Aim Of The Work

The present study was carried out to evaluate the possible effects of vitamin A and E supplementation during chemotherapy on apoptosis in breast cancer patients.

Subjects and Methods

I. Chemicals and kits:

- Serum p53 ELISA kit: MedSystems Diagnostics GmbH, Vienna, Austria.
- Serum survivin EIA kit: Assaydesigns, USA.
- QIAamp® DNA Mini and Blood Mini kit, Qiagen, Germany.
- Electrophoresis buffer, TBE (Tris-borate and EDTA), Sigma, Switzerland
- Acyrlamide, N, N'- methylene-bis-acrylamide, Sigma, Switzerland
- N, N, N', N'-tetramethylenediamine (TEMED), Sigma, Switzerland
- Tris-buffered Saline-Tween buffer (TBST), Sigma, Switzerland
- Bovine Serum Alumbin, Sigma, Switzerland.
- Monoclonal mouse anti-human cytochrome c antibody (primary Ab), and Anti-mouse IgG, Biotin antibody (Secondary Ab), eBioscience, San Diego, CA.
- Avidin-horseradish peroxidase (HRP), eBioscience, San Diego, CA.
- 3,3',5,5' tetramethyl-benzidine (TMB), Vector Lab, USA.

All other chemicals used were of an anylatical grade and needed no further purification.

II. Subjects

The present study was carried out on forty-five breast cancer patients (grade II and III), referred to Cancer Management and Research Department-Medical Research Institute-University of Alexandria, for chemotherapy. The study was approved by the Ethics Committee of Medical Research institute. According to its instructions a signed consent was obtained from all individuals enrolled in the study. They were allocated into two groups:-

Group I: Twenty breast cancer patients received the recommended chemotherapy course consisting of 6 cycles of FAC (5-Flourouracil, Adriamycin, and Cyclophosphamide), from each patient in this group a blood sample was collected after surgery and before the first cycle of chemotherapy (Group Ia). From the same patients, another blood sample was collected two weeks after the 6^{th} cycle of the chemotherapeutic course (Group Ib).

Group II: Twenty-five breast cancer patients received the recommended chemotherapy course as in group I, each patient in this group was supplemented with a daily dose of vitamin A, (10000 IU) [306], and vitamin E, (400 mg) [307], during the recommended chemotherapy course. From each patient in this group, a blood sample was collected after surgery and before the first cycle of chemotherapy (Group IIa), then another blood sample was collected from the same patients, two weeks after the 6th cycle of the chemotherapeutic course (Group IIb).

For all patients, clinical and histopathological data were collected including; Fine Needle Aspiration Cytology (FNAC), tumor size, grade, number of positive lymph node, vascular invasion, receptor status, and the presence of previous history of malignancies or any other disease (diabetes, hypertension, and bronchial asthma). Patients with cardiovascular diseases were excluded from this study, as it is well documented that prolonged vitamin A intake may contribute to increased cardiovascular mortality.

III. Methods

1-Sampling

Blood samples were allowed to clot in order to separate serum after centrifugation at 4000 rpm for 15 minutes.

2-The following markers were assessed in the collected serum samples:

* Quantitative determination of p53 concentrations by ELISA kit [308].
* Assessment of serum concentrations of Survivin by EIA kit [309].
* Determination of free circulating DNA [195].
* Determination of total cytochrome c levels by western blotting technique [310].

3- Statistical analyses:

Statistical analyses were performed using the SPSS-11.5, Wilcoxon test was used to compare the median of the different parameters for all studied groups. A significant difference was considered at ($p<0.05$).

(1) Determination of serum p53 protein by ELISA [311]

Principle

An anti-human p53 coating antibody is adsorbed onto microwells, Fig (1a).

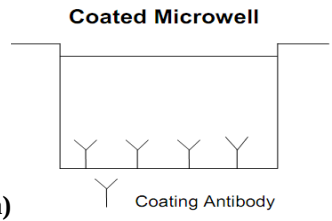

Fig (1a)

Human p53 present in the sample or in standard solution binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human p53 antibody is added and binds to human p53 which is captured by the first antibody, Fig (1b).

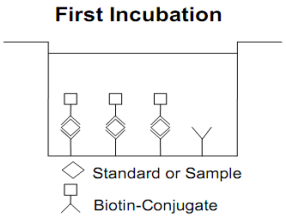

Fig (1b)

Following incubation, unbound biotin-conjugated anti-human p53 antibody is removed during a washing step. Streptavidin-Horseradish peroxidase (HRP) is added and binds to the biotin-conjugated anti-human p53 antibody, Fig (1c).

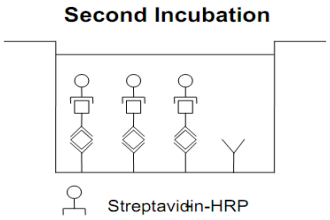

Fig (1c)

Following incubation unbound Streptavidin-HRP is removed during a washing step, and substrate solution which is reactive with HRP is added to the wells, Fig (1d).

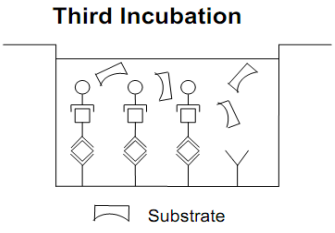

Fig (1d)

A coloured product is formed in proportion to the amount of human p53 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm, Fig (1e).

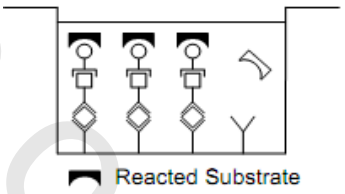

Fig (1e)

A standard curve is prepared from 7 human p53 standard dilutions and human p53 sample concentrations are determined.

Reagents:

1) Microwell plate coated with monoclonal antibody to human p53.
2) Biotin-Conjugate anti-human p53 monoclonal antibody.
3) Streptavidin-HRP.
4) Human p53 standard lyophilized: 100 U/ml upon reconstitution .
5) Sample diluents.
6) Assay buffer concentrate 20x: Phosphate Buffered Saline (PBS) with 1% Tween 20 and 10% Bovine Serum Alubmin.
7) Washing buffer concentrate 20x: PBS with 1% Tween 20.
8) Substrate solution (3,3',5,5'tetramethyl-benzidine): TMB substrate solution.
9) Stopping solution: 1M Phosphoric acid.

Preparation of Reagents

All serum samples and reagents were brought to room temperature and diluted according to manufacturer's instructions before starting the test procedure.

- Dilute washing buffer concentrate 20x to 1x with distilled water.
- Dilute assay buffer concentrate 20x to 1x with distilled water.
- Dilute biotin-conjugate with 1x assay buffer (dilution at 1:100).
- Dilute Streptavidin-HRP with 1x assay buffer (dilution at 1:100).
- Reconstitute p53 standard by addition of 1ml distilled water.

Procedure

(1) Wash the microwell strips twice with 400 µl washing buffer per well with thorough aspiration of microwell contents between washes. Allow the washing buffer to sit in the wells for about 10–15 seconds before aspiration. Then place the microwell strips upside down on a wet absorbent paper for not longer than 15 minutes.

(2) External Standard Dilution:

A) Reconstitute human p53 standard by addition of 1ml distilled water. Then allow it to sit for 10-30 minutes and mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 100 U/ml).

B) Label 7 tubes S1, S2, S3, S4, S5, S6, S7.

C) Prepare 1:2 serial dilutions for the standard curve as follows:

- Pipette 225 µl of sample diluent into each tube (S1 to S7).
- Pipette 225 µl of reconstituted standard (concentration = 100 U/ml) into the first tube (S1) and mix well (the concentration of standard 1 = 50 U/ml)
- Pipette 225 µl of this dilution into the second tube (S2) and mix thoroughly before the next transfer.
- Repeat this serial dilutions to S7 thus creating the points of the standard curve (S1=50, S2=25, S3=12.5, S4=6.25, S5=3.13, S6=1.56, S7=0.78), Fig (2).

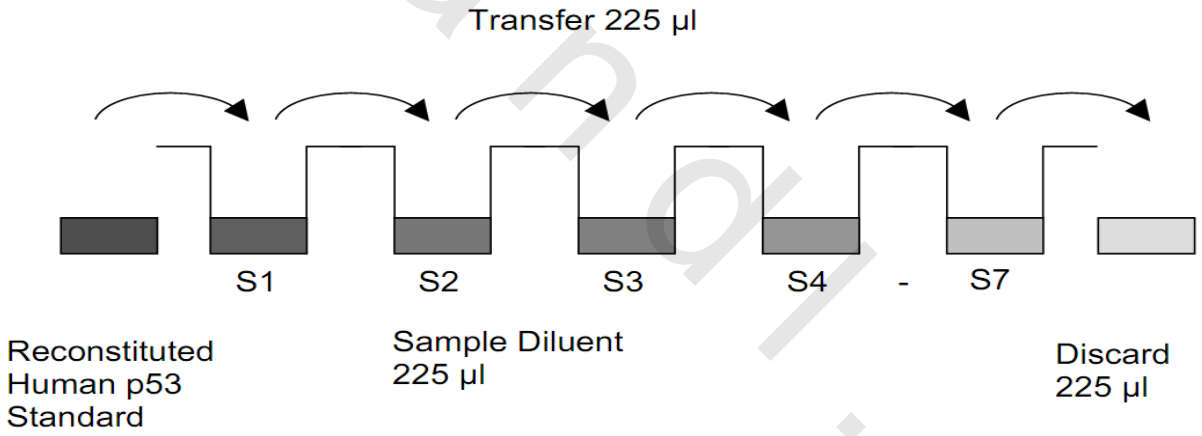

Fig (2): Serial Dilutions of p53 standard curve.

(3) Pipette 100 µl of the prepared standard dilutions (S1 - S7) in the standard wells.

(4) Pipette 100 µl of sample diluent into the blank well.

(5) Pipette 50 µl of sample diluent to the sample wells.

(6) Pipette 50 µl of each serum sample into the sample wells.

(7) Add 50 µl of biotin-conjugate to all wells.

(8) Cover the plate with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours.

(9) After incubation period remove the adhesive film and aspirate the wells content.

(10) Wash the microwell strips 3 times according to step 1 of the test procedure.

(11) Add 100 µl of diluted streptavidin-HRP to all wells, including the blank well.

(12) Cover the plate with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour.

(13) After incubation period remove the adhesive film and aspirate wells content.

(14) Wash microwell strips 3 times according to step 1 of the test procedure.

(15) Pipette 100 µl of TMB substrate solution to all wells.

(16) Incubate the plate at room temperature (18° to 25°C) for about 10 min.

(17) Stop the enzyme reaction by quick pipetting 100 µl of stopping solution into each well.

(18) Measure the absorbance of each microwell on a spectrophotometer using 450 nm.

Calculation of Results

1) Draw a standard curve of human p53 by plotting the absorbance for each standard against the concentration, Fig (3).

2) Determine the concentration of circulating human p53 for each sample from this standard curve, it should be noted that dilution factor was considered in calculating the concentration of p53 in serum samples.

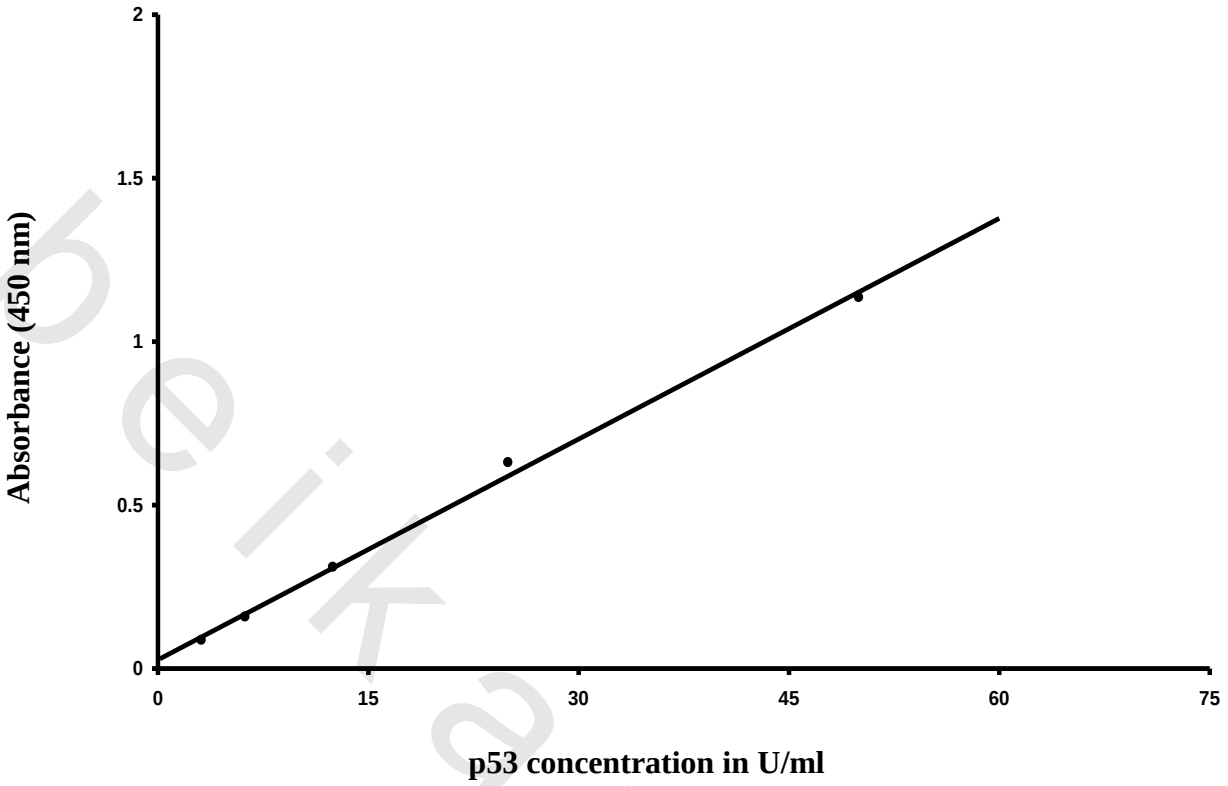

Fig (3): Standard curve of p53.

(2) Determination of serum survivin level with human Total Survivin Enzyme Immunometric Assay (EIA) kit [312]

Principle

A monoclonal antibody to survivin is immobilized on a microtiter plate binds to the survivin in the standards or samples. After a short incubation the excess sample or standard is washed out and a rabbit polyclonal antibody to survivin is added. This antibody binds to the survivin captured on the plate. After a short incubation the excess antibody is washed out and goat anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the polyclonal survivin antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of survivin in either standards or samples.

Reagents:

1. Microwell plate coated with mouse monoclonal antibody to human survivin.

2. Total survivin antibody.

3. Human survivin standard lyophilized: 1000 pg/ml upon reconstitution.

4. Total survivin conjugate.

5. Assay Buffer.

6. Washing buffer concentrate.

7. TMB substrate solution (TBM & hydrogen peroxide).

8. Stopping solution: 1N Hydrochloric acid.

Preparation of Reagents

All serum samples and reagents were brought to room temperature and diluted according to manufacturer's instructions before starting the test procedure.

- Dilute washing buffer concentrate: add 50 ml of the buffer to 950 ml distilled water.
- Reconstitute survivin standard.
- **Survivin Standards:**
  - Allow the 500 pg/vial human survivin standard to warm for no more than 10 minutes.
  - Pipette 500 µL of standard diluents (assay buffer) into survivin standard vial (The concentration of reconstituted survivin standard is 1000 pg/ml).
  - Label six tubes #1 through #6.
  - Pipette 250 µL of standard diluent into tubes #1 through #6.
  - Pipette 250 µL of the 1000 pg/mL standard survivin to tube #1 and vortex well.
  - Add 250 µL of tube #1 to tube #2 and vortex thoroughly, repeat these dilutions for tubes #3 through #6.
  - The concentration of survivin in the reconstituted human survivin standard vial and in tubes #1 through #6 is 1000, 500, 250, 125, 62.5, and 31.25 pg/mL, respectively.

Assay Procedure

(1) Bring all reagents (Except the survivin standard) to room temperature for at least 30 minutes prior to opening.

(2) Pipette 100 µl of standard diluent into the S0 (0 pg/mL standard) well.

(3) Pipette 100 µl of standards #1 through #6 into the appropriate wells.

(4) Pipette 100 µl of each serum samples into the appropriate wells.

(5) Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.

(6) Aspirate the contents of the wells and wash wells by adding 400 µL of washing solution to every well.

(7) Repeat washing for 4 more times.

(8) After the final washing, aspirate the wells well to remove any remaining washing buffer.

(9) Add 100 µl of survivin antibody into each well, except the blank.

(10) Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.

(11) Aspirate the contents of the wells and wash by adding 400 µl of washing solution to every well.

(12) Repeat washing for 4 more times.

(13) After the final washing, aspirate the wells well to remove any remaining washing buffer.

(14) Add 100 µl of survivin conjugate to each well, except the blank.

(15) Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.

(16) Aspirate the contents of the wells and wash by adding 400 µl of washing solution to every well.

(17) Repeat washing for 4 more times.

(18) After the final washing, aspirate the wells well to remove any remaining washing buffer.

(19) Add 100 µl of substrate solution into each well.

(20) Incubate the plate for 30 minutes at room temperature on a plate shaker at ~500 rpm.

(21) Add 100 µl of stopping solution 2 to each well. This stops the reaction and the plate should be read immediately at 450 nm.

Calculation of Results

1) Draw a standard curve of human survivin by plotting the optical density O.D for each standard against the concentration, Fig (4).

2) Determine the concentration of circulating human survivin for each sample from this standard curve.

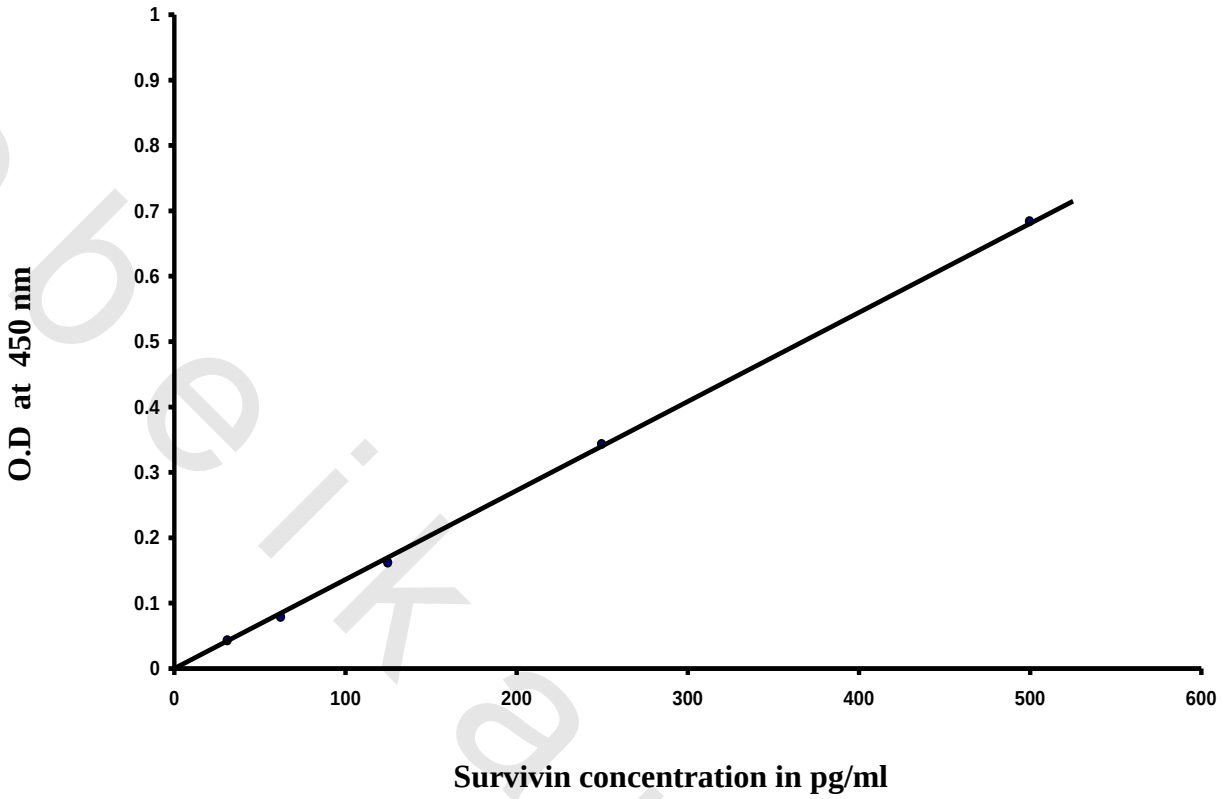

Fig (4): Standard curve of survivin.

(3) Determination of circulating DNA in serum by polymerase chain reaction (PCR)[195]

A) DNA extraction from serum:

Principle

Purification on QIAamp Mini spin columns:

The QIAamp DNA purification procedure comprises 3 steps and is carried out using QIAamp Mini spin columns in a standard microcentrifuge.

1) Adsorption to the QIAamp membrane:

DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation step.

2) Removal of residual contaminants:

DNA bound to the QIAamp membrane is washed in 2 centrifugation step. Using 2 different washing buffers AW1 and AW2, improves significantly the purity of the eluted DNA. The washing conditions ensure complete removal of any residual contaminants without affecting DNA binding.

3) Elution of pure nucleic acids:

Purified DNA is eluted from the QIAamp Mini spin column in a concentrated form in the AE buffer.

Procedure:

1. Pipette 20 µl of QIAGEN Protease into a 1.5 ml microcentrifuge tube.
2. Add 200 µl of each serum sample to the microcentrifuge tube.
3. Add 200 µl of AL buffer to each sample tube, then mix by pulse vortexing for 15 sec, then incubate at 56°C for 10 min.
4. Centrifuge all tubes to remove drops from the inside of the lid.
5. Add 200 µl of ethanol (96–100%) to all tubes, and mix by pulse vortexing for 15 sec, After mixing, centrifuge the tubes to remove drops from the inside of the lid.
6. Apply the previous mixture from step 5 to the QIAamp Mini spin columns (in a 2 ml collection tube) without wetting the rim, and then centrifuge at 8000 rpm for 1 min.
7. Add 500 µl of AW1 buffer to the columns, and then centrifuge at 8000 rpm for 1 min.
8. Add 500 µl of AW2 buffer to the columns without wetting the rim, and then centrifuge at 14,000 rpm for 3 min.
9. Place all columns in a new 2 ml collection tubes and discard the old collection tubes containing the filtrate, and then centrifuge at full speed for 1 min.
10. Add 200 µl of AE buffer to each column, then incubate the columns at room temperature (15–25°C) for 5 min, and then centrifuge at 8000 rpm for 1 min.

(B) **Polymerase Chain Reaction (PCR):**

PCR is a technique that allows exponentional amplification of a particular DNA sequence within a longer double stranded DNA molecule to produce a quantity sufficient to be investigated using conventional laboratory methods. PCR entails the use of:

- A pair of primers, each of which is short DNA fragment with a defined sequence complementary to a target DNA sequence to be amplified.
- Thermo-stable DNA polymerase that extends the primers by linking individual nucleotides together to form long DNA strands.

Principle:

The PCR method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction. The main three steps of the cycles are as follow:

1. **Denaturation step:** Heating the reaction to 94 to 98°C for 20-30 seconds. This causes separation of the two complementary strands of the DNA molecule.

2. **Annealing step:** The reaction temperature is lowered to 50–65 °C allowing annealing of the primers to the single-stranded DNA template. The polymerases then begin to attach additional complementary nucleotides at these sites, thus strengthing the bonding between the primers and the DNA.

3. **Extension step:** The temperature is again increased to 72°C. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding deoxy nucleotide triphosphate bases (dNTPs) that are complementary to the template in 5' to 3' direction.

Each time these three steps are repeated, the number of copied DNA molecules is doubled, Fig (4,5). After several rounds of amplifications, the PCR product is analyzed on agarose gel and is abundant enough to be detected with an ethidium bromide stain.

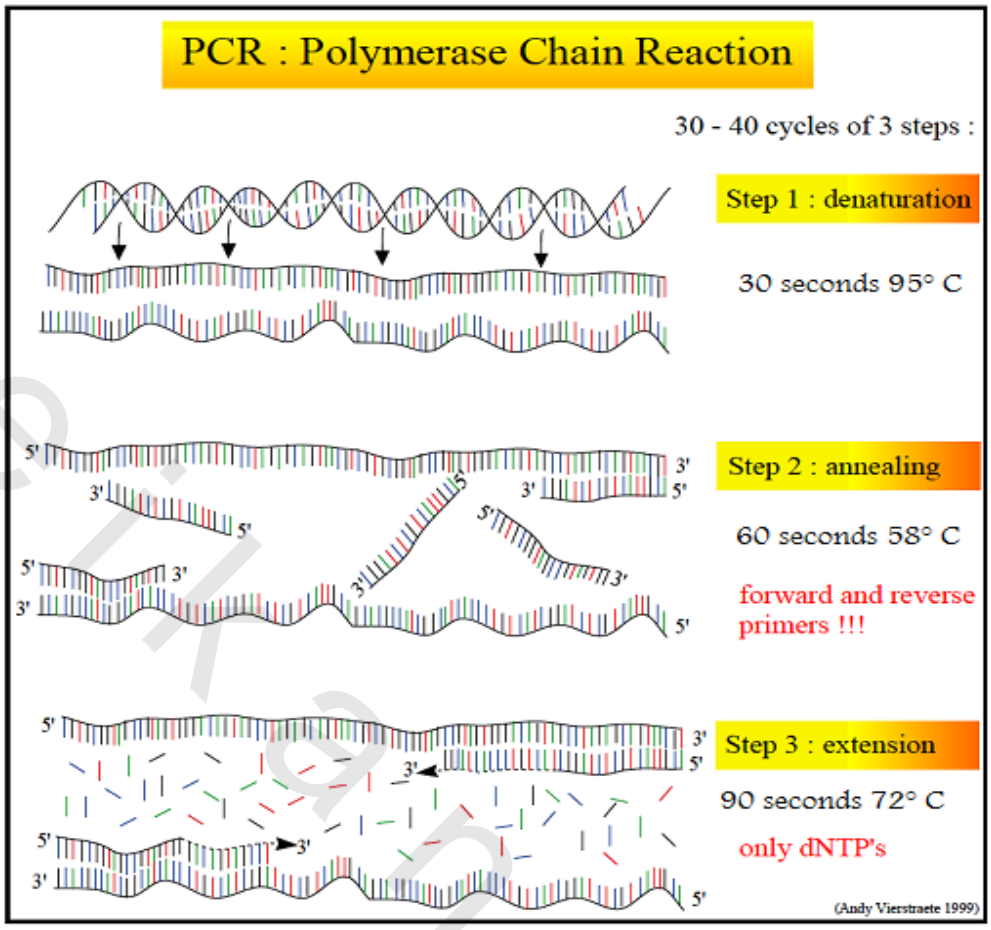

Fig (5): Schematic diagram of the main three steps in PCR.

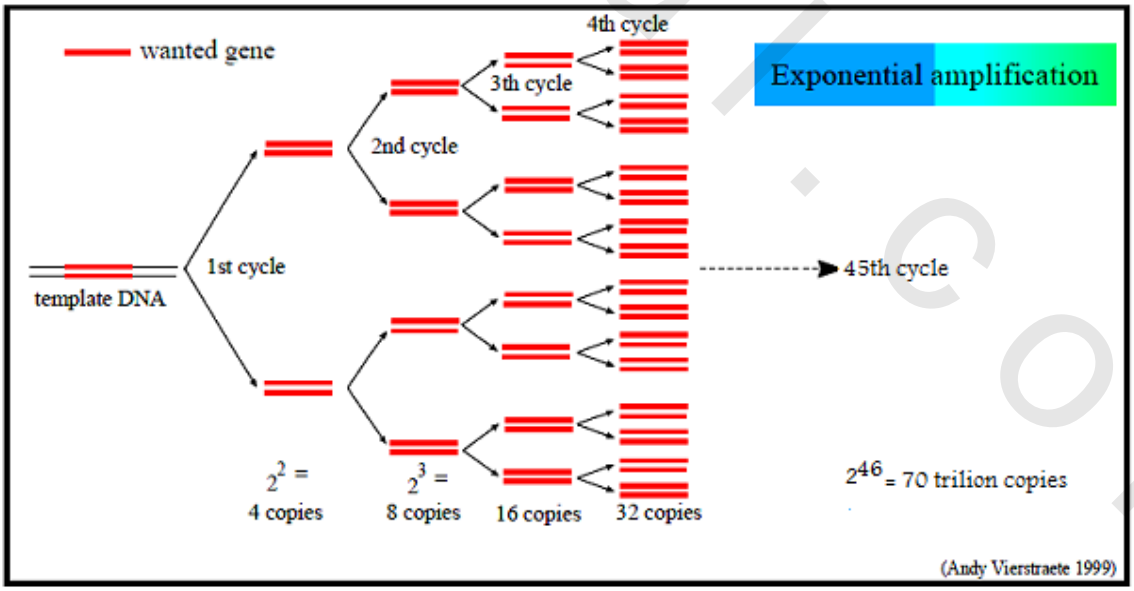

Fig (6): Schematic diagram represents the exponentional amplification of a particular DNA sequence.

Procedure:

Efficient DNA PCR was achieved by using Dream Taq® Green PCR Master Mix (Fermentas). 7 µl DNA were added to 10mM of each primer in a total volume 20 µl.

1. ***DNA integrity*** was detected via one forward primer and two reverse primers to amplify two different fragment lengths (100 and 400 bp) for β–actin gene as a target gene for amplification and the sequences were as follow [195], Fig (7):

Human β – actin	Sequence (5' – 3')
Forward	GCA CCA CAC CTT CTA CAA TGA
100 Reverse	GTC ATC TTC TCG CGG TTG GC
400 Reverse	TGT CAC GCA CGA TTT CCC

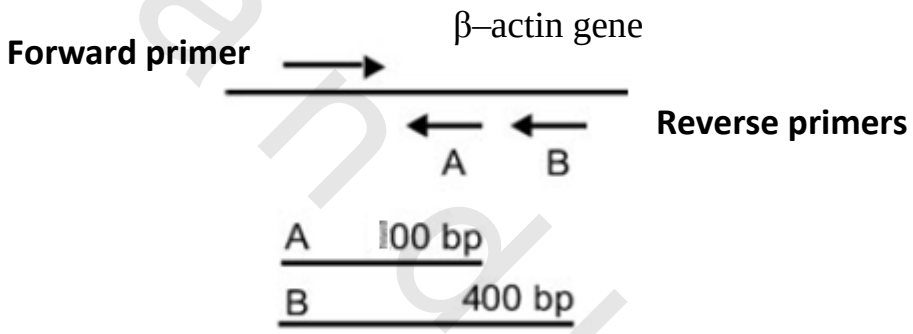

Fig (7): Schematic diagram illustrated DNA integrity analysis for PCR amplification of β–actin. One forward primer and two reverse primers (*A*, *B*) were used to amplify two different fragment lengths (100, and 400 bp).

The reaction was performed according to manufacture's instructions:

Preparing the Reaction Mix	1	Thaw reagents on ice.
	2	Gently vortex to mix the reagents.
	3	Pipette the PCR Master Mix, Primers, and deionized water into reaction tubes.
	4	Pipette DNA template into the reaction tubes.

For Human β – actin:

Reaction components	Components	Volume Reaction (µl)	Final Conc.
	Green Master Mix (2X)	**10**	1X
	Forward Pimer	**1.5**	0.4µM
	100 or 400 Reverse	**1.5**	0.4µM
	DNA template	**7**	Varied
	Total Volume	20	-

Thermal cycling optimization:

The reaction tubes were loaded on a thermal cycler and the thermal cycles were adjusted as follow:

1. Preheat at 95 °C for 4 minutes.
2. 45 cycles of the following three steps:
    - Denaturation at 95 °C for 30 seconds.
    - Annealing at 52 °C for 1 minute.
    - Extention at 72 °C for 1 minute and 30 seconds.
3. Final extention at 72 °C for 10 minutes.

Gel Electrophoresis

This technique is simple, rapid to perform, and the location of DNA within the gel can be determined directly by staining with low concentration of fluorescent interchelating dyes, such as ethidium bromide. Bands containing as little as 20 pg of double-stranded DNA can be detected by direct examination of the gel in UV.

Reagents:

1) Electrophoresis buffer (10 X): Tris-borate and EDTA (TBE).

2) Agarose solution: 2%.

3) DNA ladder: 100 bp.

Preparation of reagents:

All reagents prepared and diluted according to manfacture's instructions.

Procedure:

1) Seal the open ends of the plastic tray supplied with the electrophoresis apparatus, with tape to form a mold. Set the mold on a horizontal section of the bench.

2) Prepare sufficient electrophoresis buffer (1X TBE) to fill the electrophoresis tank and to cast the gel. It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel.

3) Prepare a solution of 2% agarose in TBE buffer. Add powdered agarose to a measured quantity of electrophoresis buffer in an Erlenmeyer flask.

4) Heat the slurry in microwave oven until the agarose dissolves. Use insulated gloves to transfere the flask, when the molten gel has cooled, add ethidium bromide to a final concentartion of 0.5µg/ml. Mix the gel solution throughly by gentle swiring.

5) While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.

6) Pour the warm agarose solution into the mold.

7) Allow the gel to set completely (30-45 minutes at room temprature), then pour a small amount of Tris-Boric EDTA (TBE) buffer on the top of the gel, and carefully remove the tape. Mount the gel in the electrophoresis tank.

8) Add enough TBE buffer to cover the gel to a depth of approx. 1mm.

9) Slowly load the 10µl of the DNA ladder in the first slot, and 10 µl of each DNA sample into the slots of the submerged gel.

10) Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V/cm.

11) Run the gel until the green dye migrates an appropriate distance through the gel.

12) When the DNA samples or dyes migrate sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank.

13) Examine the gel by UV light and photograph the gel.

14) Intenisty of the bands on the photographed gel was converted to arbitrary units (AU) by using scion image program.

(4) Determination of serum cytochrome C by western blotting technique [313]

Principle:

Western blotting (WB) is a method for identifying a specific protein in a complex mixture. In western blotting, proteins are electrophoresed into a gel, as the proteins migrate through the gel they are separated based upon size and charge. Characteristically, smaller proteins migrate through the gel faster than larger proteins. Sufficiently separated proteins by Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), can be transferred to a solid membrane (polyvinlidene fluoride PVDF or Nitrocellulose) for WB analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel onto the membrane. To detect the antigen blotted on the membrane, a primary antibody (serum) is added at an appropriate dilution and incubated with the membrane. Antibodies present in the serum bind to the protein(s). In order to detect the bound antibodies, anti-immunoglobulin antibodies coupled to an enzyme alkaline phosphatase or horseradish peroxidase are added. This anti-IgG enzyme is commonly called a "second antibody" or "conjugate". Finally, after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody is bound to the protein.

The procedure can be broken down into a series of steps:

1. **SDS-DPAGE**: by which proteins are separated according to their size. SDS binds strongly to most proteins, causes them to unfold to a random, rod-like chain, and makes them net negative in charge. No covalent bonds are broken in this process. Therefore, the amino acid composition and sequence remains the same. Proteins that loose their specific folding patterns and biological activity but their polypeptide chains remain intact, are called denatured. High concentrations of reducing agents, such as 2-mercaptoethanol, break disulfide bonds. This allows the SDS to completely dissociate and denature the protein. During electrophoresis, the SDS denatured proteins migrate through the gel towards the positive electrode at a rate that is inversely proportional to their molecular weight (the smaller the protein, the faster it migrates).

2. **Electroblotting**: which is the transfer of the separated proteins to a membrane while retaining their relative position, this can be done electrophoretically in a specially designed chamber.

Proteins are adsorbed to the membrane by hydrophobic bonds. Membranes are much stronger and more pliable than gels and can undergo many manipulations without tearing. A specific protein is detected by immunodetection method, Fig (8).

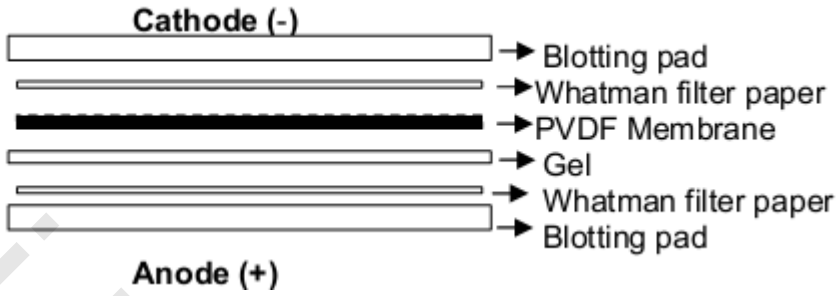

Fig (8): Arrangements of accessories for protein transfer for western blot.

3. **Immunodetection**, it is the detection of protein of interest by its specific reaction with an antibody and determination of its size relative to standard proteins of known size. For this method, the membrane is placed in blocking buffer, which contains blocking proteins that bind to all unoccupied sites on the membrane. The membrane is then probed with primary antibody specific to the protein of interest. The primary antibody binds to the adsorbed protein antigen. Then membrane is washed to remove excess, unbound antibody. This antigen-antibody complex is then detected by treating the membrane with secondary antibody linked with enzyme (such as alkaline phosphatase or horseradish peroxidase). Again, membrane is washed to remove excess secondary antibody. In the next step, the membrane is incubated in a solution containing, phosphatase or peroxidase substrates that yield chromogenic products. Areas containing antigen-antibody conjugates will develop color, depending on the type of substrate used and product formed.

Reagents:

1. Laemmli Sample buffer (62.5 mmol/l Tris HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromphenol blue, and 5% 2-mercaptoethanol).
2. Acyrlamide stock solution (30 % acrylamide & 8 % N,N'-methylene-bis-acrylamide)
3. Tris-HCl buffer (pH, 6.8)
4. Ammonium persulfate (10%)
5. Sodium dodecyl sulphate SDS, (10 %)

6. N,N,N' ,N '-tetramethylenediamine (TEMED)
7. Electrophoresis buffer (Glycine Tris-HCl, pH=6.8)
8. Transfer buffer (Tris-HCl, glycine, SDS, methanol, pH= 8.3)
9. Tris-buffered Saline-Tween buffer (TBST, pH=8)
10. Blocking buffer (3 % Bovine Serum Albumin (BSA), in TBST)
11. Nitrocellulose membranes.
12. Primary antibody (Monoclonal mouse anti-human cytochrome C Ab).
13. Secondary antibody (Anti-mouse IgG Biotin Ab).
14. Avidin-HRP.
15. TMB detection kit.

Procedure, Fig (9):

1. Determine the concentration of total proteins in all serum samples.
2. Add 50ul of each serum sample to 50 ul Laemmli sample buffer, heat the samples at 95^{o} C for 10 minutes.
3. For standard protein, prepare it such as serum samples in step (2).
4. Load 35ul of each prepared sample on 20% SDS-polyacyramide gel, run the gel at 110 volts for 120 minutes, apply equal volume of the standard and run it along with the samples on the gel.
5. Transfer the proteins separated on the gel to nitrocellulose membrane (Protran ®).
6. Block the membrane by incubating it in 3 % BSA in TBST buffer, overnight at 4^{o} C.
7. Wash the membrane for 5 min with enough TBST buffer. Then repeat the washing for 3 successive times (15 min each)
8. Incubate the membrane with primary Ab, at 1:1000 dilution (10 ul Ab is added to l0 ml blocking buffer), overnight at 4^{o} C.
9. Repeat the washing as in step 7.
10. Incubate the membrane with the secondary Ab, at 1:5000 dilution (2 ul Ab is added to 10 ml blocking buffer), overnight at 4^{o} C.
11. Repeat the washing as in step 7.
12. Incubate the membrane with Avidin-HRP, at 1:1000 dilution (10 ul avidin-HRP is added to l0 ml TBST buffer), for 2 hours at 4^{o} C.
13. Repeat the washing as in step 7.

14. Incubate the membrane with TMB substrate for 20-30 min at room temperature till the blue precipitate is formed on the membrane.
15. Wash the membrane with distilled water, then allow it to dry in air.
16. Photograph the bands on the membrane.

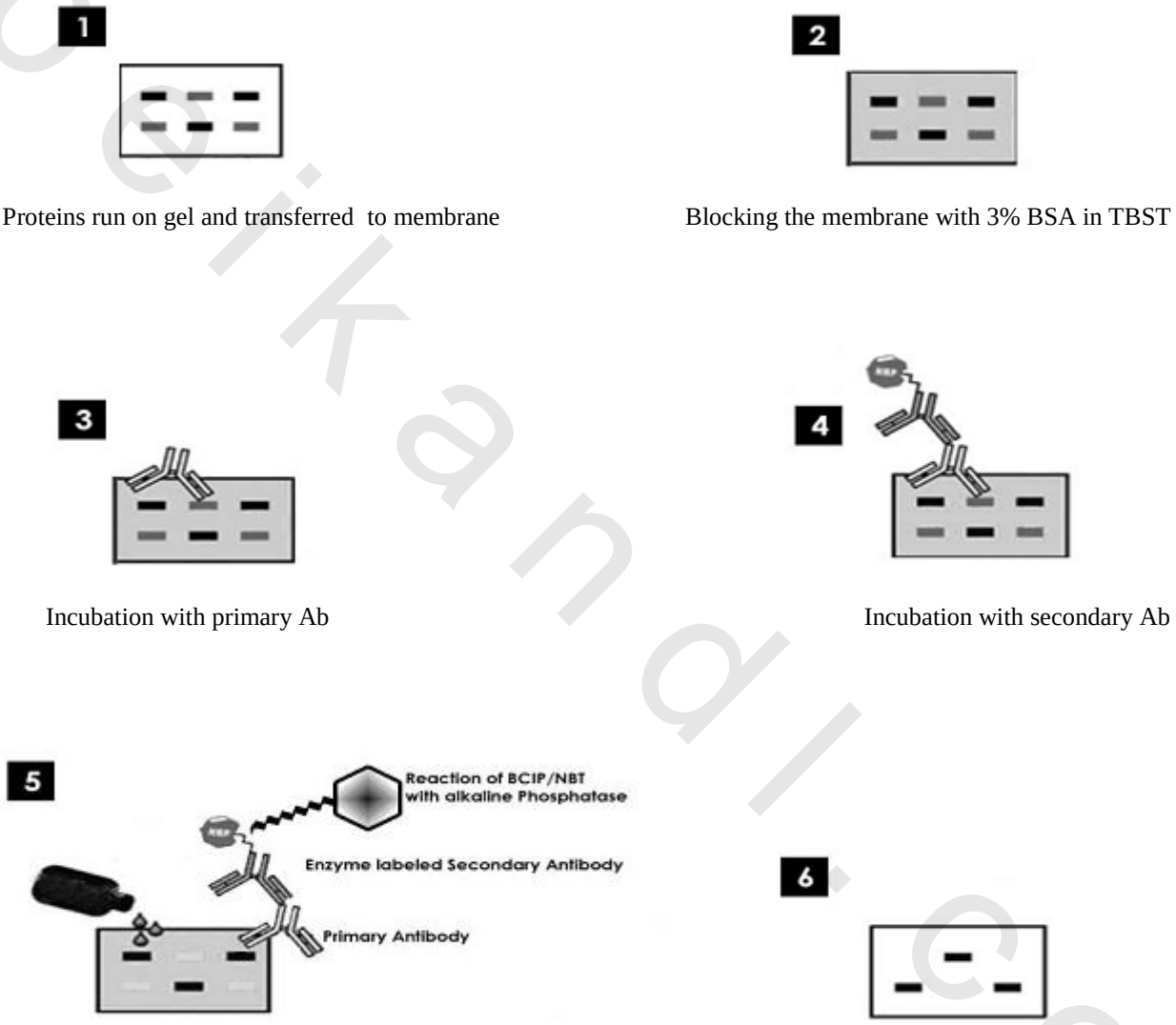

Fig (9): A schematic diagram of western blotting technique

Results

I. Clinicopathological parameters and characteristic of patients in control and vitamins-treated groups:

The general characteristics of all patients in control and vitamins-treated groups are represented in Tables (1 & 2), these tables showed that 11 patients (55%) had a negative family history and 9 patients (45%) had positive family history in control group, while 18 patients (72%) had negative family history and 7 patients (28%) patients had positive family history in vitamins-treated group. Concerning tumor size, 3 patinets (15%) had T1 tumor, 13 patients (65%) had T2 tumor, and 4 patients (20%) had T3 tumor in the control group, whereas in vitamins-treated group, 2 patients (8%) had T1 tumor, 16 patients (64%) had T2 tumor, and 7 patients (28%) had T3 tumor. Negative lymph nodes were observed in 5 patients (25%) in the control group, while 15 patients (75%) had positive lymph nodes, in vitamins-treated group, 6 patients (30%) had negative lymph nodes and 14 patients (70%) had positive lymph nodes. 13 patients (76%) in the control group were of histological grade II, and 4 patients (24%) were of grade III, whereas in vitamins-treated group 22 patients (88%) were of grade II, and 3 patients (12%) were of grade III. With respect to pathology type, all 20 patients (100%) in control group had invasive ductal carcinoma, while in the vitamins-treated group, 23 patients (92%) had invasive ductal carcinoma, and the remaining 2 patients (8%) had invasive lobular carcinoma. In the control group, 16 patients (80%) had positive ER, and one patient (5%) had negative ER, also 14 patients (70%) had positive PR, and 2 patients (10%) had negative PR, 8 patients (40%) had negative HER-2, and one patient (5%) had positive HER-2. In vitamins-treated group, 18 patients (72%) had positive ER and PR, and one patient (4%) had negative ER and PR, 8 patients (32%) had negative HER-2, while one patient (4%) had positive HER-2. Vascular invasion was presented in 12 patients (60%) and no vascular invasion was found in the rest of patients in the control group, while it was found in 10 patients (40%) in vitamins-treated group, and it was absent in one patient (4%). According to menopause status, 8 patients (40%) were premenopause and 12 patients (60 %) were posmenopause in the control group, in vitamins-treated group, 7 patients (28%) were premenopause and the remaining 18 patients (72 %) were postmenopause.

The mean age of all patients in this study was represented as mean±S.E.M, in the control group the age ranged from 34 to 64 years with a mean value of 49.21 ± 1.86. While in vitamins-treated group the age ranged from 36 to 77 years with a mean value of 52.72 ± 2.08

Table (1): Clinicopathological parameters and characterstics of patients in control group.

Clinicopathological parameters and characterstics		Patient number (N=20)	Percent (%)
Family history	Negative	11	55
	Positive	9	45
Tumor size	< 2 (T1)	3	15
	2-5 (T2)	13	65
	>5 (T3)	4	20
Lymph Node status (LN)	Negative	5	25
	Positive		
	• (1-3)	4 } 15	20 } 75
	• (4-9)	5 } 15	25 } 75
	• ≥ 10	6 } 15	30 } 75
Histological grade	II	13	76
	III	4	24
Pathology type	Invasive ductal carcinoma	20	100
	Invasive lobular carcinoma	0	0
ER satatus	Negative	1	5
	Positive	16	80
	Unknown	3	15
PR status	Negative	2	10
	Positive	14	70
	Unknown	4	20
HER-2 status	Negative	8	40
	Positive	1	5
	Unknown	11	55
Vascular invasion	Negative	0	0
	Positive	12	60
	Unknown	8	40
Menopausal status	Premenopause	8	40
	Postmenopause	12	60

Table (2): Clinicopathological parameters and characterstics of patients in vitamins-treated group.

Clinicopathological parameters and characterstics		Patient number (N=25)	Percent (%)
Family history	Negative	18	72
	Positive	7	28
Tumor size	< 2 (T1)	2	8
	2-5 (T2)	16	64
	>5 (T3)	7	28
Lymph Node status (LN)	Negative	6	30
	Positive		
	• (1-3)	9	45
	• (4-9)	1 } 14	5 } 70
	• ≥ 10	4	20
Histological grade	II	22	88
	III	3	12
Pathology type	Invasive ductal carcinoma	23	92
	Invasive lobular carcinoma	2	8
ER satatus	Negative	1	4
	Positive	18	72
	Unknown	6	24
PR status	Negative	1	4
	Positive	18	72
	Unknown	6	24
HER-2 status	Negative	8	32
	Positive	1	4
	Unknown	16	64
Vascular invasion	Negative	1	4
	Positive	10	40
	Unknown	14	56
Menopausal status	Premenopause	7	28
	Postmenopause	18	72

II. Results of the biochemical parameters:

In this study, concentrations of serum p53, survivin, and levels of cytochrome C and free circulating DNA were determined in the control Group (Group Ia & Ib) and vitamins-treated Group (Group IIa & IIb), before and after chemotherapy.

The results are represented as range, and mean values ± S.E.M. Statistical analyses were made to compare all parameters in all studied groups (Tables 3, 4,5, and 6) and Figures (1- 11).

Correlation studies between all biochemical parameters with each others and with all clinopathological characteristics of all patients in the two studied groups, were done using Spearman correlation, ($r > 0.3$ at $P < 0.05$ was considered significant), Tables (7 &8).

(1) Results of serum p53 concentrations:

The level of serum p53 concentration in the control group ranged from 0.38-4.03 U/ml with a mean value ± S.E.M of 2.25±0.24 U/ml before chemotherapy (group Ia), and from 1.45-10.34 U/ml with a mean value of 3.92±0.57 U/ml after chemotherapy (group Ib). In the vitamins-treated group, the level ranged from 0.55-5.42 U/ml with a mean value of 2.13±0.29 before chemotherapy (group IIa), and from 1.00-9.45 U/ml with a mean value of 3.3±0.41 U/ml after chemotherapy (group IIb), Table (3).

The statistical analyses of these results, revealed that:

(1) The level of serum p53 concentration showed a significant increase (P=0.01*) in Group Ib as compared to Group Ia, also when comparing Group IIb with Group IIa (P1=0.004*).

(2) The level of serum p53 concentration didn't show any significant difference when comparing Group IIb with Group Ib, Table (3) and Fig (1).

Table (3): Statistical analyses of serum p53 concentrations (U/ml) in the control and the vitamins-treated groups before and after chemotherapy.

Groups	Control Group (Group I)		Vitamins-treated Group (Group II)	
	Before Chemotherapy (Group Ia) (N=20)	After Chemotherapy (Group Ib) (N=20)	Before Chemotherapy (Group IIa) (N=25)	After Chemotherapy (Group IIb) (N=25)
Range	0.38-4.03	1.45-10.34	0.55-5.42	1.00-9.45
Mean±S.E.M	2.25±0.24	3.92±0.57	2.13±0.29	3.3±0.41
P	.01*			
P1			.004*	
P2		.3		

P: Values when comparing the control group before and after chemotherapy.

P1: Values when comparing the vitamins-treated group before and after chemotherapy.

P2: Values when comparing the vitamins-treated group with the control group after chemotherapy.

N: Total number of patients in each group.

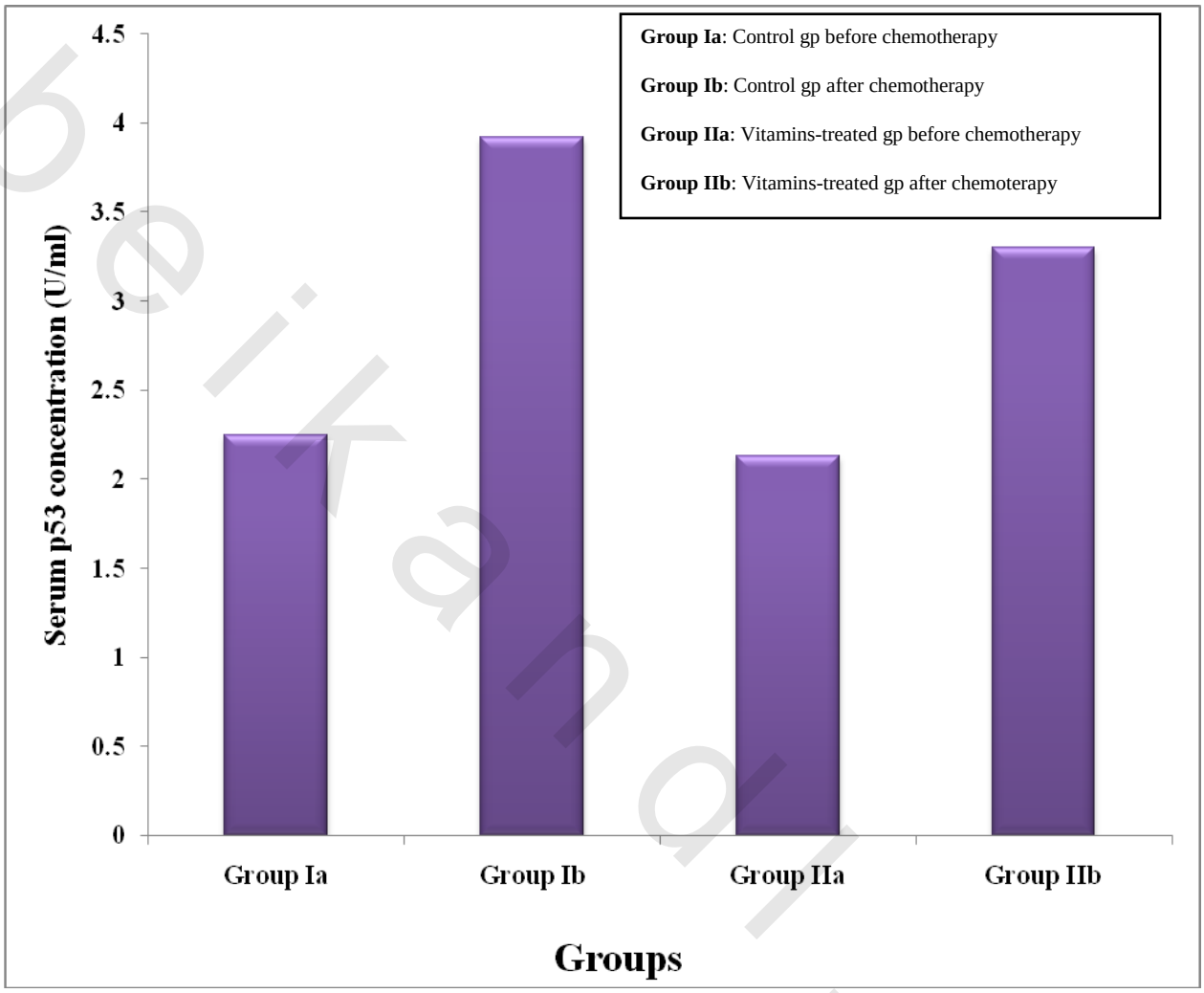

Fig (1): Serum p53 concentrations (U/ml) in all studied groups.

(2) Results of serum survivin concentartions:

The level of serum survivin concentration ranged from 14.29-60.71 pg/ml with a mean value ± S.E.M of 21.49±2.58 pg/ml in group Ia, and from 13.57-31.43 pg/ml with a mean value of 21.80±1.07 pg/ml in group Ib. The level ranged from 17.86-50 pg/ml with a mean value of 28.14±1.48 in group IIa, and from 15-27.86 pg/ml with a mean value of 18.93±0.70 pg/ml in group IIb, Table (4).

The statistical analyses of these results, revealed that:

(1) The level of serum survivin concentration didn't show any significant diffrence when comparing Group Ib with Group Ia.

(2) The level of serum survivin concentration showed a significant decrease when comparing Group IIb with both Group IIa (P1=0.001*) and Group Ib (P2=0.04*), Table (4) and Fig (2).

Table (4): Statistical analyses of serum survivin concentrations (pg/ml) in the control and the vitamins-treated groups before and after chemotherapy.

Groups	Control Group		Vitamins-treated Group	
	Before Chemotherapy Group (Ia) (N=20)	After Chemotherapy Group (Ib) (N=20)	Before Chemotherapy Group (IIa) (N=25)	After Chemotherapy Group (IIb) (N=25)
Range	14.29-60.71	13.57-31.43	17.86-50	15-27.86
Mean±S.E.M	21.49±2.58	21.80±1.07	28.14±1.48	18.93±0.70
P	.90			
P1			.001*	
P2		.04*		

P, P1, P2 & N are as indicated in Table (3).

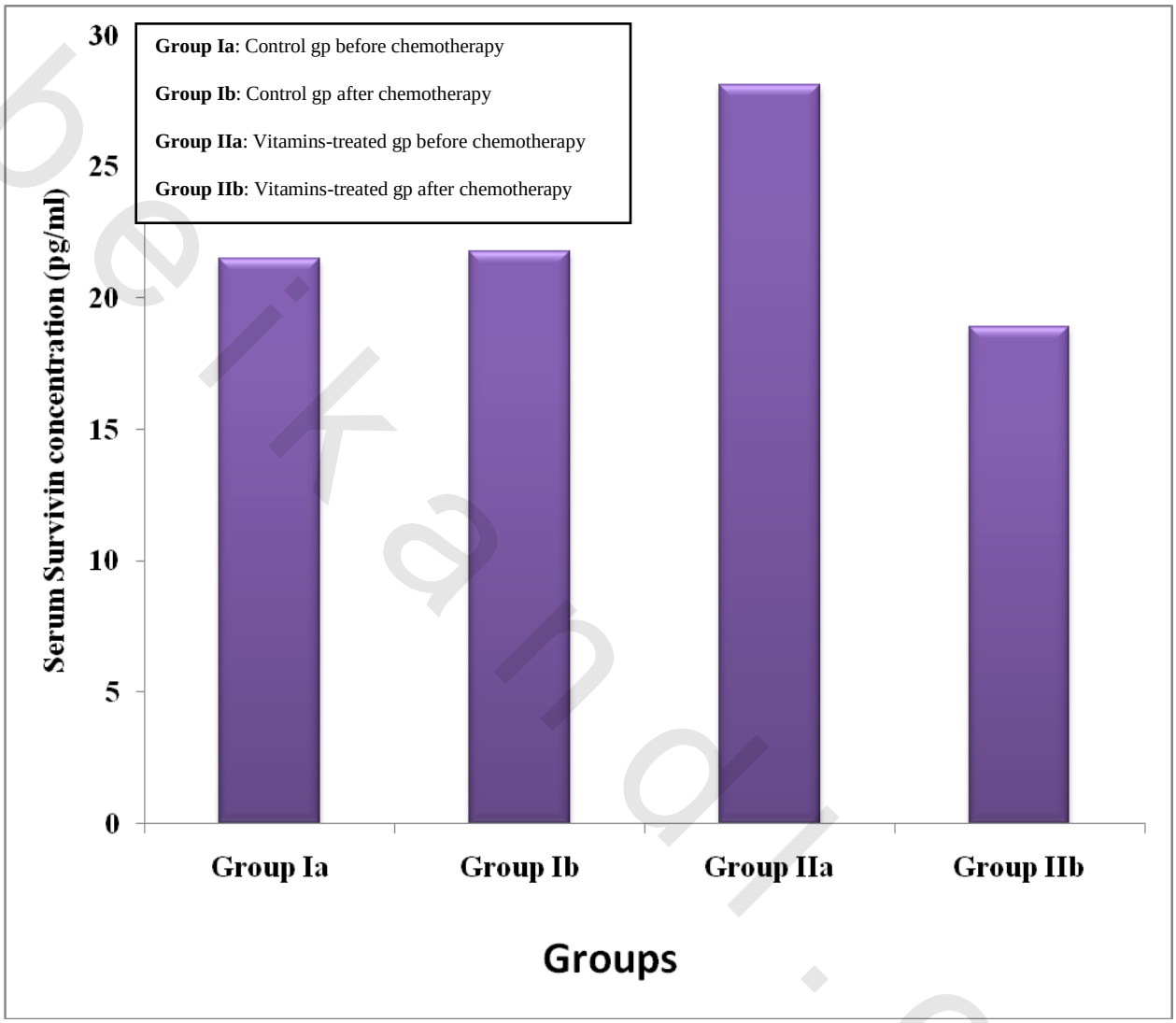

Fig (2): Serum Survivin Concentrations (pg/ml) in all studied groups.

(3) Results of Serum circulating DNA integrity and DNA integrity index.

The band intensity mass in AU at 100 and 400 bp and DNA integrity index (400/100) in both control and vitamins-treated groups before and after chemotherapy are as follows:

A) Band intensity of cfDNA at 100 bp:

The band intensity of cfDNA at 100 bp ranged from 1.37-17.09 with a mean ± S.E.M of 9.69± 1.19 in Group Ia, and from 3.14-13.68 with a mean value of 8.51 ± 0.99 in Group Ib.While, the band intensity in Group IIa ranged from 3.25-21.11 with a mean value of 13.25±1.52, and from 10.81-27.81 with a mean value of 17.59±1.09 in Group IIb, Table (5).

B) Band intensity of cfDNA at 400 bp:

The band intensity at 400 bp ranged from 3.68-12.81 with a mean ± S.E.M of 7.81± 0.78 in Group Ia, and from 1.41-14.26 with a mean value of 6.38 ± 0.91 in Group Ib. While, the band intensity in Group IIa ranged from 1.6-7.41 with a mean value of 4.56±0.39, and from 0.88-6.53 with a mean value of 3.04± 0.41 in Group IIb, Table (5).

C) cfDNA integrity index (400/100):

The DNA integrity index ranged from 0.43-1.04 with a mean value 0.76±0.06 in Group Ia, and from 0.11-1.26 with a mean value of 0.83±0.09 in Group Ib. While it ranged from 0.12-1.71 with a mean value of 0.39±0.08 in Group IIa and from 0.06-0.51 with a mean value of 0.22±0.03 in Group IIb, Table (5).

The statistical analyses of these results revealed that:

(1) The band intensity of cfDNA at 100 and 400 bp and DNA integrity index didn't show any significant difference when comparing Group Ib with Group Ia, Tables (5) and Fig (3 & 4 &5).

(2) The band intensity of cfDNA at 100 bp showed a significant increase when comparing Group IIb with both Group IIa (P1=0.015) and Group Ib (P2= 0.001), Table (5) and Fig (3)

(3) The band intensity at 400 bp showed a significant decrease when comparing Group IIb with both Group IIa (P1=0.03) and Group Ib (P2=0.04), Table (5) and Fig (4).

(4) DNA integrity index showed a significant decrease when comparing Group IIb with both Group IIa (P1= 0.03) and Group Ib (P2=0.02), Table (5) and Fig (5).

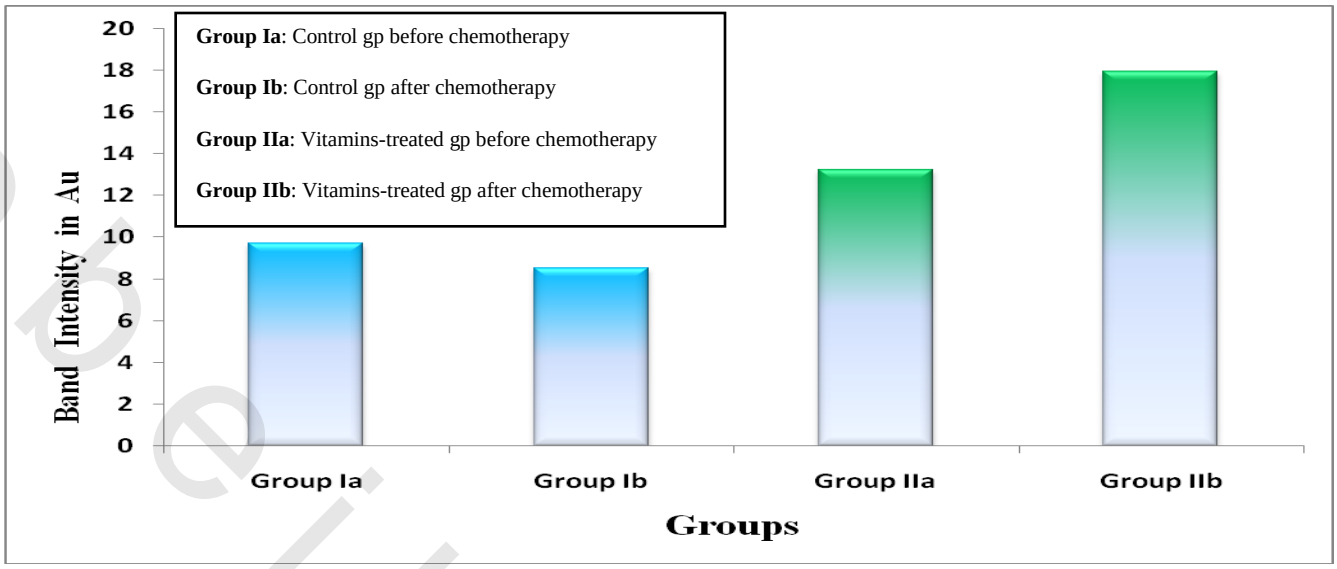

Fig (3): Band intensity of serum cfDNA at 100 bp in all studied groups.

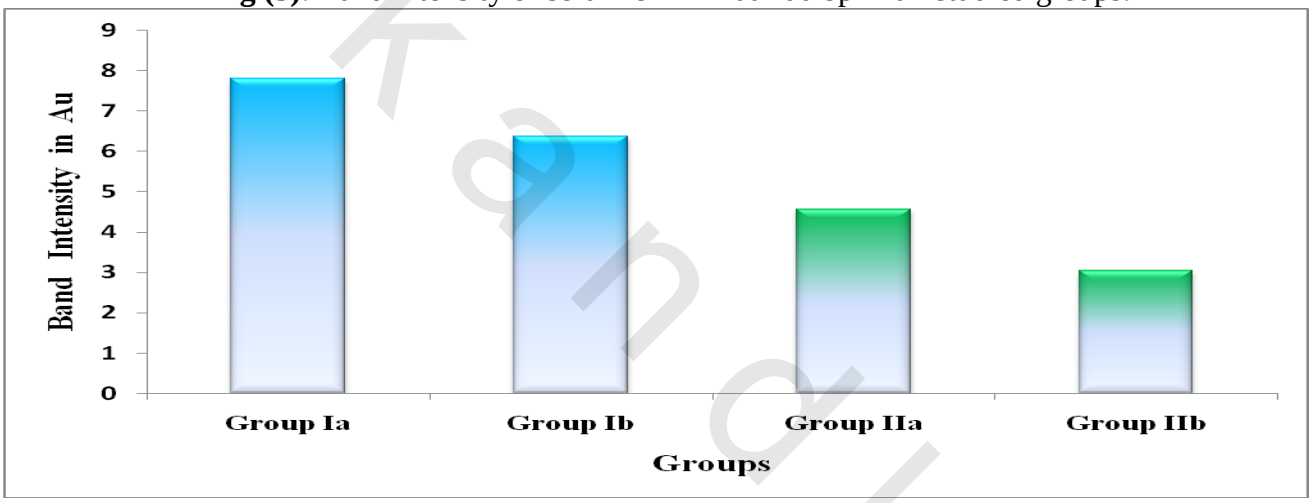

Fig (4): Band intensity of serum cfDNA at 400 bp in all studied groups.

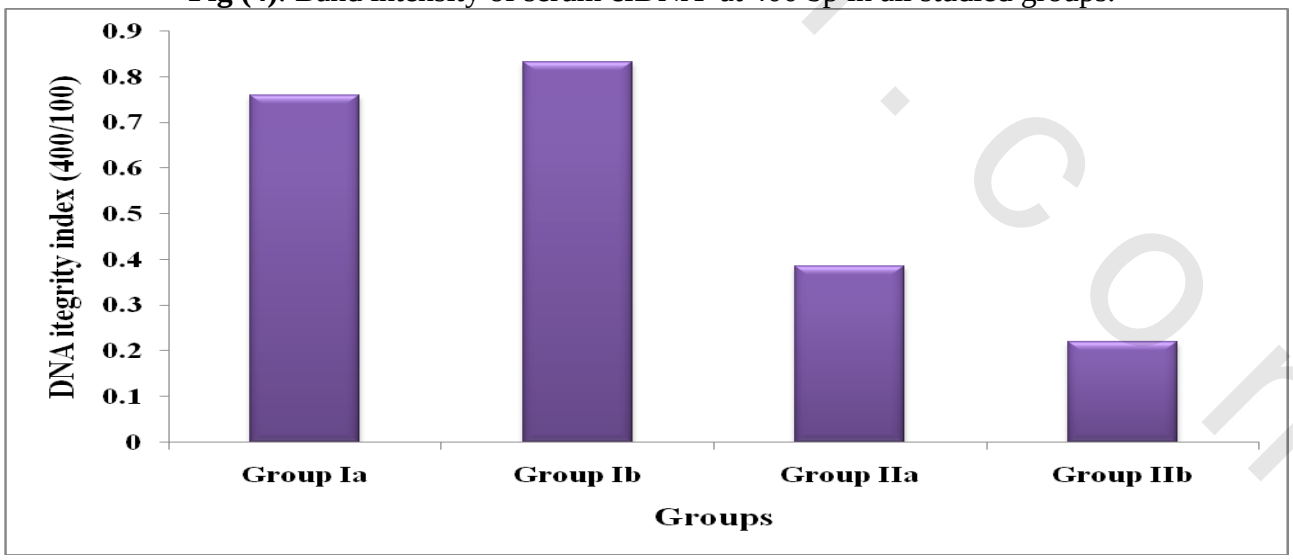

Fig (5): Serum DNA integrity index (400 bp/100 bp) in all studied groups.

Gel electrophoresis of serum DNA samples

The following photographs represent the PCR products of two amplification for each patient sample, one for β-actin at 100 bp and the other at 400 bp, which illustrate the followings:

- In Groups (Ib & IIb), It was noticed that the shorter fragments released from apoptotic cells are the main bands rather than the longer fragments of necrosis.
- It was found that the apoptotic bands in the vitamins-treated group was more intense and predominant rather than that in the control group.

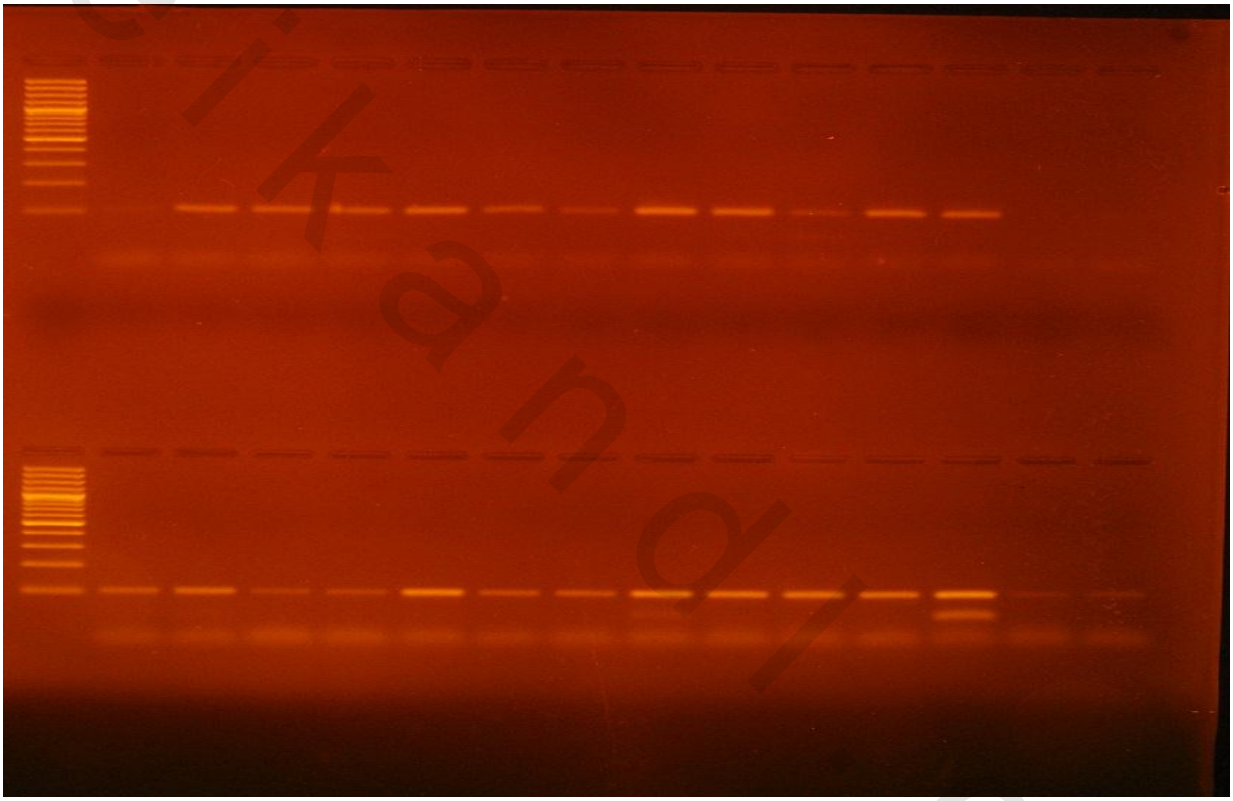

Fig (6): A photograph of the electrophoresis for PCR products for the control group at 100 bp. The upper lanes represent amplification product in patients before chemotherapy, while the lower lanes represent amplification product after chemotherapy.

Lane 1 is DNA ladder while lanes (2 - 15) are amplifications for patients in the control group (before and after chemotherapy) showing bands at 100 bp.

Most of patients after chemotherapy, showed the apoptotic bands at 100 bp with increased intensities as compared with the corresponding bands before chemotherapy, in which some patients didn't show this band.

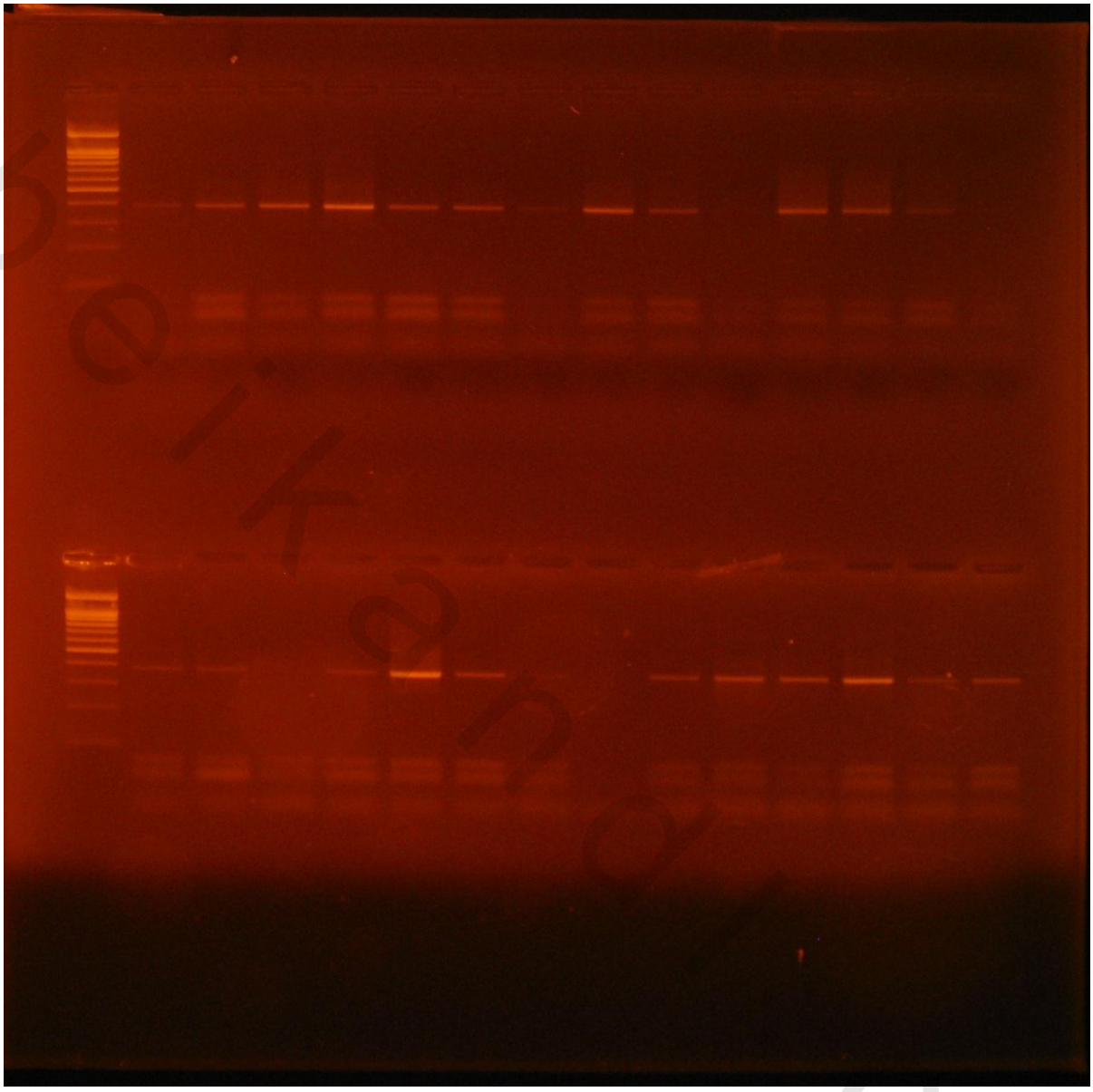

Fig (7): A photograph of the electrophoresis for PCR products for the control group at 400 bp. The upper lanes represent amplification product in patients before chemotherapy, while the lower lanes represent amplification product after chemotherapy.

Lane 1 is DNA ladder while lanes (2 - 15) are amplifications for patients in the control group (before and after chemotherapy) showing bands at 400 bp.

Most of patients before chemotherapy, showed the non-apoptotic (necrotic) bands at 400 bp with increased intensities as compared with the corresponding bands after chemotherapy, in which some patients didn't show this band.

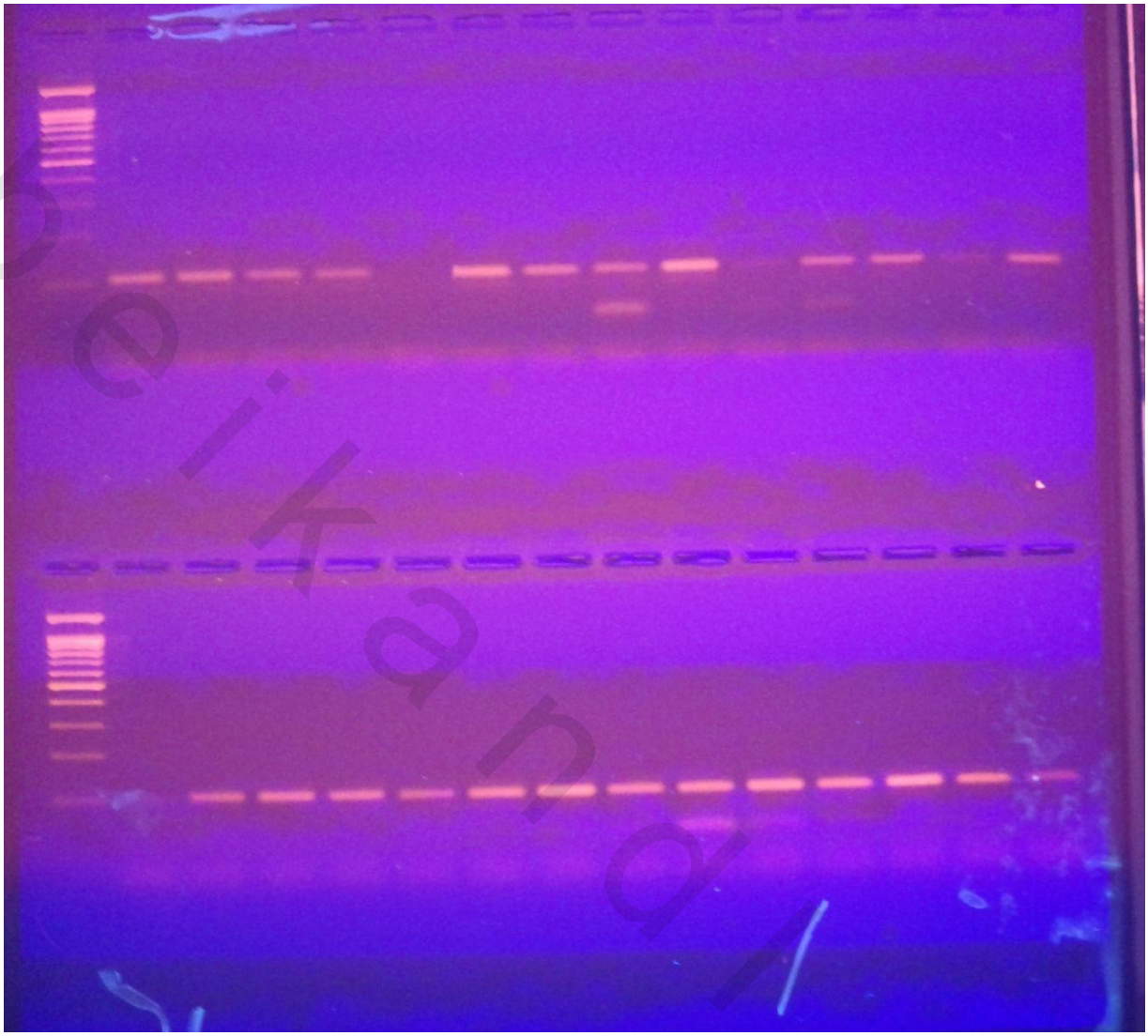

Fig (8): A photograph of the electrophoresis for PCR products for the vitamins-treated group at 100 bp. The upper lanes represent amplification product in patients before chemotherapy, while the lower lanes represent amplification product after chemotherapy.

Lane 1 is DNA ladder while lanes (2 - 15) are amplifications for patients in vitamins-treated groups (before and after chemotherapy) showing bands at 100 bp.

Most of patients after chemotherapy, showed the apoptotic bands at 100 bp with increased intensities as compared with the corresponding bands before chemotherapy, in which some patients didn't show this band. Also, this figure illustrates that band intensity at 100 bp was increased in this group as compared with those of control group presented in Fig (6).

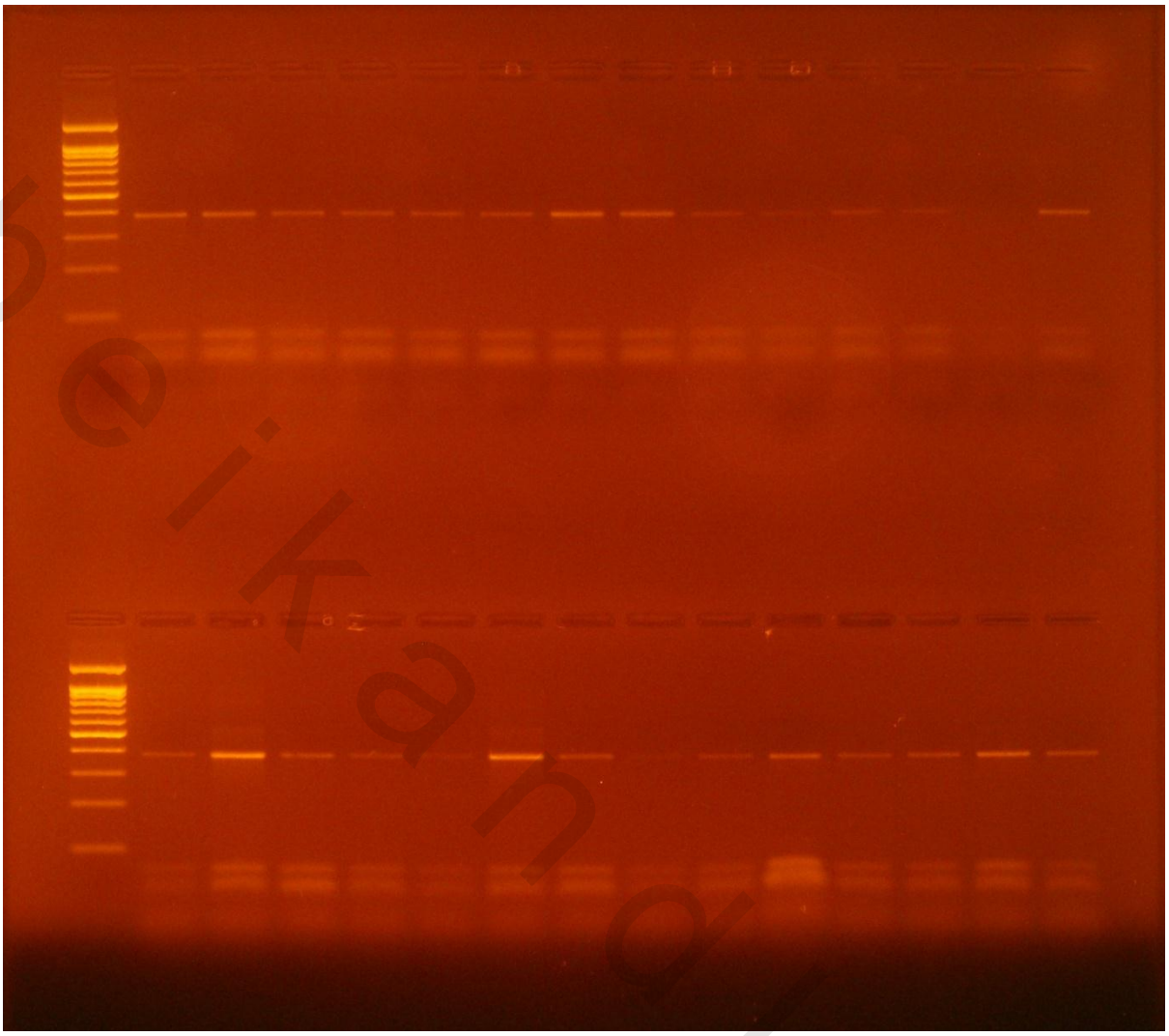

Fig (9): A photograph of the electrophoresis for PCR products for the vitamins-treated group at 400 bp.The upper lanes represent amplification product in patients before chemotherapy, while the lower lanes represent amplification product after chemotherapy

Lane 1 is DNA ladder while lanes (2-15) are amplifications for patients in vitamins-treated groups (before and after chemotherapy) showing bands at 400 bp.

Most of patients before chemotherapy, showed the necrotic bands at 400 bp with increased intensities as compared with the corresponding bands after chemotherapy, in which some patients didn't show this band.

(4) Results of serum Cytochrome C:

The relative intensity of bands obtained on the membrane were analysed using image gel software, this intensity can give an approximate indication of serum cytochrome c.

Serum levels of cytochrome c ranged from 4.56-11.28 with a mean value of 7.54±0.56 in group Ia, and ranged from 1.68-11.51 with a mean value of 5.73±0.78 in group Ib. While in Group IIa ranged from 3.78-11.73 with a mean value of 7.77±0.48, and ranged from 4.50-12.65 with a mean value 8.25±0.53 in Group IIb, Table (6).

The statistical analyses of these results revealed that:

(1) The levels of serum cytochrome C didn't show any significant difference when comparing Group Ib with Group Ia, and when comparing Group IIb with Group IIa.

(2) The levels of cytochrome C showed a significant increase when comparing Group IIb with Group Ib (P2=0.009), Table (6) and Fig (11).

Table (6): Serum Cytochrome c levels in control and vitamins-treated groups before and after chemotherapy.

Groups	Control Group		Vitamins-treated Group	
	Before Chemotherapy Group (Ia) (N=20)	After Chemotherapy Group (Ib) (N=20)	Before Chemotherapy Group (IIa) (N=25)	After Chemotherapy Group (IIb) (N=25)
Range	4.56-11.28	1.68-11.51	3.78-11.73	4.50-12.65
Mean±S.E.M	7.54± 0.56	5.73 ± 0.78	7.77±0.48	8.25±0.53
P	.1			
P1			.19	
P2		.009*		

P, P1, P2 & N are as indicated in Table (3).

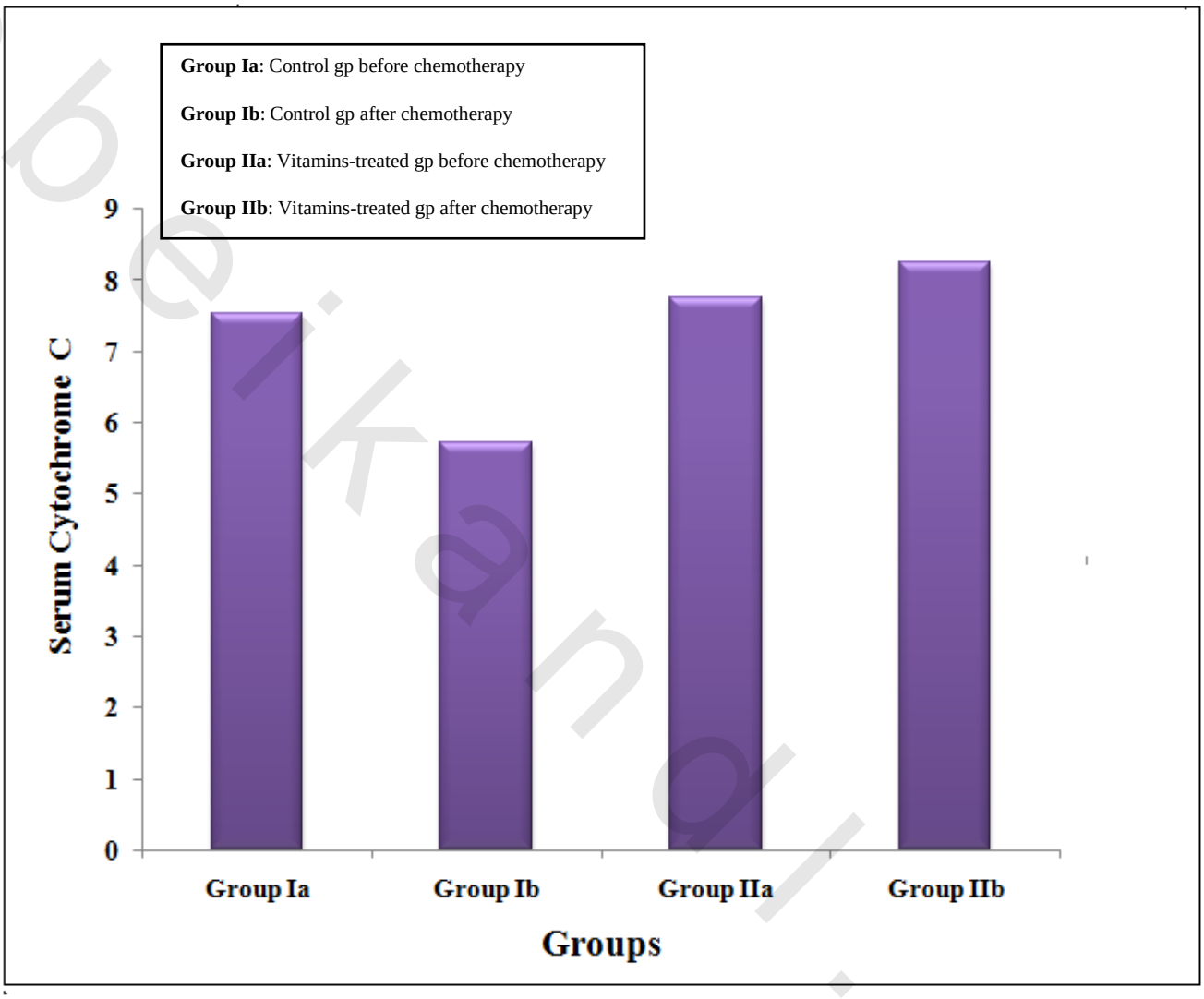

Fig (10): Serum Cytochrome c levels in all studied groups.

Western blotting photographs of serum cytochrome C

After chemotherapy, in the vitamins-treated group, the release of cytochrome c was increased Fig (11d). While, there was no change in its release in the control group (I1b), as compared to their corresponding groups before chemotherapy, Fig (11c & 11a).

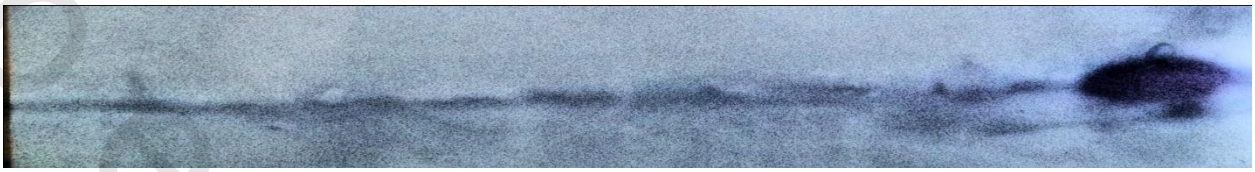

Fig (11a)

Fig (11b)

Fig (11c)

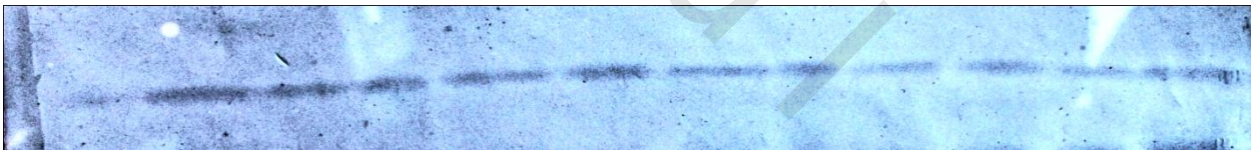

Fig (11d)

Fig (11a): The release of cytochrome c in the control group before chemotherapy.

Fig (11b): The release of cytochrome c in the control group after chemotherapy.

Fig (11c): The release of cytochrome c in the vitamins-treated group before chemotherapy.

Fig (11d): The release of cytochrome c in the vitamins-treated after chemotherapy.

(5) Correlation studies:

(A) Correlation between all biochemical parameters in all studied groups:

- There was a significant negative correlation only between DNA integrity index and cytochrome c level, (r = -0.49, P = 0.01), Table (7).
- No correlation was found between all the other biochemical parameters, Table (7).

(B) Correlation between all biochemical parameters and clinicopathological characteristics:

- There was a significant negative correlatin between the level of p53 concentration and the age of patients (r = -0.33, P = 0.04), Table (8).
- There was a significant positive correlation between DNA integrity index before and after chemotherapy and the vascular invasion (r = 0.49, P = 0.02 & r = 0.35, P = 0.04), respectively, Table (8).
- No correlation found between all the biochemical parameters and the clinicopathological charaterstics of the patients in the two studied groups:

Table (7): Correlations between all biochemical parameters in the control and the vitamins-treated groups.

		Biochemical parameters			
		P53	**Survivin**	**DNA integrity index**	**Cytochrome C**
P53	r		-0.01	0.08	-0.03
	p		0.97	0.62	0.67
Survivin	r			0.19	-0.35
	p			0.33	0.06
DNA integrity index	r				-0.49*
	p				0.01

r: Spearman Coefficient

Diffrences were considered statistically significant at p < 0.05 and r > 0.3.

Table (8): Correlations between the studied biochemical parameters and clinicopathological parameters and patients characteristics in the control and vitamins-treated groups before and after chemotherapy:

Clinicopathological parameters and patient characteristics		Biochemical parameters							
		P53		Survivin		DNA integrity index		Cytochrome C	
		Before	After	Before	After	Before	After	Before	After
Age	r	-0.33*	-0.16	0.07	-0.33	-0.23	-0.08	0.16	0.22
	p	0.04	0.34	0.66	0.07	0.18	0.64	0.36	0.20
Tumor Size	r	0.18	0.31	0.25	-0.16	-0.02	-0.03	0.14	0.24
	p	0.28	0.7	0.14	0.59	0.93	0.89	0.42	0.18
Lymph node	r	0.04	-0.14	0.09	0.10	0.05	0.05	0.17	-0.05
	p	0.81	0.42	0.61	0.58	0.79	0.79	0.34	0.81
ER	r	-0.19	0.13	-0.06	0.03	0.20	0.22	-0.18	0.09
	p	0.28	0.45	0.73	0.87	0.29	0.21	0.34	0.64
PR	r	-0.13	0.18	0.09	-0.09	0.01	0.09	0.02	0.17
	p	0.47	0.31	0.60	0.61	0.94	0.61	0.89	0.36
Her-2	r	-0.39	0.22	-0.47	0.26	-0.22	0.48	0.17	-0.41
	p	0.47	0.49	0.11	0.41	0.49	0.11	0.63	0.27
Vascular invasion	r	-0.04	0.08	-0.01	0.02	0.49*	0.35*	0.11	-0.09
	p	0.81	0.66	0.93	0.93	0.02	0.04	0.55	0.62
Menpausal status	r	-0.42	-0.26	0.10	-0.15	-0.19	-0.06	-0.09	0.03
	p	0.1	0.12	0.56	0.41	0.25	0.74	0.64	0.89
Family history	r	-0.17	-0.26	-0.17	-0.02	0.41	0.30	-0.29	-0.28
	p	0.31	0.13	0.33	0.93	0.62	0.18	0.09	0.12

r: Spearman Coefficient

Diffrences were considered statistically significant at $p < 0.05$ and $r > 0.3$.