MATERIAL & 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 where described here in.

3.1. Material

3.1.1. Chemicals and kits

Bacillus thuringiensis subsp. *Kurstaki* (active ingredient 9.4%, inert ingredients 90.6%) was supplied from Kafr El-Zayat Pesticides and Chemicals Company, while methomyl (S-methyl N-(methylcarbamoyloxy) thioacetimidate, 90% purity) was purchased from Shandong Huayang Technology Ltd. Company, China. All other reagents used were of analytical reagent grade. Kits of Triglycerides, Cholesterol, Albumin, LDL, HDL, AST, ALT, Urea, Creatinin, Protein, LDH, ALP were purchased from Biodiagnostic.

3.2. Methods

3.2.1. Experimental design

Twenty eight male albino rats weighing 150-170g 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 (National Institutes of health) guide for laboratory animal welfare. The rats were housed in stainless steel bottomed wire cages and maintained at a temperature of 22 ± 2°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 (*Yousef *et al*., 2002).

Table (4): Composition of the experimental diet (Yousef *et al*., 2002)

Ingredient	**Amount (g/kg)**
Dried skim milk	375
Corn oil	90
Corn starch	300
Sucrose	225
Cellulose	5
Vitamin mixture	1
Mineral mixture	4

After two weeks of acclimatization, rats were randomly assigned to four groups with seven animals each. The test substances were orally administered to the animals according to the following experimental protocol:

Group I (control): Control rats were orally administered with distilled water daily for 21 days.

Group II (methomyl; MET): Rats were orally treated with 3.4 mg methomyl /kg body weight per day (equivalent to 1/10 LD_{50}) for 21 days (El-Demerdash et al., 2013a). The acute oral LD_{50} for male rats is 34 mg/ kg and for female rats it is 30 mg/kg (Tomlin, 2006)

Group III (*Bacillus thuringiensis; Bt*): Rats were orally given 267 mg biopesticide, *B. Thuringiensis* /kg b.w daily for 21 days (Shaban *et al.*, 2003).

Group IV (MET+*Bt*): Rats were orally treated with both MET (3.4 mg/kg BW) plus *Bt* (267 mg/kg BW) daily for 21 days (El-Demerdash *et al.*, 2013a ; Shaban *et al.*, 2003).

At the end of the experimental period, rats were starved overnight and then were sacrificed by cervical decapitation. Blood samples were taken from the aortic plexus and collected into glass tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and the resultant plasma was used for biochemical analyses. The abdominal cavity of each rat was opened where the liver and kidney were removed. These tissues were further used for enzyme and histological analyses.

3.2.2. Sampling

3.2.2.1. Plasma samples

Blood portions collected into glass tubes containing EDTA, were centrifuged at 3000 xg for 15 min to obtain plasma used for biochemical analyses.

3.2.2.2. Tissue samples preparation

The dissected organs were washed in ice-cold saline to remove the blood. Liver and kidney tissues were homogenized separetly with (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 9000 xg for 30 min at 4°C and the clear supernatant was used for various biochemical assays.

3.2.2.3. Histological analysis

Dissected liver and kidney tissues of rats were immediately fixed into 10% formaldehyde saline. Tissues were processed by embedding in paraffin. Sections were cut by rotatory microtome and mounted on glass slides. The sections were stained by conventional Hematoxylin & Eosin (H&E) stain. The sections were examined by light microscope (Bancroft and Stevens, 1990).

3.2.3. Biochemical parameters

3.2.3.1 oxidative stress and antioxidant parameters

3.2.3.1.1. Determination of thiobarbituric acid reactive substances concentration:

Principle

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 wavelength 530 nm. MDA was estimated by the method of Mesbah *et al.*, (2004) in plasma, liver and kidney.

Reagents

1- KCl (1.15 M) : 85.7 g of KCl were dissolved in distilled water and the volume adjusted to 1000 ml (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 at 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 wavelength 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 nmole/ml) of stock solution of 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 9.

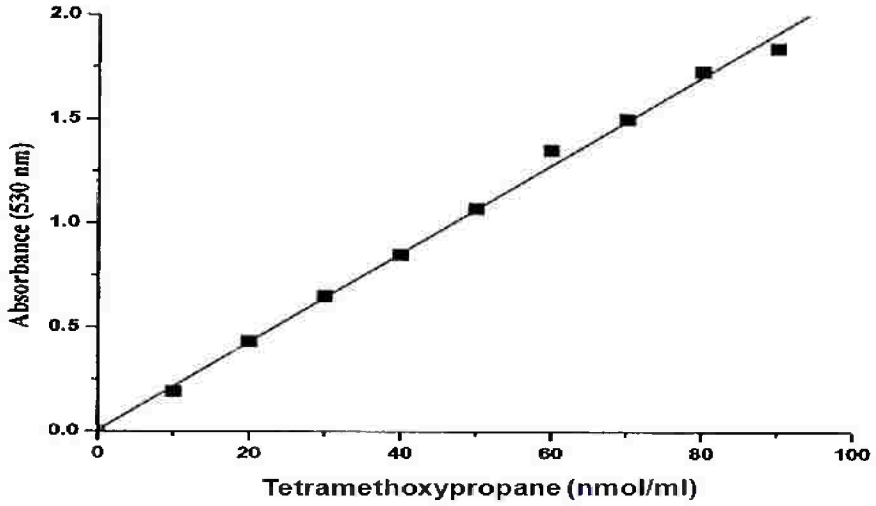

Fig. 9. MDA standard curve

Calculation

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

Where :

AA: Absorbance of recorded sample

Slope : obtained from the standard curve

3.2.3.1.2. Determination of reduced glutathione content:

Principle

Reduced glutathione content was assayed in plasma, 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 wavelength 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 pepared 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 Biomedicals, 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 µL of distilled standared samples were mixed with 1.7 ml of the disodium hydrogen phosphate solution. To the above mixture, 250 µL of DTNB reagent was added and the absorbance of the sample was measured at wavelength 412 nm.The standared curve of GSH was constructed as showed in Table 5 and Figure 10. All measurements were duplicated.

Table (5): 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

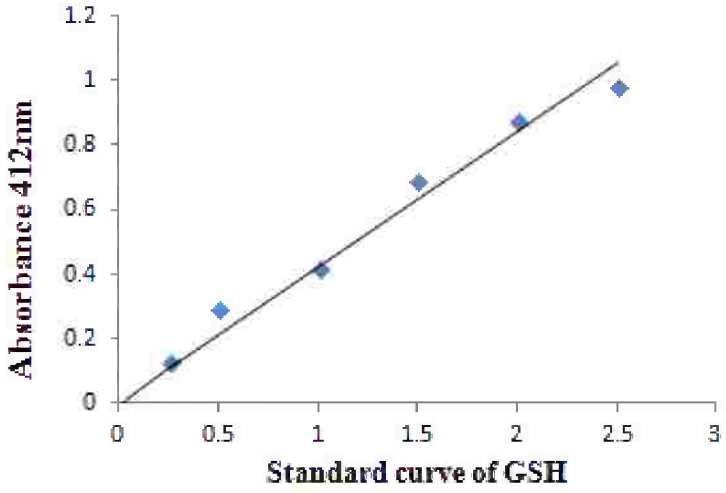

Fig. 10. Standared curve of GSH

3.2.3.1.3. Determination of superoxide dismutase (SOD; EC 1.15.1.1) activity:

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 (Nishikimi *et al.*, 1972). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate –mediated reduction of nitroblue tetrazotium dye. SOD activity was determined in rat plasma, liver and kidney.

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

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

Procedure:

R4 should be diluted 1000 times immediately befor use (10 µ + 10 ml 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:

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

3.2.3.1.4. Determination of catalase (CAT; EC 1.11.1.6) activity

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.

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

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 . The 0.10 ml of the reagent 2 were 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 absorbance of sample (A_{sample}) were read against sample blank and standard ($A_{standard}$) against standard blank at wavelength 240 nm.

Calculation:

The activity of catalase was calculated using the following equations:

1. In plasma (U/l) = $A_{standard}$ - A_{sample} / $A_{standard}$ * 1000
2. In tissue (U/g) = $A_{standard}$ - A_{sample} / $A_{standard}$ * 1/ gm tissue used per test

3.2.3.1.5. Determination of glutathione S-transferase (GST; EC 2.5.1.18) activity:

Principle:

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

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

The assay procedure is based on the formation of glutathione nitrobenzyl.

Reagents:

1. Phosphate buffer, 0.1 M, pH 6.5.
2. Phosphate buffer, 0.1 M, pH 7.4.
3. Reduce glutathione (GSH), 5 mM.
4. 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 wavelength 310 nm.

Calculation:

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

$$\text{GST activity} = \frac{\text{At}}{1.9 \times \text{time} \times \text{mg protein}}$$

Where;

At: absorbance of sample (test)

Specific activity = µmol / min. / mg of protein.

Extinction coefficient was 1.9

3.2.3.2. Liver function parameters

3.2.3.2.1. Determination of alanine amino transferase (ALT; EC 2.6.1.2) activity:

Principle:

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

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

ALT activity is measured by measured by monitoring the concentration of pyruvate hydazone formed with 2,4-dinitophenylhrdrazine (Reitman and Frankel ,1975).

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 (bank) 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:

Table (6): Determination of ALT activity in the sample

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

3.2.3.2.2. Determination of aspartate amino transferase (AST; EC 2.6.1.1) activity:

Principle:

The amino group is 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 is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitophenylhrdrazine (Reitman and Frankel ,1975).

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 (bank) 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:

Table (7): Determination of AST activity in the sample

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

3.2.3.2.3. Determination of Lactate dehydrogenase (LDH; EC 1.1.1.27) activity

Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate by NADH, to form lactate and NAD^+ (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 wavelength 340 nm.

Reagents

1. Reagent 1 (R1 Buffer) (Tris buffer pH 7.5, 50 mmol/L; Pyruvate,3.0 mmol/L; Sodium Azide, 8.0 mmol/L)
2. Reagent 2 (R2 Coenzyme) (NADH > 0.18 mmol/L and Sodium azide, 8.0 mmol/L)
3. Working solution (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 ℃ or 1 week at 15 -25 ℃).

Procedure

In one test tubes at 37 ℃, 1 ml working solution was added to 20µl sample (test). The solution was mixed. The absorbance was read after 30,1, 2 and 3 minutes. The mean absorbance change per minute (ΔA/min) was determined.

Calculation

The LDH specific activity can be calculated using the following formula

U/mg protein = 8095 x ΔA at 340 nm/min/mg protein.

3.2.3.2.4. Determination of alkaline phosphatase (ALP; EC 3.1.3.1) activity

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 (Belfield and Goldberg, 1971).

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 (bank) 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 tostand 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/L) = At / As *75

3.2.3.2.5. Determination of protein content:

The protein content of the plasma, liver and kidney were determined by following the method described by Lowry *et al.* (1951).

Principal:

Protein gives violt – blue complex with copper salts in alkaline medium.

Reagents

1- Modified protein assay reagent : 3 g $CuSO4.5H_2O$ and 5g KI were dissolved in 500ml of (0.2N) NaOH containing 9g sodium potassium tartarate, then the volume of this solution was raised up to one litre by 0.2 N NaOH. The working reagent was freshly prepared by adding 2.3 % sodium carbonate to stock reagent in the ratio 7: 1

2- Folin Ciocaltu reagent (2N).

3- Stock standard protein solution (200 mg /dl)

4- Working standared protein solution (2mg /ml)

Set of standard protein concentration of bovin serum albumin (BSA) were prepared from the working standared protein solution in the concentration of (0.2- 2 mg / ml) phosphate buffer (Fig.11)

Procedure

One hundred micro liter of homogenate (10% w/v) and 0.9 ml phosphate buffer were added to 4ml working biuret reagent and vortexed. The mixture was allowed to stand for 10 min at room tempreture.One hundred twenty – five µL of folin were added, vortexed and left for 30 min at room temperature. A blank tube (1ml of phosphate buffer instead of sample) was prepared in a simillar manner.The absorbance was measured at wavelength 650 nm.

Calculation

$$\text{Concentration of protein (mg /ml)} = \frac{\mathbf{AA}}{\text{slope}}$$

AA: Aborbance of sample.

Slope: obtained from the standard curve.

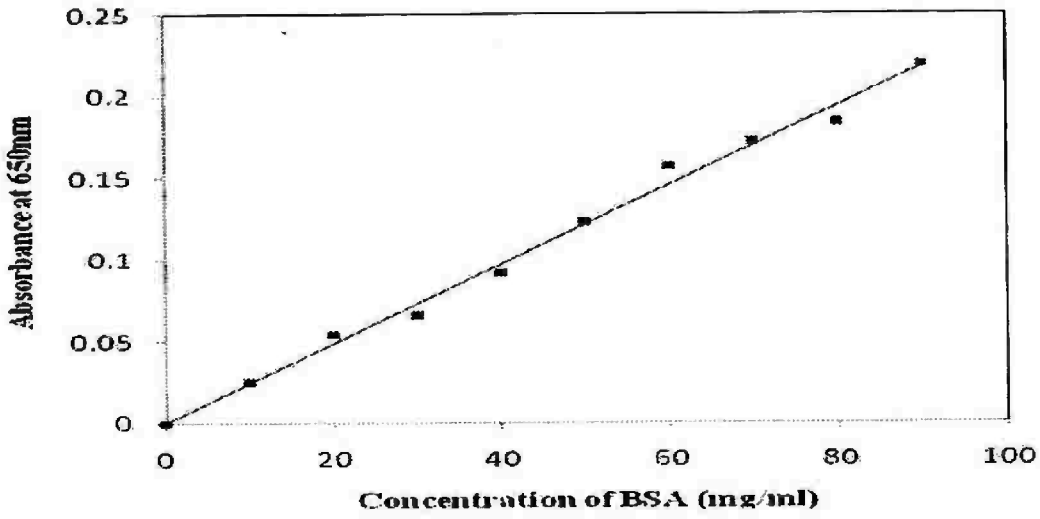

Fig. 11. Total protein standared curve

3.2.3.2.6. Determination of plasma albumin concentration:

Principle:

Albumin at PH 4.2 is sufficiently cationic to bind dye bromcresol green (BCG) to form a blue-green colored complex (Doumans *et al.*, 1971).

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

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 605 nm.

Calculation:

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

Plasma albumin concentration (g/dl) = At / As * concentration of standard.

3.2.3.3. Kidney function parameters

3.2.3.3.1. Determination of urea concentration

Principle:

Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide .

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

The free ammonia in an alkaline PH and in the presence of indicator forms colored complex proportional to the urea concentration in the specimen (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), 10 µl standard urea solution (standard) or empty test tube (bank) 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.

3.2.3.3.2. Determination of creatinine concentration

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 wavelength 500 nm using spectrophotometer (Henry *et al.*, 1974). After deproteinization creatinine in alkaline solution, forms a yellow-red complex with picrate.

$$\text{Creatinine + Picric acid} \xrightarrow{\text{Alkaline PH}} \text{Creatinine-Picric acid complex}$$

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

Reagents

1. Reagent 1 (Creatinine standard, 2.0 mg/dl)
2. Reagent 2 (Picric acid, 38 mmol/l)
3. Reagent 3 (Sodium hydroxide, 1.2 mol/l)
4. Reagent 4 (Trichloroacetic acid (TCA),1.2mol/l)
5. 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

In labelled centrifuge tubes,0.5 ml of sample (test) was added to 0.5 ml reagent 4. The tubes were mixed well and centrifuged at 2500 r.p.m. for 10 minutes and the supernatants were separated off (protein free filterate).

In three test tubes, 1.0 ml of sample supernatants (protein free filterate), 0.5 ml of reagent 1 and 4 (standard) or 0.5 ml of distilled water and reagent 4 (blank) were added to 1ml of working solution. The solutions were mixed well. The absorbance of sample and standard were read against blank at wavelength 525 nm.

Calculation

Calculate the creatinine concentration by using the following formulae:

Creatinine Concentration mg/dl=

Absorbance of Specimen/Absorbance of Standard * Standard value (2.0 mg/dl)

3.2.3.4. Lipids profile

3.2.3.4.1. Determination of plasma cholesterol concentration:

Principle:

Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase (CE) to cholesterol and fatty acids. Free cholesterol, including that originally present, is then oxidizied by cholesterol oxidase (CHOD) to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). In the presence of peroxidase, the formed hydrogen peroxide formed affect the oxidative coupling of phenol and 4- Aminoantripyrine to form a red colored quinoneimine dye.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol + Fatty acids.}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2.$$

$$2\ H_2O_2 + \text{4- Aminoantripyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine dye} + 4\ H_2O$$

The intensity of the color produced is directly proportional to Cholesterol concentration. It is determined by measuring the increase in the absorbance at wavelength 500-550 nm (Allain *et al.*, 1974)

Reagents:

1. Reagent 1 (pipes buffer PH 6.9, 90mmol/l; phenol, 26 mmol/l; Cholesterol oxidase, 500 U/l; peroxidase,1250 U/l; 4- Aminoantripyrine,0.4 mmol/l)
2. Standard Cholesterol, 200mg/dl

Procedure:

In three test tubes, 10 µl of sample (test), standard cholesterol solution (standard) or empty test tube (blank) were mixed with 1ml of reagent 1. The solutions were mixed well and incubated at 25 ºC for 5 minutes. The absorbance of samples (At) and standard (As) were read against blank at wavelength 525 nm.

Calculation:

The concentration of cholesterol concentration was estimated according to the following equation:

Cholesterol concentration (mg/dl) = At / As * standard value

3.2.3.4.2. Determination of triglycerides (TAG) concentration:

Principle:

Sample triglycerides incubated with lipproteinlipase (LPL),liberate glycerol and free fatty acids . Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and Adenosine triphosphate (ATP). Glycerol-3-phosphate (G3P) is then converted by glycerolphosphate (GPO) to dihyroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). In the last reaction, H_2O_2 react with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red colored dye:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{glycerol} + \text{free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{G3P} + \text{ADP}$$

$$\text{G3P} + O_2 \xrightarrow{GPO} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AP} + \text{p} - \text{chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the triglycerides concentration in the sample (Fossati and Prencipe, 1982).

Reagents:

1. Reagent 1 : buffer and enzymes (GOOD PH 7.5, 50mmol/L; P-Chlorophenol, 2 mmol/L; lipoprotein lipase, 150000 U/ L; glycerolkinase ≥ 120U/ L;glycerol-3-oxidasa, 500 U/L; peroxidase, 2500 U/L; 4-aminophenazone, 440U/L; ATP, 0.1 mmol/L)
2. Standard triglycerides solution, 200 mg/dl.

Procedure:

In three test tubes,10 µl of sample (test),10 µl standard triglycerides solution (standard) or empty test tube (blank) were added to 1 ml reagent 1 . The tubes were incubated at 25 ºC for 10 minutes. The absorbance of sample (At) and standard (As) were read against blank at wavelength 546 nm.

Calculation:

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

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

3.2.3.4.3. Determination of High density lipoprotein (HDL) cholesterol concentration:

Very Low density lipoprotein (VLDL) and Low density lipoprotein (LDL) in the sample precipitate with phosphotungstate and magnesium ions .The supernatant contains high density lipoprotein (HDL) (Grove, 1979; Burstein *et al.*, 1980). The HDL cholesterol is then spectrophotometrically measured of the coupled reaction described below:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + {}^1/_2\, O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholestenone} + H_2O_2$$

$$2\, H_2O_2 + \text{4- Aminoantripyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\, H_2O$$

Reagents:

1. Reagent 1 (cholesterol HDL kit: phosphotungstate, 0.4 mmol/l and magnesium chloride, 20 mmol/l).
2. HDL cholesterol standard, 15mg/dl

Procedure:

In labelled centrifuge tubes,0.2 ml of sample (test) were added to 0.5 ml reagent 1. The tubes were mixed well and allowed to stand for 10 minutes at room temperature then centrifuged at 4000 r.p.m. for 10 minutes and the supernatants were separated off. In three test tubes, 100µl of sample supernatants, 100µl of HDL cholesterol standard (standard) or 100µl of distilled water (blank) were added to 1ml of reagent 1. The solutions were mixed well and incubated at 25 ºC for 30 minutes. The absorbance of sample and standard were read against blank at wavelength 500 nm.

Calculation:

The HDL cholesterol concentration in the sample was calculated using the following equation:

HDL cholesterol concentration (mg/dl) = A_{sample} / $A_{standard}$ *concentration of standard.

3.2.3.4.4. Determination of Low density lipoprotein (LDL) cholesterol concentration:

Principle:

Low density lipoprotein (LDL) in the sample precipitate with polyvinyl sulphate. Their concentration is calculated from the difference between the serum total cholesterol and the cholesterol in the supernatant after centrifugation (Assmann *et al.*, 1984). The cholesterol is spectrophotometrically measured by means of the coupled reaction described below:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + {}^{1}/_{2}\ O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholestenone} + H_2O_2$$

$$2\ H_2O_2 + \text{4- Aminoantripyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\ H_2O$$

Reagents:

1. Reagent 1 (cholesterol LDL kits : polyvinyl sulphate, 3g/l and polyethyleneglycol, 3g/l)
2. Reagent 2 (pipes buffer PH 6.9, 90mmol/l; phenol, 26 mmol/l; Cholesterol oxidase, 500 U/l; peroxidase,1250 U/l; 4- Aminoantripyrine,0.4 mmol/l)
3. Standard cholesterol, 200mg/dl

Procedure:

In labelled centrifuge tubes,0.2 ml of sample (test) were added to 0.5 ml reagent 1. The tubes were mixed well and allowed to stand for 15 minutes at room temperature then were centrifuged at 4000 r.p.m. for 15 minutes and the supernatants were separated off. In three test tubes, 20 µl of sample supernatants, 20µl of cholesterol standard (standard) or 20µl of distilled water (blank) were added to 1ml of reagent 2. The solutions were mixed well and incubated at 25 ºC for 30 minutes. The absorbance of sample and standard were read against blank at wavelength 500 nm.

Calculation:

The LDL cholesterol concentration in the sample was calculated according to the following equation:

LDL cholesterol= total cholesterol- cholesterol in the supernatant

The cholesterol concentration in the supernatant was calculated using the following general equation:

Cholesterol concentration in the supernatant (mg/dl) = $A_{sample} / A_{standard}$ *concentration of standard cholesterol.

3.2.3.4.5. Determination of Very Low density lipoprotein (VLDL) cholesterol concentration:

VLDL cholesterol concentration in the sample was calculated according to the following equation (Yousef *et al.*, 2003).

VLDL cholesterol concentration (mg/dl) = Triglycerides concentration / 5

3.2.3.5. Determination of plasma acetylcholinesterase (AChE; EC 3.1.1.7) activity:

Principle:

The enzyme assay is based on the breakdown of acetylthiocholine iodide substrate to produce thiocholine that reacts with dithiobisnitrobenzoic acid (DTNB) to form yellow colour (Ellman *et al.*, 1961).

Reagents:

1. Phosphate buffer, 0.1 M, pH 7.4.
2. DTNB (0.01 M).
3. Acetylthiocholine iodide 0.075 M (21.67 mg/ml).

Procedure:

At a test tube, 50 µl of sample were added to 3 ml phosphate buffer, 20 µl Acetylthiocholine iodide, and 100 µl DTNB. The test tube was incubated at 37ºC for 20 minute and at this pH level, there is an appreciable non-enzymic hydrolysis of the substrate. The blank consists of buffer, substrate, and DTNB solutions. The obtained color was estimated at wavelength 412 nm and the specific activity was calculated.

Calculation:

Specific Activity = [A] / ([Molar extinction coefficient of DTNB] * [Protein concentration (mg/ml)] *time)

Where:

Specific activity = moles of substrate hydrolyzed / minute / mg of protein

A = absorbance

Molar extinction coefficient of DTNB = $1.36 * 10^4$ (Ellman *et al.*, 1961).

3.2.4. 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 $P<0.05$.