MATERIALS AND METHODS

1. MATERIALS

a. Sampling and source of samples

Raw peanut (52 samples) and roasted peanut (52 samples) were randomly collected from different regions in Great Cairo, Egypt. The samples were sent to the laboratory as soon as they were collected and stored at -20°C before analysis. One kilogram of every studied commodity was collected weekly from March 2006 to March 2007. One hundred and four samples of raw peanut and roasted peanut, fifty-two samples for each commodity were determined for the four types of aflatoxins (B_1, B_2, G_1 and G_2).

b. Chemicals

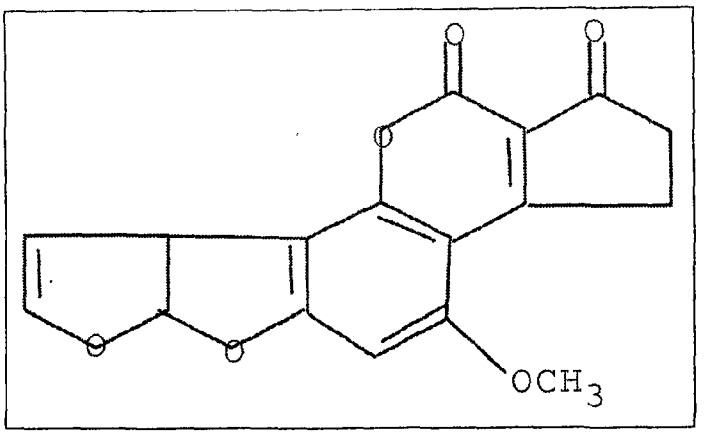
Standard Aflatoxin was purchased from Acrose, Chemical Company, Nitherland.

HPLC grade solvents and chemicals were purchased from Lab-scan Company represents in Egypt.

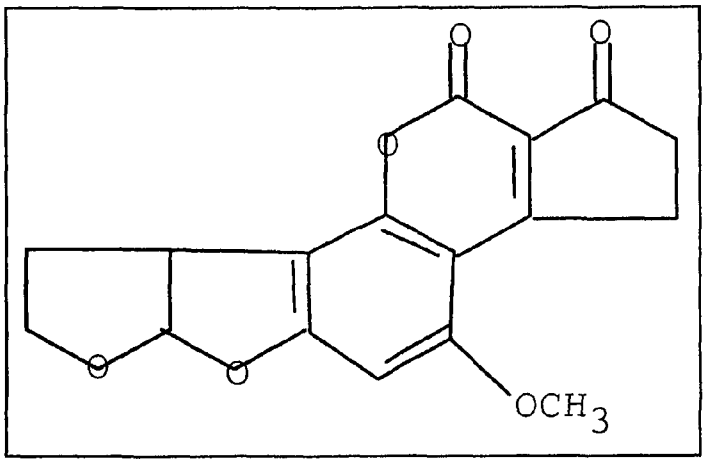
Reagent kits were purchased from Bio–diagnostic Company, Dokki, Giza, Egypt.

Investigated aflatoxins

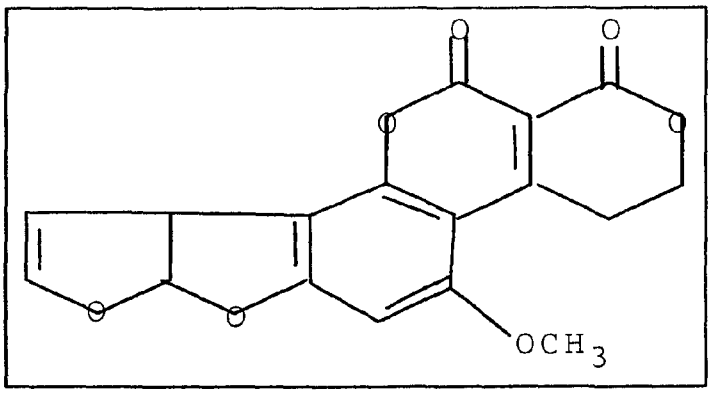
Four types of aflatoxin were investigated in this study (i.e. B_1, B_2, G_1 and G_2).The chemical structures of these toxins (**Fig. 1.**).



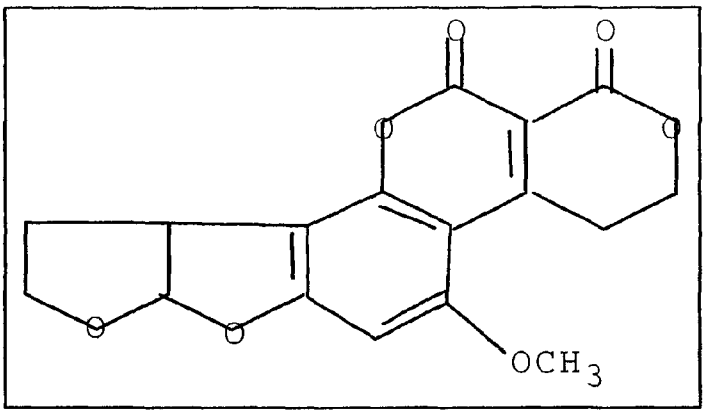
Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

Fig. 1. The chemical structures of aflatoxin (B_1, B_2, G_1 and G_2)

2. METHODS

a. Extraction and determination of aflatoxins

1. Preparation of sample

a. Sample Pre-treatment and homogenization

The samples were prepared according to the guidelines of Codex Alimentarius (1992) and AOAC (2005). One-kilogram of each sample was complete homogenized. Two replicates of 500 g were taken; one for extraction and the second was kept in the freezer at -20°C. Analysis was carried out soon after sub-sampling.

b. Extraction

Fifty g of homogenized sample were weighted into spherical flask 500 ml with stopper. Then 200 ml of methanol-water (80:20) solution were added and the flask was shacked for 30 min. The extract was filtered through medium fast filter paper (Whatman No.1).

c. Partitioning

Fourty ml of filtrate were transferred into 500 ml separating funnel. Fourty ml (10%) sodium chloride solution and 50 ml n-hexane were added to the funnel. The funnel was shacked gently for one min. and the phases were let to separate. The aqueous lower layer was drained into another 500 ml separating funnel and 50 ml chloroform were added and shacked gently for 1min. The phases were let to separate and lower layer was drained through anhydrous sodium sulfate (15 g) into 250 ml flask. The aqueous layer was washed with two portions of 25 ml chloroform and shacked gently for one min. each time. The received chloroform was evaporated to dryness by using

rotary evaporator. The dry film residue was dissolved in 2ml dichloromethane and transferred to silica gel clean up column.

d. Clean-up column

Glass wool was put into 5 ml plastic syringe to make a bed for 0.5g sodium sulfate. Three ml of dichloromethane were added to the syringe then 0.5 g of silica gel was added and dichloromethane was rinsed out of the column, after that another 0.5 g of sodium sulfate was added. One ml of dichloromethane was added to the column (AOAC, 2005).

e. Derivatization of the sample extracts

Two hundred μl of hexane and 50 μl Trifluroacetic acid (TFA) were added to the column extract. The tube was capped and shacked on vortex-mix vigorously for 30 sec. (exactly). The tube was let stand for 5 min and 1.950 ml acetonitril-water (1:9) were added. The tube was shacked on vortex-mix for 30 sec. (exactly) and the layers were let to separate for 10 min. The lower aqueous layer was used for HPLC (AOAC, 2005).

2. Preparation of aflatoxins standard solutions

a. Aflatoxin stock solution

Individual stock solutions was prepared in benzene- CH_3CN (98:2) approximately 20 μg/ml and stored in deep freezer. The concentration was measured by using spectrophotometer according to AOAC (2005).

b. Aflatoxin mixture working solution

Working solutions were prepared by diluting the individual stock solutions to get the final concentration of approximately 1μg/ml

of each and stored in small portion in deep freezer according to AOAC (2005).

3. Determination of aflatoxins by high performance liquid chromatography (HPLC)

Determination of total aflatoxin and aflatoxins B_1, B_2, G_1 and G_2 were carried out by high performance liquid chromatography (HPLC) as the procedure of AOAC (2005).

Hewlet purchased (HP) 1100 equipped (HPLC) with reversed phase Chrompack C_{18} column (5 um, 250 mm Length, and 4.6 mm id.), pump, auto sampler and programmable fluorescence detector. Mobile phase water- methanol-acetonitrile (60: 25: 15 v/v /v) was also used and the amount of water might be changed for improving the resolution between G_{2a} and B_{2a}. Flow rate of mobile phase was ranged from 0.8 – 1 ml/min with maximum pressure 3000 psi. Twenty five μl loop of injection ware also used with detection conditions (excitation 360 nm, emission 440 nm with high gain).

Calculation

Concentration of sample Cs (μg/kg) was calculated as follows:

$$Cs = \frac{Hs}{Hst} \times \frac{Ast}{Ws} \times \frac{V}{VA} \times \frac{D}{I}$$

Where

Cs = sample concentration

Hs = Peak height of sample

Hst = Peak height of standard

Ast = Standard amount (μg)

Ws = Sample weight (g)

V = Extract volume (ml)

Va = Taken volume for partitioning (ml)

D = Final dilution (ml)

I = Injected volume (ml).

b. Animal feeding experiments

1. Experimental animals

A total of 28 male albino rats, (Sprague Dawely strain), weighing 200 to 250 g, were obtained from Helwan Station for experimental animals, Helwan, Cairo, Egypt. The animals were housed in stainless steel cages and raised in the animal house of biochemistry department, Faculty of Agriculture, Cairo University. The rats were kept under normal healthy laboratory conditions. The animals were adapted on free access of water and fed basal diet for two weeks before the initiation of the experiments.

2. Diets (Basal Diets)

The composition of the standard diet was made according to AOAC (2005) as follow:

Ingredient	%
Corn starch	65
Casein	10
Corn seed oil	10
***Vitamins mixture**	1
****Salt mixture**	4
Cellulose	10
Total	100%

*The vitamin mixture was recommended by Campbell (1961). The following amounts of vitamins were added to 100 g of diet:

Vitamin	Quantity
Vitamin A	200 I.U.
Vitamin D	100 I.U.
Vitamin E	10 I.U.
Vitamin K	0.5 mg
Thiamin	0.5 mg
Riboflavin	1.0 mg
Pyridoxine	0.4 mg
Pantothenic acid	4.0 mg
Choline chloride	200.0 mg
Inositol	25 mg
Vitamin B12	2.0 mg
Biotin	0.02 mg
Folic acid	0.20 mg
Cellulose (powder)	Up to 1.0 g

**The salt mixture was suggested by Hegsted *et al.* (1941) as follows:

Salt	Quantity
$CaCO_3$	50.0 g
K_2HPO_4	64.5 g
$CaHPO_4$	11.8 g
$MgSO_4.7H_2O$	20.4 g
NaCl	33.5 g
$Fe(C_6H_5O_7).6H_2O$	5.5 g
KI	0.16 g
$MnSO_4.4H_2O$	1.00 g
$CuSO_4.5H_2O$	0.05g
$ZnCl_2$	0.06 g

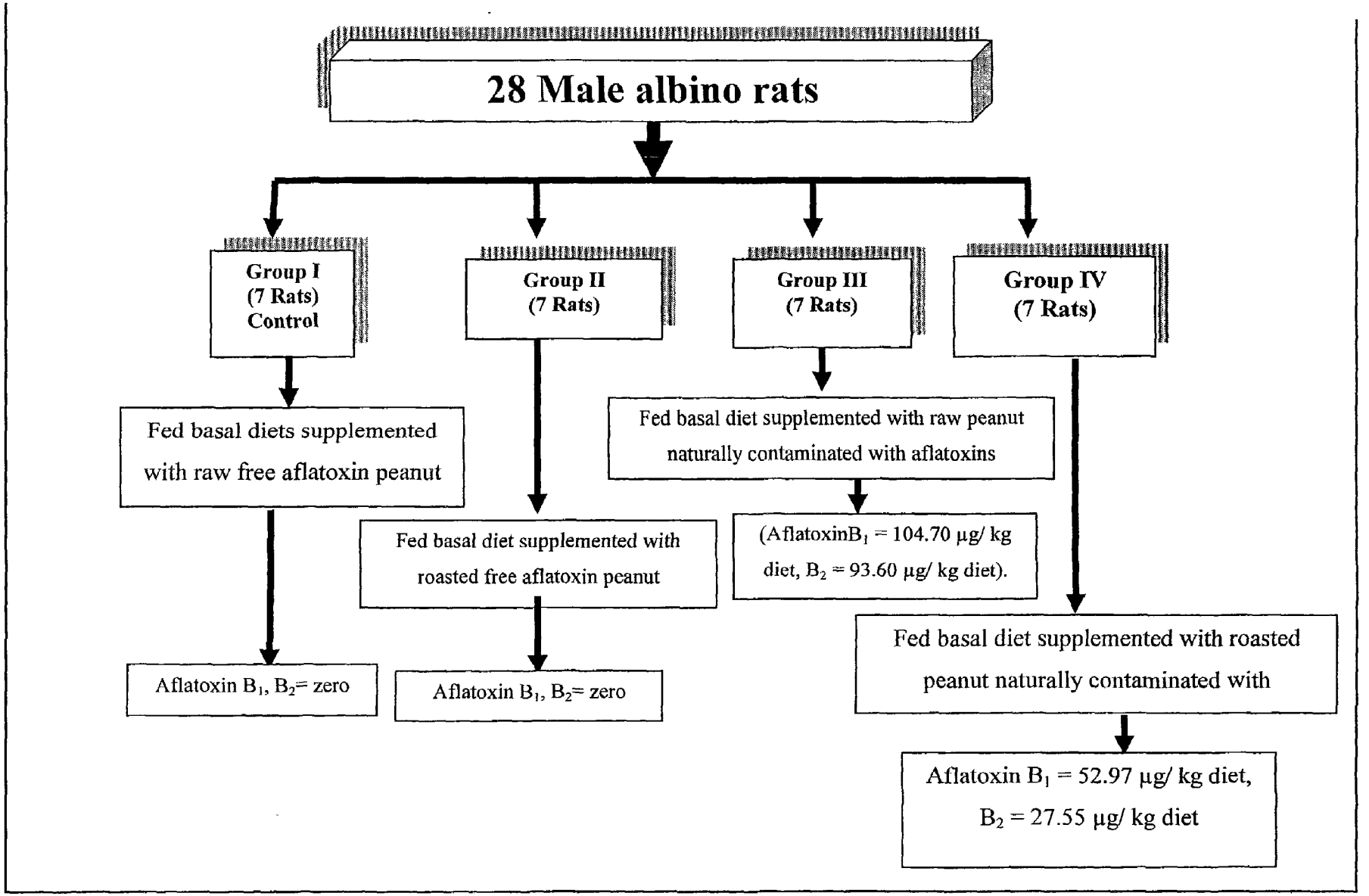


Fig. 2. Scheme of Experimental Design

3. Experimental design

A total of 28 rats were divided into four groups as follows:

Group I Control (7 Rats): Fed basal diets supplemented with raw free aflatoxin peanut. (Aflatoxin B_1, B_2= zero).

Group II (7 Rats): Fed basal diet supplemented with roasted free aflatoxin peanut. (Aflatoxin B_1, B_2= zero).

Group III (7 Rats): Fed basal diet supplemented with raw peanut naturally contaminated with aflatoxins. (Aflatoxin B_1 = 104.70 μg/ kg diet, B_2 = 93.60 μg/ kg diet).

Group IV (7 Rats): Fed basal diet supplemented with roasted peanut naturally contaminated with aflatoxins. (Aflatoxin B_1=52.97 μg/ kg diet, B_2 = 27.55 μg/ kg diet). The feeding experiment continued for 45 days. The experimental design is summarized in the Fig. (2).

4. Experimental procedure

a. Samples

Blood samples were collected in eppendorf tubes (1.5 ml) under ether anesthesia from orbital sinus vein from each of seven of the surviving rats by hyparinized capillary tubes every two weeks and at the end of the experiment. Samples were centrifugated at 3500 rpm for 15 minutes, in a refrigerated centrifuge to separate serum. Separated serum was kept in a deep freezer (-40°C) for selected biochemical analysis by using commercial reagent kits.

b. Organ samples

At the end of experimental period (45 days) the animals (3 rats of each group) were killed and dissected to obtain samples of the blood and

internal organ (liver). The livers were rinsed with formalin solution (10%) and kept at 4°C for histopathological analysis.

5. Biochemical analysis

a. Serum total protein

Total protein was determined by biuret method according to Gornal *et al.* (1949).

Buffered solution: It contains biuret reagent: cupric sulfate (6mmol/L), sodium potassium tartrate (21mmol/L), sodium hydroxide (750mmol/L) and potassium iodide (6mmol/L).

Procedure: Serum (25μl) was added to 1.0 ml biuret reagent in a clean dry test tube. The solution was mixed and allowed to stand for 10 minutes at 37°C or30 minutes at room temperature. The product colour was read against blank (biuret reagent) at 550nm. (520-570nm.), the absorbance of sample and standard was measured against reagent blank. Color stable for one hour. Linearity up to 10 g/dl.

Calculation

$$\text{Protein concentration (g/dL)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times 5$$

b. Serum albumin

Serum albumin was determined according to Doumas *et al.* (1971).In a buffered solution bromcresol green forms with albumin a green colored complex whose intensity is proportional to the amount of albumin present in the sample.

Buffered solution: It contains bromcresol green (0.12mmol/L) and citrate buffer pH 4.2 (50mmol/L)

Procedure: Serum (10μl) was added to2.0 ml buffer solution in a clean dry test tube. The solution was mixed and incubated for 5 minutes at 25-30°C.Absorbance of sample (A_{sample}) and standard ($A_{standard}$) against reagent blank were measured at 630nm. (620-640nm.).

Calculation

$$\text{Albumin concentration (g/dL)} = \frac{A_{Sample}}{A_{Standard}} \times 4$$

c. Liver function enzymes

1. Serum alanine aminotransferase ALT (GPT) activity

The colorimetric determination of ALT activity was carried out according to the method of Reitman and Frankel (1957).In general; the method is based on the following equation:

L-alanine + α-ketoglutarate $\xrightleftharpoons{ALT}$ pyruvate + glutamate

The liberated pyruvate was measured in the presence of 2,4 dinitrophenylhydrazine at 505nm.

Calculation

The number of units/ml of ALT of sample was calculated using the standard curve.

2. Serum aspartate aminotransferase AST (GOT) activity

The colorimetric determination of AST activity was carried out according to the method of Reitman and Frankel (1957).In general; the method is based on the following equation:

α-ketoglutarate + L-aspartate $\xrightleftharpoons{AST}$ L-glutamate + oxaloacetate

The liberated oxaloacetate was measured in the presence of 2,4 dinitrophenylhydrazine at 505nm.

Calculation

The number of units/ml of AST of sample was calculated using the standard curve.

6. Histopathological analysis

Tissue specimens were taken from the liver of experimental and control groups of rats during the post mortem examination then fixed in 10% formol saline for 24 hours. Trimming was done on the fixed tissue specimens and washed in tap water for 12 hours. Serial alcohol (Methyl, Ethyl and Absolute) were used fro dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 3 micron thickness by slidge microtome. The obtained tissue sections were collected on the glass slides and stained by hematoxylin and eosin stain for histopathological examination by the light microscope (Banchroft *et al.,* 1996).

7. Statistical analysis

All data were expressed as mean± standard error. Data analysis was made by analysis variance (ANOVA $P < 0.05$). The statistical packaged for social science (S.P.S.S., 1999) Program version 10 was used for all analysis.