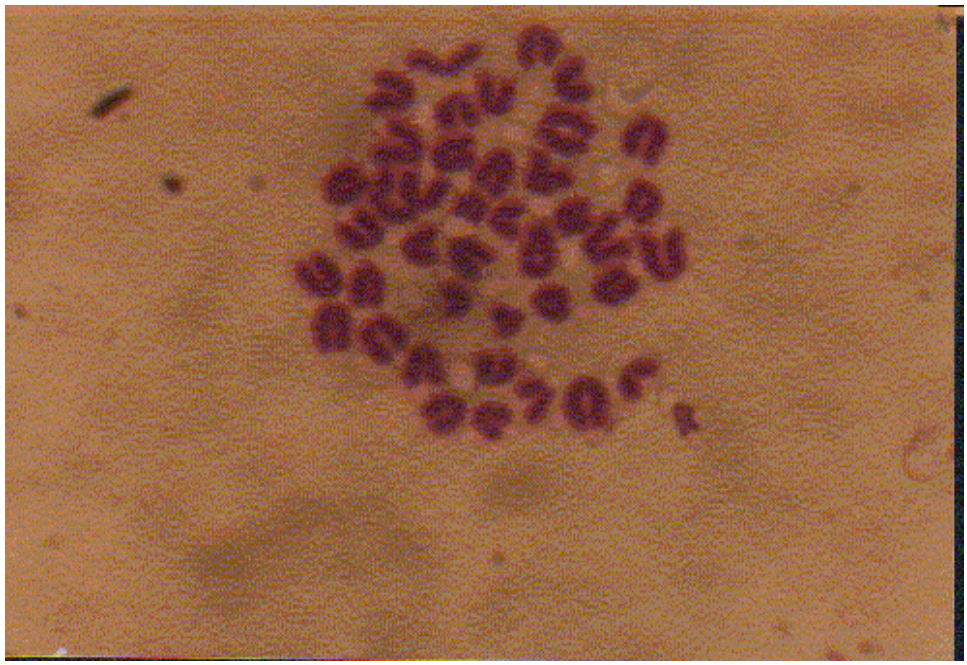

Figure (10): Normal mice chromosomes.

Depending on the previous results, black seed is effective in treating the body as a whole and fights the actual cause of the symptoms, provides quick energy, encourages hair growth and retards hair fall out. This means that the defense activity in the animal body was increased because black seed is considered as a potent natural immuno-stimulant (Ayoub, 1999).

Most of the CA occur in the G_1 phase of the cell cycle, thus, ochratoxin A obstruct against the DNA repair system. In addition ochratoxin A causes damage of the DNA molecule & this leads to accumulation of the breaks in the DNA single strand inside the cell. These effects occurred as a result of ochratoxicosis which stops the activity of DNA polymerase enzyme that is specific for the DNA repair by the excision repair system (Moutschen & Ehrenberg, 1985). After treatment with black seed it was found that black seeds keep DNA-breaks away from accumulation in the strand. This proved that the black seed shared in complete the gaps in the nucleotides by synthesizing it in the DNA strand because of its protein content. For this reason it might be played an important role in the repair system in the S phase (DNA synthesis phase) (Jones, 2000).

These results confirmed earlier findings that black seed has a positive stimulating effect on the immune system. These findings are of great practical significance since it was mentioned that a natural immune enhancer like the black seed could play an important role in

the treatment of cancer, AIDS, and other disease conditions associated with immune deficiency states (Nooruddin, 2003).

It was suggested that treatment of mice with 1 μg black seed proved to be effective in reduction of SCE and in increasing the blastogenic index, MI, and replicative index. These effects are an indication of the natural immuno-stimulant activity of black seed (Al-Azzawi, 1999).

Black seeds are rich in oil and have a high level of true protein (Abdel-Aal and Attia 1993a; 1993b). Black seed meal is a novel protein source which has been used in poultry feeds (Khalifah, 1995; Zewil, 1996).

Different active ingredients that found in black seeds are considered as a promoter of good health.

Thymoquinone is one of the important active ingredients of black seed which is known to have anti-tumour activity *in vivo* and *in vitro* experiments (Salomi *et. al.*, 1992) together with other therapeutic effects like anti-inflamatory and anti-microbial activity (Houghton *et. al.*, 1995). Moreover, thymoquinone has been shown to have a remarkable impact on the suppression of doxorubicin-induced renal diseases in rats (Badary *et. al.*, 2000; Al-Shabana *et. al.*,1998).

β-carotene is another active ingredient found in black seed, which is well known to destroy cell damaging substances that produce cancer,

thus it is considered as an anti-oxidant compound (Randhawa & Al-Ghamdi, 2002).

El-Sherbeny, (2001) reported the protective effect of black seeds against the genotoxic action of an herbicide, 2,4-D on mitosis and meiosis of the mouse.

Also, *in vivo* studies have shown that tumour development in mouse skin could be inhibited by the active principles of black seeds, that black seeds are mainly responsible for the anti-tumor (Kumara and Huat, 2001; Salomi *et. al.,* 1992).

Other studies revealed the benefit of pretreatment with black seed was Al-Azzawi, (1999). Giving black seed before exposure to gamma-radiation could saturate the system of black seed with natural constitute that might act as an antioxidant or scavengers for the free radicals produced by radiation.

These pharmacological properties of black seed support the traditional use of black seed and its derivatives as a treatment and curative agent against many diseases.

Conclusion

1. Ochratoxin A is considered as one of the potent fungal toxins that have many genotoxic effects.
2. Even low doses of ochratoxin A can cause CA in animal blood and spleen cells.
3. Black seed is a promising plant that could be used for detoxification of mycotoxins such as ochratoxin A and as curing plant.
4. Black seeds act as a prophelacting agent more than curing agent.
5. Mouse blood and spleen cells could be cultivated *in vitro* before and after the exposure to mutagenic or carcinogenic mycotoxic agents *in vivo*.

Recommendation

1. Further studies are needed on the effect of ochratoxin A on the chromosomes of other organs such as brain, testis, & bone morrow.
2. Further study on the teratogenicity and mutagenicity effect of toxin and the toxin residue in different organs is very necessary.
3. Further study on the immunization against ochratoxin A by selective immunity agents and antitoxins.
4. The black seeds are used for prevention or treatment of acute problems in a correct dosage.
5. Isolation, identification and characterization of the main active compounds responsible for the effect of black seed as a detoxificant drug.

Summary

Ochratoxin A is a natural secondary product by several fungi of the genera *Aspergillus* & *Penicillium* in different agriculture commodities. It causes great economic losses and health hazard to human and farm animals.

This work was planned to study the induction of some chromosomal abnormalities that caused by ochratoxin A such as ring chromosome, acentric chromosome, dicentric chromosome, deletion, aneuploidy, and polyploidy and diminish these variations by using the black seed (*Nigella sativa*) in Balb/c mice.

The results of the in vivo study are summarized as follows:

1. The amount of ochratoxin A produced by the isolate of *Aspergillus ochraceus* on corn as a substrate ranged between 47 – 50 ppb.
2. The treatment of mice with crude ochratoxin A by oral route caused signs of marked increase in body weight, blindness, hair loss, dyspnea, emaciation, irritation around the neck and shivering before death.
3. Short term culture for blood and spleen cells was used for chromosomal analysis to estimate the genotoxic effect of ochratoxin A and the treatment and the prophelacting effect of the black seed.

4. Genotoxic effects due to ochratoxicosis that observed on blood and spleen cells of mice included many chromosomal aberrations such as (chromosome & chromatid breaks, ring chromosome, acentric chromosome, dicentric chromosome, aneuploidy, polyploidy & deletion), these genotoxic effects of ochratoxin A observed in blood and spleen cells of mice were dose dependent.
5. Ochratoxin A contaminated diet also showed genotoxic effects in blood and spleen cells of mice including (ring chromosome, deletion, acentric chromosome, dicentric chromosome, & polyploidy).
6. Black seeds contained high activity in diminishing the percentage of different chromosomal aberrations (ring chromosome, chromatid braek, and acentric chromosome) detected in mice blood and spleen cells.

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List of Abbreviations

Code	Word
OTA	Ochratoxin A
BEN	Balkan Endemic Nephropathy
PN	Porcine Nephropathy
CNS	Central Nervous System
HEN	Human Endemic Nephropathy
bw	Body weight
CIN	Chronic Interstitial Nephritis
NK	Natural killer cells
CA	Chromosomal Aberrations
SCE	Sister Chromatid Exchange
UVL	Ultra Violet Light
USD	Unscheduled DNA Synthesis
mM	milli Molar
nM	nano Molar
μ M	micro Molar
ROS	Reactive Oxygen Species
I.P.	Intraperitoneal
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Hr	Hour
Min	Minutes
PHA	Phytohaemagglutinine
AIDS	Aquired Immune Deficiency Syndromes
IL	Inter leukin
TLC	Thin Layer Chromatography
D.W.	Distilled Water
ppb	Part per billion
PBS	Phosphate Buffer Saline

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

دراسة بعض التغيرات الكروموسومية الناتجة عن التلوث بالأوكراتوكسين A في الفئران

رسالة مقدمة

الى

كلية العلوم في جامعة النهرين

وهي جزء من متطلبات نيل درجة الماجستير علوم في التقانة الأحيائية

من قبل

نور الحكم حسن التكريتي

بكالوريوس ٢٠٠١

كلية العلوم جامعة النهرين

ذلك في

صفر ١٤٢٦

آذار ٢٠٠٥

الخلاصة:

يعد الأوكراتوكسين A من النواتج الثانوية الطبيعية المفرزة من عدد من الفطريات التي تعود لجنسي الأسبيرجيلس و البنسليوم (*Aspergillus* and *Penicillium*) على عدد من المحاصيل الزراعية مسبباً خسائر أقتصادية كبيرة وخطرة على صحة الأنسان والحيوان.

صُمّمت هذه الدراسة للتحري عن بعض التغيرات الكروموسومية الناتجة عن التلوث بالأوكراتوكسين A مثل تكسر الكروماتيد والكروموسوم، الكروموسوم الحلقي، الكروموسوم فاقد السنترومير والثنائي السنترومير، وحذف جزء من الكروموسوم ،والتغيرات العددية في الكروموسومات ومعالجة تلك التغيرات بواسطة بذور بواسطة الحبة السوداء *Nigella sativa* .

يمكن أيجاز النتائج التي تمَّ التوصل أليها بما يأتي:

١- تراوحت كمية الأوكراتوكسين A المنتج من العزلة المستخدمة لنوع الـ*Aspergillus ochraceus* بين ٤٧ - ٥٠ جزء بالبليون.

٢- أظهرت المعاملة الفموية للفئران بالأوكراتوكسين A المنتج زيادة واضحة في وزن الحيوان أضافةً الى العمى، وفقدان الشعر،وسوء في التنفس ،وهزال ، والتهيج حول العنق، والرجفان قبل الموت.

٣- أستخدمت طريقة الزرع القصيرة الأمد لخلايا الدم والطحال في الفئران لأستخلاص الكروموسومات وذلك لحساب التأثير الجيني للأوكراتوكسين A وكذلك في تأثير المعالجة والوقاية بواسطة الحبة السوداء.

٤- أدّت التـأثيرات السـمية بالأوكراتوكسـين A الـى ظهـور العديـد مـن التغيرات الكروموسومية في خلايا الدم والطحال للفئران المعاملة مثل تكســر الكروماتيــد، الكروموســوم الحلقــي، الكروموســوم فاقــد السـنترومير والثنـائي السـنترومير، وحـذف جـزء مـن الكروموسـوم، والتغيـرات العدديـة فـي الكروموسـومات، وأتضـح أنَّ هـذه التـأثيرات السمية الخلوية تتناسب طردياً مع كمية الجرعة المعطاة للفئران.

٥- أظهـرت المعاملـة بالعليقـة الملوثـة بالأوكراتوكسـين A تـأثيرات سـمية خلويــة أيضــاً فــي خلايــا الــدم والطحــال للفئــران المعاملــة تضــمّنت الكروموســوم الحلقــي، وتكســر الكروماتيــد، و الكروموســوم فاقــد الســنترومير والثنــائي الســنترومير، والتغيــرات العدديــة فــي الكروموسومات.

٦- أظهـرت المعاملـة العلاجيـة بالحبـة السـوداء نقصـاً فـي نسـبة التغيـرات الكروموســومية (الكروموســوم الحلقــي، وتكســر الكروماتيــد، و الكروموسوم فاقد السنترومير) فـي خلايـا الـدم والطحـال فـي الفئـران المعاملة.

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