4. MATERIALS AND METHODS

The present research was conducted in the Environmental Toxicology Laboratory, Institute of Graduate Studies and Research, Alexandria University, Egypt. Materials used and the experimental protocol followed were described herein.

4.1. Materials

(a) Cobaltus Chloride (Cobalt II Chloride hexahydrate) was purchased from BDH Chemicals Company
Dose: 35 mg/kg (material safety data sheet)

(b) Ethylene diamine tetraacetic acid (EDTA) was purchased from BDH Chemicals Company
Dose: 55 mg/kg

(C) 1, 4, 8, 11-tetraazacyclotetradecane (Cyclam) was purchased from BDH Chemicals Company
Dose: 30 mg/kg

4.2. Animals and experimental design

Twenty eight male albino rats aged 8-10 weeks and weighting 107-143g were obtained from the animal house of the Faculty of Medicine, Alexandria University. Animals were handled in accordance with the principles of laboratory animal care as contained in NIH guide for laboratory animal welfare. The rats were housed in stainless steel bottomed wire cages and maintained at a temperature of 22 ± 2^{o}C, relative humidity of 40-60%, with a 12 h/12 h light/dark cycle and free access to pellet diet and water ad libitum. After two weeks of acclimatization, rats were randomly assigned to four groups with seven animals for each group. The tested substances were intraperitoneally injected to the animals according to the following experimental protocol:

- Group I(Control) : served as control.
- Group II(Cobalt Chloride) : Rats were injected intraperitoneally with Cobalt Chloride dissolved in distilled water (35mg/kg), for 24 hours (Amudha and Pari, 2011).
- Group III (Cobalt Chloride (Co) + Ethylene diamine tetraacetic acid (EDTA): Rats were treated with both Cobalt Chloride (35mg/kg) and EDTA (55mg/kg) by intraperitoneal injection for 24 hours (Ogutcu et al., 2006).
- Group IV (Cobalt Chloride (Co) + 1, 4, 8, 11-tetraazacyclotetradecane (Cyclam): Rats were treated with both Cobalt Chloride (35mg/kg) and Cyclam (30mg/kg) by intraperitoneal injection for 24 hours (Ogutcu et al., 2006).

At the end of experimental period, blood samples were taken from the aortic plexus; a portion was taken into tubes containing EDTA for plasma analysis. Rats were dissected and the abdominal cavity of each one was opened where the liver and kidney were immediately removed, these tissues were further used for enzyme analyses.

4.3. Biological samples and assay

4.3.1. Blood and tissue-sampling

Blood was collected from all examined rats at EDTA tubes to get plasma. The collected blood was centrifuged at 3000 r.p.m for 15 min to obtain plasma that was kept at -80°C until used for biochemical analyses.

4.3.2. Tissue preparation

The dissected organs were washed in ice-cold saline to remove the blood. The tissues (liver and kidney) were homogenized (10% W/V) in 1.15%KCl, 0.01M sodium, potassium phosphate buffer (pH 7.4) using a Potter-Elvehjem type homogenizer. The homogenized tissues were centrifuged at 10.000 r.p.m. for 30 min at 4°C and the clear supernatants were used for various biochemical assays.

4.4. Principles of biochemical and enzymatic assays

4.4.1. Thiobarbituric acid reactive substances

Quantitative measurement of lipid peroxidation was performed in tissue homogenate (10%) based on the formation of thiobarbituric acid reactive substances (TBARS) and expressed as the extent of malondialdehyde (MDA) production. The basis of this method is the reaction of malondialdehyde with thiobarbituric acid (TBA) at low pH and high temperature to form a colored complex. This complex is called Thiobarbituric acid reactive substances (TBARS) which is measured spectrophotometrically at 535 nm.(Esterbauer and Chesseman, 1990).

Reagents

1- KCl (1.15 M) : 85.7 g of KCl were dissolved in distilled water and the volume adjusted to (1L).
2- Thiobarbituric acid (TBA) 0.67 %: 0.679 g of TBA was dissolved in distilled water and the volume adjusted to 100 ml.
3- Trichloroacetic acid (TCA) 20%: 20 g of TCA were dissolved in distilled water and the volume adjusted to 100 ml.

Procedure

One gram of the tissue was added to 3 ml of KCl solution (1.15 M), Grinded by homogenizer which fitted with a Teflon piston. Cosquently, 0.5 ml of TCA and 1 ml TBA were added into 0.5 ml of homogenate. The mixture was heated to 100 ^{o} C for 15 minutes, after cooling, 4 ml butanol were added. The specimens were centrifuged at 1500 rpm for 15 min. Finally, the optical density of the suprrnatant measured using UV-160IBC, UV Visible double beam spectrophotometer, Shimadzu, at 530 nm. MDA is expressed in n mole/g tissue. All measurements were duplicated**.**

Preparation of MDA standard curve

Gradual concentration ranging from (10 - 100 nmol/ml) of stock solution of 1.1.3.3 – tetramethoxypropane (fluka chemical company) (1000 nmol) was prepared for standard

curve. The concentration of MDA in test samples was calculated using a standared curve Figure (4)

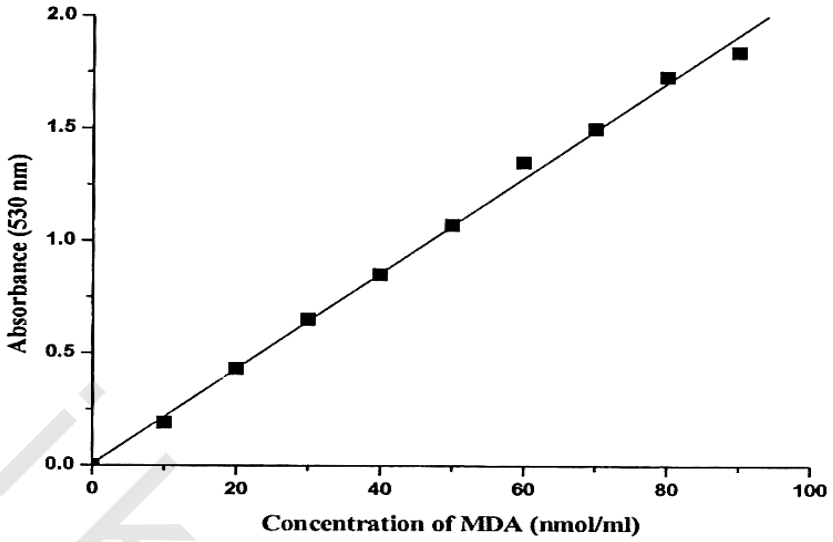

Figure (4): MDA standard curve

Calculation

$$\text{Concentration of MDA (nmol / ml)} = \frac{A}{slope}$$

Where:

A: Absorbance of recorded sample

Slop: obtained from the standard curve

4.4.2. Reduced Glutathione (GSH)

Reduced glutathione content was assayed in liver and kidney by the method which utilized metaphosphoric acid for protein precipitation and 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) for color development and its intensity was measured at 412 nm using U.V. double beam spectrophotometer (Ellman, 1959).

Reagents

1. Disodium hydrogen phosphate solution (0.3 M). It was prepared by dissolving 4.23 g of Na_2HPO_4 in 100 ml distilled water.
2. DTNB reagent. It was prepared by dissolving 4 mg of (5.5′- dithiobis-2-nitrobenzoic acid) in 10 ml of 1% (w/ v) sodium citrate.

Reduced glutathione standard curve

A- Stock standard glutathione

Glutathione (GSH) (MP Biomedical, LTC) gama glutamyl- cysteinylglycine, (MW = 307.3). 5mmol /L solution of GSH was prepared by dissolving 0.015 g of the GSH in 10 ml distilled water.

B- Serial dilutions of the standard GSH solution

GSH (3mmol / L) solution was prepared by taking 1.2 ml of the stock solution and completing the volume to 2 ml with distilled water. This step was repeated by taking 1, 0.8, 0.6, 0.4, 0.2 and 0.1 ml to obtain 2.5, 2, 1.5, 0.5 and 0. 25 µg GSH solution, respectively.

C- Procedure

Exactly 50 µ L of distilled standard samples were mixed with 1.7 ml of the disodium hydrogen phosphate solution. To the above mixture, 250 µ L of DTNB reagent was added and The absorbance of the sample was measured at 412 nm.The standard curve of GSH was constructed as showed in **Table (3) and Figure (5)**. All measurements were duplicated.

Table 3: GSH concentration and the corresponding absorbance used to construct the standard curve

Tube number	Concentration (mmol/L)	Absorbance
1	0.25	0.127
2	0.5	0.292
3	1	0.415
4	1.5	0.69
5	2.0	0.874
6	2.5	0.98

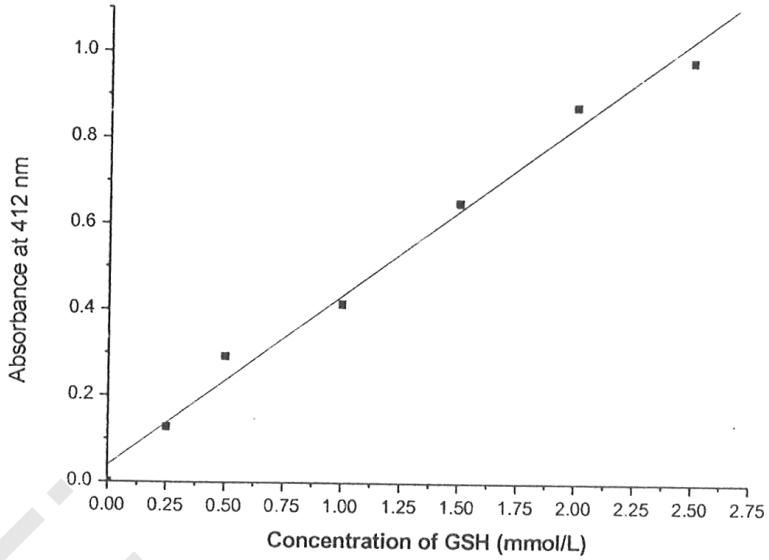

Figure (5): Standared curve of GSH

4.4.3. Superoxide dismutase (SOD)

Principle

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form acrucial part of the cellular antioxidant defense mechanism (Misra and Fridovich, 1972). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate –mediated reduction of nitrobl ue tetrazotium dye. SOD activity was determined in rat liver and kidney.

$$2O_2^{\bullet} + 2H^{+} + SOD \longrightarrow H_2O_2 + O_2$$

Reagents:

1	Phosphate Buffer pH 8.5	50 mM/l
2	Nitroblue tetrazolium (NBT)	1 mM/l
3	NADH	1 mM/l
4	Phenazine methosulphate (PMS)	0.1 mM/l
5	Extraction Reagent	

- R4 should be diluted 1000 times immediately befor use (10 µ + 10 m l dist. Water) , discard after use.
- Sample should be diluted to give an inhibition percent between 30 and 60**.**
- Working Reagent: Mix R1 + R2 + R3 in ratio of (10+1+1 ml) , immediately befor use.

Procedure:

R4 should be diluted 1000 times immediately befor use (10 µ + 10 m l dist. Water) , discard after use. Sample should be diluted to give an inhibition percent between 30 and 60**.** Working Reagent: Mix R1 + R2 + R3 in ratio of (10+1+1 ml) , immediately befor use.

For sample test tubes , 0.1 mLof the samples were added with 1mL of working reagent but for control 0.1 mL of the the sample is replaced by 0.1 mL distilled water.the test tubes were mixed well to inititiate the reaction by addition of 0.1 mL of PMS (R_4). The increase in the absorbance is measured at 560 nm for 5 minute for control (Δ A control) and for sample (Δ A sample) at 25 ^{o} C. All measurements were duplicated.

Calculation

$$\text{Percent inhibition} = \frac{\Delta A \text{ control} - \Delta A \text{ sample}}{\Delta A \text{ control}} \times 100$$

Where

$\Delta A_{control}$ = The change in absorbance at 560 nm over 5 min.following the additionof PMS to the reaction mixture in the absence of sample.

ΔA_{sample} = The change in absorbance at 560 nm over 5 min.following the additionof PMS to the reaction mixture in the presence of sample

Purified SOD was shown to inhibit the initial rate of photo activated phenazine meth - osulphate mediated reduction of $O_2^{\bullet}$ to O_2 which then reduced nitroblue tetrazolium.

SOD activity:

$$\text{U / gm tissue} = \% \text{ inhibition} \times 3.75 \times \frac{1}{gm\ tissue\ used}$$

4.4.4 .Catalase

Principle:

Catalase reacts with a known quantity of Hydrogen peroxide (H_2O_2). The reaction is stopped after exactly one minute with catalase inhibitor (Aebi, 1984)

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

In the presence of peroxidase (HRP), remaining H_2O_2 react with 3,5-Dichloro-2-hydroxybenzenesulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample. CAT activity was determined in liver and kidney.

$$2\ H_2O_2 + \text{DHBS} + \text{AAP} \xrightarrow{\text{HRP}} \text{Quinoneimine Dye} + 4\ H_2O$$

Reagents:

1. Reagent 1 (phosphate buffer PH 7.0, 100 mm/l)
2. Reagent 2 (Hydrogen peroxide , 500 mm/l) was diluted 1000 times immediately before use
3. Reagent 3 (chromogen-inhibitor)
4. Reagent 4 (enzyme : perxidase, >2000 mm/l and 4-aminoantipyrine, 2 mm/l)

Procedure:

In four test tubes, 0.05 ml sample blank and 0.05 ml distilled water (sample blank) , 0.05 ml sample (sample) , 0.10 ml distilled water (standard bank) , 0.05 ml standard (standard) were mixed with 0.50 ml of reagent one . 0.10 ml of the reagent 2 was added to the sample and standard , mixed well and incubated exactly at 25ºC for 1 minute. 0.20 ml of reagent 3 and 0.50 ml reagent 4 were added to the solutions and incubated at 37ºC for 10 minutes. The absorbances of sample (Asample) were read against sample blank and standard ($A_{standard}$) against standard blank at wavelength 510 nm. All measurements were duplicated.

Calculation:

The specific activity of catalase was calculated using the following equations:

$$(U/mg\ protein) = A_{standard} - A_{sample} / A_{standard} * 1/ mgprotein\ used\ per\ test$$

4.4.5. Glutathione S-transferase

Principle:

The measurement of the activity of GST in liver and kidney is determined by using para-nitrobenzylchloride as a substrate (Habig *et al.*, 1974). The absorbance was measured spectrophotometrically at 310 nm using U.V. double beam spectrophotometer. GST catalyzes the following reaction:

$$\text{p-Nitrobenzyl chloride + Glutathione} \xrightarrow{GST} \text{Glutathione nitrobenzyl}$$

Reagents:

1. Phosphate buffer, 0.1 M, pH 6.5.
2. Reduce glutathione (GSH), 5 mM.
3. P-nitrobenzyl chloride, 1 mM in ethanol.

Procedure:

In a test tube, 100 µl GSH, 10 µl p-nitrobenzyl chloride and 25 µl sample were added to 1.365 ml phosphate buffer, pH 6.5 and vortex then incubated for 20 minutes at room temperature. The absorbance of sample was read against air at 310 nm.

Calculation:

The activity of GST for sample was calculated with the following equations:

$$GST\ specific\ activity = At/1.9* time * mg\ protein$$

Where;

Extinction coefficient was 1.9

4.4.6. Lactate dehydrogenase

Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate by NADH, to form lactate and NAD^+. The catalytic concentration of the enzyme is determined from the rate of decrease of NADH measured spectrophotometrically at 340nm (Cabaud and Wroblewski, 1958)

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{LDH} \text{Lactate} + NAD^+$$

The initial rate of the NADH oxidation is directly proportional to the catalytic LDH activity. It is determined by measuring the decrease in absorbance at 340 nm.

Reagents

Reagent 1 (R1 Buffer)

Tris buffer (pH 7.5)	50 mmol/L
Pyruvate	3.0 mmol/L
Sodium Azide	8.0 mmol/L

Reagent 2 (R2 Coenzyme)

NADH	> 0.18 mmol/L
Sodium azide	8.0 mmol/L

Reagent Preparation

Prepare the working solution according to the number of tests required by mixing 9 volumes of reagent 1 (R1) and 1 volume of reagent 2 (R2), e.g. 900 ml R1 +100 ml R2.

Working solution is stable for 2 months at 2 – 8 ºC or 1 week at 15 -25 ºC.

Procedure

Pipette into cuvette (37 ºC):

Add 1 ml working solution in test tube

Add 20µl serum specimen

Mix, read initial absorbance after 30 secnods and start timer simultaneously. Read again after 1, 2 and 3 minutes. Determine the mean absorbance change per minute (DA/min).

Calculation

The LDH specific activity can be calculated using the following formula

Specific acU/mg protein = 8095 x DA at 340 nm/min/mg protein.

4.4.7. Alkaline phosphatase

Principle:

Colorimetric determination of alkaline phosphatase depends on the following reaction:

$$\text{Phenyl phoshate} \xrightarrow[\text{PH 10.0}]{\text{Alkaline phosphatase}} \text{phenol} + \text{phosphate}$$

The liberated phenol is measured colorimetrically in the presence of 4-aminophenazone and potassium ferricyanide (Principato et al., 1985). ALP activity was measured in liver and kidney.

Reagents:

1. Reagent 1 (standard phenol,1.59 mmol/L)
2. Reagent 2 (buffer PH 10.0, 50 mmol/L and phenyl phosphate, 5 mmol/L)
3. Reagent 3 (enzyme inhibitor : EDTA,100 mmol/L and 4-aminophenazone, 50 mmol/L)

4. Reagent 4 (color reagent: potassium ferricyanide, 200 mmol/L)

Procedure:

In three test tubes, 0.025 ml sample (test) or 0.025 ml reagent 1(standard) or empty test tube (blank) were added to 0.50 ml reagent 2, mixed well and incubated at 37ºC for 20 minutes. 0.25 ml of reagent 3 mixed with the solutions then added 0.25 ml of reagent 4 and mixed well. The solutions allowed standing at room temperature in the dark for 5 minutes. The absorbance of standard (As) and test (At) were read against blank at wave length 510 nm.

Calculation:

Alkaline phosphatase activity (IU/mg protein) = At / As *75/mg protein

4.4.8. Alanine amino transferase

Principle:

The amino group was enzymatically transferred by ALT present in the sample from alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate

$$L-Alanine + 2-Oxoglutarate \xrightarrow{ALT} Pyruvate + L\text{-}Glutamate$$

ALT activity was measured in liver and kidney by monitoring the concentration of pyruvate hydazone formed with 2,4-dinitophenylhrdrazine (Reitman and Frankel, 1957).

Reagents:

1. Reagent 1 (R1 buffer : phosphate buffer, 100 mmol/L; DL-Alanine, 200 mmol/L; 2-oxoglutarate, 6 mmol/L; sodium azide, 12 mmol/L)
2. Reagent 2 (2,4-dinitophenylhrdrazine, 2.0 mmol/L)
3. Sodium hydroxide, 0.4 mol/L

Procedure:

In two test tubes, 100 µl sample (test) or 100 µl distilled water (blank) were added to 0.5 ml reagent 1, mixed well and incubated at 37ºC for 30 minutes. 0.5 ml of reagent 2 was mixed with the solutions and incubated at 25ºC for 20 minutes. 5.0 ml of sodium hydroxide was mixed with the solutions. The absorbance of standard (As) and test (At) were read against blank at wave length 546 nm after 5 minutes.

Calculation:

The ALT activity in the sample can be determined from the following Table:

Absorbance	Concentration (U/L)
0.025	4
0.050	8
0.075	12
0.100	17
0.125	21
0.150	25
0.175	29
0.200	34
0.225	39
0.250	43
0.275	48
0.300	52
0.325	57
0.350	62
0.375	67
0.400	72
0.425	77
0.450	83
0.475	88
0.500	94

4.4.9. Aspartate amino transferase

Principle:

The amino group was enzymatically transferred by AST present in the sample from L-aspartate to the carbon atom of 2-oxoglutarate yielding oxaloacetate and L-glutamate

$$\text{L-Aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate}$$

AST activity was measured in liver and kidney by monitoring the concentration of oxaloacetate hyrazone formed with 2,4-dinitophenylhrdrazine (Reitman and Frankel, 1957).

Reagents:

1. Reagent 1 (R1 buffer : phosphate buffer, 100 mmol/L; L- aspartate, 100 mmol/L; 2-oxoglutarate, 5mmol/L; sodium hydroxide, 140 mmol/L; sodium azide, 12 mmol/L)

2. Reagent 2 (2,4-dinitophenylhrdrazine, 2.0 mmol/L)
3. Sodium hydroxide, 0.4 mol/L

Procedure:

In two test tubes, 100 µl sample (test) or 100 µl distilled water (blank) were added to 0.5 ml reagent 1, mixed well and incubated at 37ºC for 30 minutes. 0.5 ml of reagent 2 was mixed with the solutions and incubated at 25ºC for 20 minutes. 5.0 ml of sodium hydroxide was mixed with the solutions. The absorbance of standard (As) and test (At) were read against blank at wavelength 546 nm after 5 minutes.

Calculation:

The AST activity in the sample can be determined from the following table:

Absorbance	Concentration (U/L)
0.020	7
0.030	10
0.040	13
0.050	16
0.060	19
0.070	23
0.080	27
0.090	31
0.100	36
0.110	41
0.120	47
0.130	52
0.140	59
0.150	67
0.160	76
0.170	89

4.4.10. Total protein

Principle:

Protein in an alkaline cupric sulfate (biuret reagent) produces a violet color complex. The intensity of colour in serum was directly proportional to protein concentration and measured at 546 nm spectrophotometrically (Armstrong and Carr, 1964).

Reagents:

1. Standard albumin, 5 g/dl
2. Biuret reagent (cupric sulfate, 6 mmol/l; sodium potassium tartrate, 21 mmol/l; sodium hydroxide 750 mmol/l; potassium iodide, 6 mmol/l)

Procedure:

In three test tubes, 0.025 ml sample (test), 0.025 ml standard albumin (standard) or empty test tube (blank) were added to 1ml biuret reagent. The solutions were mixed well and incubated for 10 minutes at 37 ºC. The absorbance of standard (As) and test (At) were read against blank at wavelength 546 nm.

Calculation:

Protein concentration was calculated using the following equation:
Protein concentration (g/dl) = At / As * 5

4.4.11. Albumin

Principle:

Serum albumin at PH 4.2 is sufficiently cationic to bind dye bromcresol green (BCG) to form a blue-green colored complex. The intensity of the colour is measured spectrophotometrically at 630 nm (Doumas *et al*., 1977).

$$\text{Albumin} + \text{BCG} \xrightarrow{\text{PH 4.2}} \text{Albumin-BCG complex}$$

The intensity of blue-green color is directly proportional to albumin concentration in the specimen.

Reagents:

1. Standard Albumin solution (4 g/dl).
2. Colour reagent (succinate buffer PH 4.2, 75 mmol/l; bromcresol green , 0.26mmol/l)

Procedure:

In three test tubes, 10µl sample (test), 10µl Standard albumin solution (standard) or empty test tube (blank) were added to 2 ml colour reagent, the absorbance of standard (As) and test (At) were read after 5 minutes against blank at wavelength 630 nm.

Calculation:

The concentration of Albumin concentration was estimated according to the following equations:

Serum albumin concentration (g/dL) = At / As * concentration of standard.

4.4.12. Urea content

Principle:

Serum urea was hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

In an alkaline medium, the ammonium ions produced react with salicylate and hypochlorite to form green coloured indophenols. The produced colour is assessed spectrophotometrically at 578 nm (Patton and Crouch, 1977).

Reagents:

1. Reagent 1 (R1 buffer :phosphate buffer PH 8.0, 100 mmol/l; sodium salicylate, 80 mmol/l; sodium nitroprusside, 6.0 mmol/l; EDTA, 30.0 mmol/l)
2. Reagent 2 (Urease > 6000 U/l)
3. Reagent 3 (sodium hydroxide, 400 mmol/l; sodium hypochlorite, 20.0 mmol/l)
4. Standard urea (50 mg/dl)

Procedure:

In three test tubes, 10 µl sample (test) or 10 µl standard urea solution (standard) or empty test tube (blank) were mixed with 50 µl reagent 2 and 1.0 ml of the reagent 1. The solutions were mixed well and incubated at 25ºC for 5 minutes. 200 µl of reagent 3 was mixed with the solutions and incubated at 25ºC for 10 minutes. The absorbance of standard (As) and test (At) were read against blank at wavelength 578 nm.

Calculation:

The urea concentration was calculated using the following equation:

Urea concentration (mg/dl) = At / As * concentration of standard.

4.4.13. Creatinine

Principle

Plasma creatinine in an alkaline medium reacts with picrate forming a colored complex. The colour intensity is proportional to the creatinine concentration in the specimen and is determined by measuring the absorbance at 500 nm using spectrophotometer (Henry, 1974).

After deproteinization creatinine in alkaline solution, forms a yellow-red complex with picrate.

Alkaline pH
Creatinine + Picric acid >>>>>>>>>> Creatinine-Picric acid complex

The intensity of the color produced is directly proportional to creatinine concentration. It is determined by measuring the increase in absorbance at 500 – 550 nm.

Reagents

R1 Creatinine standard	2.0 mg/dl
R2 Picric acid	38 mmol/l
R3 Sodium hydroxide	1.2 mol/l
Trichloroacetic acid	1.2 mol/l

Working solution (R2 + R3):

According to requirements, prepare the working solution by mixing equal volumes of R2 and R3. The working solution is stable for 6 hours at 20-25 0C, when stored in a dark bottle.

Procedure

Pipette into centrifuge tubes 0.5 ml serum and 0.5 ml TCA

Mix, well, centrifuge at 2500 rpm for 10 min. Collect the supernatant (protein free filtrate). (PFF). The PFF can be stored up to 7 days at 4ºC.

Add 0.5 ml Dist. H_2O in Blank only

Add 0.5 ml TCA 1.2 M in Blank and Standard tubes

Add 0.5 ml standard in the standard tubes

Add 0.5 ml PFF in the specimen tubes

Add 1 ml of the working solution in all tubes.

Mix well, measure the absorbance of specimen and standard against blank after exactly 20 minute at 20 - 25ºC.

Calculation

Calculate the creatinine concentration by using the following formulae:

Creatinine Concentration mg/dl=

Absorbance of Specimen/Absorbance of Standard * Standard value

4.4.14. Lipid profile

4.4.14.1. High density lipoprotein (HDL) in plasma

Enzymatic determination of HDL was measured by (Fruchart, 1982).

Principle

Chylomicron, very low density lipoproteins (VLDL) and low density lipoprotein(LDL) of plasma are precipitated by phosphotungstic acid and magnesium ions. After centrifugation, high density lipoprotein(HDL) was in the supernatant. Cholesterol included in this phase, is measured by enzymatic method.

Reagents

Reagent	Component	Concentration
Reagent 1	phosphotungstic acid	14 mMol/L
Reagent 2	Magnesium Sulphate	1 mol/L
Standard	Cholesterol	50 mg/dl
Cholesterol PAP Kit is used to HDL determination	-	200mg/dl

Procedure

In test tube, 500 µL of samples were added with 40 µL of R_1 and 10 µL of R_2 (precipitating reagent). Test tubes were mixed, waited for 10 minutes and centrifuged at 5000 r.p.m for 15 minutes. The supernatant was collected for HDL determination.

Reagents were added as follow:

Reagent	Blank	Standard	Samples
Cholesterol reagent	1 ml	1 ml	1 ml
Distilled water	50µL	-	-
Standard(50mg/dl)	-	50µL	-
Supernatant	-	-	50µL

All tubes were mixed at 500 nm and read the optical density (OD) at 500 nm after 5 minutes incubation the final color is a stable for at least 1 hour.

Calculations

$$\text{HDL level (mg/dl)} = \frac{\text{OD Sample-OD Blank}}{\text{OD Standard-OD Blank}} \times 50 \times 1.1$$

50 = Standard concentration (mg/dl)

1.1 =dilution factor of sample

4.4.14.2. Low density lipoprotein (LDL)

Enzymatic determination of LDL was measured by (NCEP, 2001).

Principle

Low density lipoprotein (LDL) in sample precipitate with polyvinyl sulphate. Their concentration is calculated from the difference between the plasma total cholesterol and cholesterol in the supernatant after centrifugation. The cholesterol is spectrophotometrically measured by the means of coupled described below:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + 1/2\ O_2 + H_2O \xrightarrow{\text{peroxidase}} \text{Choleserone} + H_2O_2$$

$2H_2O_2$ + 4Aminoantipyrine + phenol ⟶ Quanonemine $+4H_2O$

Reagent A (Cholesterol LDL Kit)

1*20ml polyvinyl sulphate (3g/L), polyethylene glycol (3g/L)

Procedure

A- Precipitating step:

In labeled centrifuge tubes, 0.4 ml sample and 0.2 ml of reagent A (cholesterol LDL Kit) Were added. All tubes were mixed thoroughly and waited for 15 minute at room temperature. All tubes were centrifuged at 4000 r.p.m. for 15 min. after that the supernatants were collected carefully.

B- Colorimetric measurement:

Reagents were added as follow

Reagent	Blank	Standard	Samples
Distilled water	20µL	-	-
Cholesterol standard (200mg/dl)	-	20µL	-
Sample supernatant	-	-	20µL
Cholesterol LDL Kit	1 ml	1ml	1 ml

All tubes were mixed thoroughly and incubated the tubes at room temperature. The absorbance (A) of standard and samples were measured against blank at 500 nm. The color was stable for at least 30 minutes.

Calculation

$$\text{LDL –C (mg/dl)} = \frac{\text{OD Sample –OD Blank}}{\text{OD Standard –OD Blank}} \times 200 \text{ (Standard conc.)}$$

4.4.14.3. Triglycerides (TG) in Plasma:

Enzymatic determination of triglycerides was measured by (Annoni et al., 1982).

Principle

Enzymatic determination of triglycerides according to the following reactions

Glycerol +Fatty acid $\xrightarrow{\text{Lipoprotein Lipase}}$ Triacylglycerol $+H_2O$

Glycerol-3-phosphate +ADP $\xrightarrow{\text{Glycerol Kinase\&Mg++}}$ Glycerol+ATP

Dihydroxyacetone phosphate $+H_2O_2$ $\xrightarrow{\text{GPO}}$ Glycerol-3-phosphate $+O_2$

Red quinine + $4H_2O$ $\xrightarrow{\text{peroxidase}}$ $2H_2O_2$ +4-aminoantipyrine +ADPS

GPO = Glycerol-3-phosphate Oxidase.

ADPS = N-Ethyl-N-Sulfopropyl-n-methoxyaniline

Reagent	Component	Concentration
Reagent 1	Pipes buffer	pH 7.5,50 mMol/L
	ADPS	1mMol/L
	Magnesium salt	15mMol/L
Reagent 2	Lipoprotein lipase	100U/L
	Glycerolkinase	800U/L
	Glycerol-phosphate kinase	5000U/L
	peroxidase	350U/L
	Amino antipyrine	0.7mMol/L
	ATP	0.3mMol/L
Standard	Glycerol(Triglycerides equivalent).	200mg/dl

Procedure

1- Reagent were added as follows

Reagent	Blank	Standard	Sample
Working reagent	1ml	1ml	1ml
Distilled water	10µL	-	-
Standard	-	10µL	-
Sample	-	-	10µL

2- All tubes were mixed and optical density (OD) after 5 minutes of incubation at 546nm. The final color is stable for at least 30 minutes**.**

Calculation

$$\text{TG conc. (mg/dl)} = \frac{\text{OD sample-OD Blank}}{\text{OD Standard-OD Blank}} \times 200(\text{standard conc})$$

4.4.14.4. Total Cholesterol in plasma

Principle

Enzymatic colorimetric determination of total Cholesterol was measured by (Allian et al., 1974).

Colorimetric determination of total Cholesterol level occurs according to the following reactions:

Cholesterol +Fatty acids $\xrightarrow{\text{Cholesterol esterase}}$ Cholesteryl ester +H_2o

4-Cholesten-3one +H_2O_2 $\xrightarrow{\text{Cholesterol oxidase}}$ Cholesteryl ester +O_2

Quinoneimine +4H_2O $\xrightarrow{\text{Peroxidase}}$ 2H2O2 +phenol+4-aminoantipyrine

Reagent	pipes buffer	50mMol/L
	phenol	24mMol/L
	Sodium cholate	0.5mMol/L
	4aminoantipyrine	0.180U/L
	Cholesterol esterase	200U/L
	Cholesterol oxidase	1000U/L
	Peroxidase	
Standard	Standard Cholesterol	200mg/dl

Procedure

1- All reagent were added as follows

Reagent	Blank	Standard	Sample
Working reagent	1ml	1ml	1ml
Distilled water	10µL	-	-
Standard	-	10µL	-
Sample	-	-	10µL

2- All tubes were mixed and the optical density (OD) was measured at 500 nm after 5 minutes of incubation at room temperature. The final color is stable for at least 1 hour.

Calculation

$$\text{TC Conc. (mg/dl)} = \frac{\text{OD Sample- OD Blank}}{\text{OD Standard- OD Blank}} \times 200 \text{ (Standard conc)}$$

4.5. Statistical analysis

All measurements were duplicately performed in independent experiments for all the treatments. The results were expressed as mean ± standard error (SE). Statistical analyses were made with one-way analysis of variance (ANOVA) using SPSS 17. When differences were found, post hoc multiple comparisons by Duncan multiple range test (DMRT) was used to determine the differences between specific treatments. The criterion for statistical significance was $P<0.05$.