AIM OF THE WORK

The present study is undertaking to explore the possibility that vitamin A and E supplementation during 5-fluorouracil, Adriamycin, and cyclophosphamide (FAC) therapy will have its impact on chemotherapy-induced oxidative stress in breast cancer patient.

SUBJECTS AND METHODS

Subjects

The present study was carried out on 45 females breast cancer patients (stage II/III), referred to the Department of Cancer Management and Research - Medical Research Institute, University of Alexandria in (2009-2011). The study was carried after the approval of Medical Research Institute Esthetics Committee and assigned consent was received from all the participants in the study. Cancer patients were divided into two groups.

Group I:

Comprised of twenty female breast cancer patients who underwent mastectomy and subjected to chemotherapeutic course (6 Cycles; 18 weeks). The recommended therapy was FAC (5- Fluorouracil, Adriyamicin and cyclophosphamide).

Group II:

Comprised of twenty five female breast cancer patients who underwent mastectomy and then subjected to chemotherapeutic course (6 Cycles; 18 week). The recommended therapy was FAC (5- Fluorouracil, Adriyamicin and cyclophosphamide). In association with the recommended chemotherapy course, patients of this group received vitamin (A) 1000 (IU)[160] and vitamin E (400 mg)[161] daily during the course of chemotherapy for 18 weeks.

For all patients, clinical and histopathological data were recorded including fine needle aspiration cytology (FNAC), tumor size, grade, stage, number of positive lymph node, vascular invasive and receptor status, and the presence of previous history of malignancies or any other diseases.

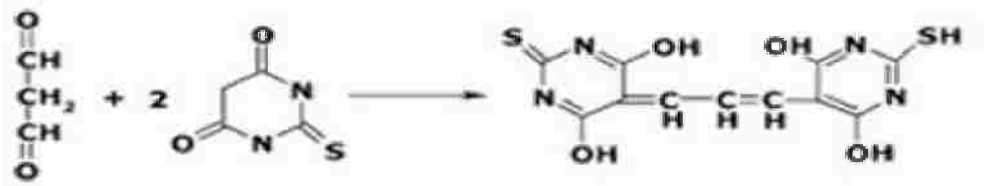
Sampling

Two blood samples were collected from all patients in this study; the first sample was collected after surgery and before the first cycle of chemotherapy and the second sample was collected two weeks after the 6^{th} cycle of chemotherapeutic course (Group I), and chemotherapeutic course plus vitamin treatment (Group II). Blood samples were collected and sera separated and stored at – 80 °Ctill analysis.

1. Colorimetric determination of malondialdehyde (MDA) [162]:

Principle:

Serum MDA was determining according to method based on the observation that thiobarbituric acid reacts with lipid peroxide, hydroperoxide, and oxygen-labile double bond to form the colour adducts with maximal absorbance at 530 nm.



Reagents:

1. 0.67% TBA (thiobarbituric)
2. 20% tricloroacetic acid

Procedure:

1. 100 µl of (serum, Standard) was mixed with 1000 µl of 0.67%TBA.
2. 500 µl of 20% tricloroacetic acid was added to the previous mixture.
3. The mixture was incubated at 100 °C for 20 minutes.
4. After cooling, the tubes were centrifuged at 12000 X g for 5 minutes.
5. After decentition, the absorbance was measured at 530 nm against distilled water.
6. The malondialdhyde (MDA) concentration of each serum sample was determined from the standard curve (500 nmol/ml) (Fig 8) and expressed in the term of nmol/ml.

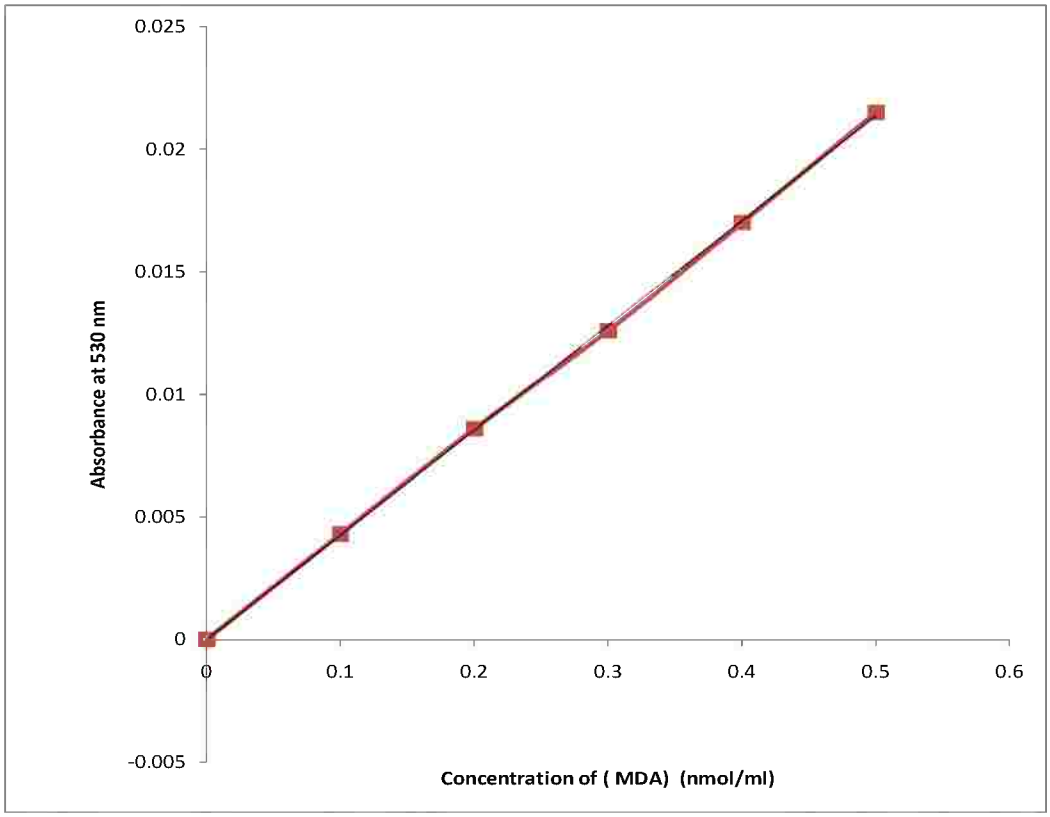


Figure (8): Standard curve of MDA

2. Quantitative determination of protein carbonyl:

Principle:

Several approaches have been taken to detect and quantitate the carbonyl content in protein preparations. The most convenient procedure is the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically[163].The licit for quantitative analysis of protein carbonyl was purchased from Cayman Chemical Company , Ann Arbor , MI , All rights reserved .printed in U.S.A.

Reagents:

1. Hydrochloric acid (2.5 M)

   The vial contains 12 M hydrochloric acid (HCL) .Slowly add the content of vial into 40 ml HPLC grade water to yield 2.5 M HCL .

2. DNPH

   The vial contains 2.4 dinitrphenylehydrazine .The content of vial was dissolved into 10 ml of 2.5 M HCL. DNPH was stable at 4 °C and stored in dark .Don't freeze.

3. 10% trichloroacetic acid (TAC) solution.

4. Guanidine hydrochloride

5. Ethanol

6. Ethyl acetate

   Each vial contains 30 ml of ethyl acetate .Mix the content of ethyl acetate into 30 ml of ethanol, for a 1:1 mixture of ethanol: ethyl acetate.

Procedure:

1. 200 µl of serum was transferred into two plastic tubes. One tube for the sample (S) and the other for the control (C).

2. 800 µl of DNPH was added to the sample tube and 800 µl of HCL was added to the control tube.

3. Both tubes (S & C) were incubated at room temperature for one hour in the dark. Each tube was vortex briefly every 15 minutes during the incubation.

4. 1 ml of 20% TCA was added to each tube and vortex. Tubes were placed on ice and were incubated for five minutes.

5. The tubes were centrifuged at 10,000 x g for 10 minutes at 4°C in a micro centrifuge.

6. The supernatant was discarded and the pellet was manually resuspend in 1 ml of 10%

TCA. Tubes were placed on ice and incubated for five minutes.

7. The tubes were centrifuged at 10,000 x g for 10 minutes at 4°C in a micro centrifuge.
8. The supernatant was discarded and the pellet was resuspend in 1 ml of (1:1) ethanol/ethyl acetate mixture. Manually suspend the pellet with spatula, vortexes thoroughly, and the tubes were centrifuged at 10,000 g for 10 minutes at 4°C in a micro centrifuge.
9. Step 8 was repeated for two more times.
10. After the final washing the protein pellets were resuspend in 500 µl of guanidine hydrochloride and vortex.
11. The tubes were centrifuged at 10,000 x g for 10 minutes at 4°C in a micro centrifuge to remove any left over debris.
12. The absorbance of the supernatant was measured at a wavelength 370 nm using quartz cuvette.

Calculations:

Protein Carbonyl (nmol/ml) = $[(CA)/ (*0.022\ \mu M^{-1})]$ (500 µl/200 µl)

* = Is the actual extinction coefficient for dinitrophenylhydrazine at 370 nm is 22,000 M^{-1} cm^{-1}.

- (CA) is corrected Absorbance (A) = $Ab_S - Ab_C$.
- 500 µl = The final volume
- 200 µl = The original volume

3. Colorimetric determination of total antioxidant capacity [164].

Principle

The total antioxidant activity was mesured by the phospho molybdate method according to the procedure, describes by Prieto and his associated (1999) [164], and modified by Pendyala and his associated (2008)[165]. The assay is based on reduction of molybdate (Mo) VI to V by the extract and, subsequent formation of a green phosphate Mo (V) complex at acid pH.

Reagents

1. Methanol.
2. 0.6 M sulfuric acid.
3. 4 mM ammonium molybdate.
4. 28 mM sodium phosphate.

-The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid (20µg/ml).

Procedure

1. 0.1 ml of serum or standard were deproteinized by adding 1 ml of methanol.
2. Deproteinized serum was vortex for 30 sec and centrifuged at 300 rpm for 30 min to precipitate the proteins.
3. 0.1 ml of deprotenized serum or standard was mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 4 mM ammonium molybdate and, 28 mM sodium phosphate) , then incubated at 95 C°for 90 min.
4. The mixture was cooled to room temperature.
5. The absorbance of the solution was measured at 695 nm using spectrophotometer against distilled water.
6. Vitamin C concentration of each sample was determined from the standard curve (Figure 9) and expressed as termed µg/ml.

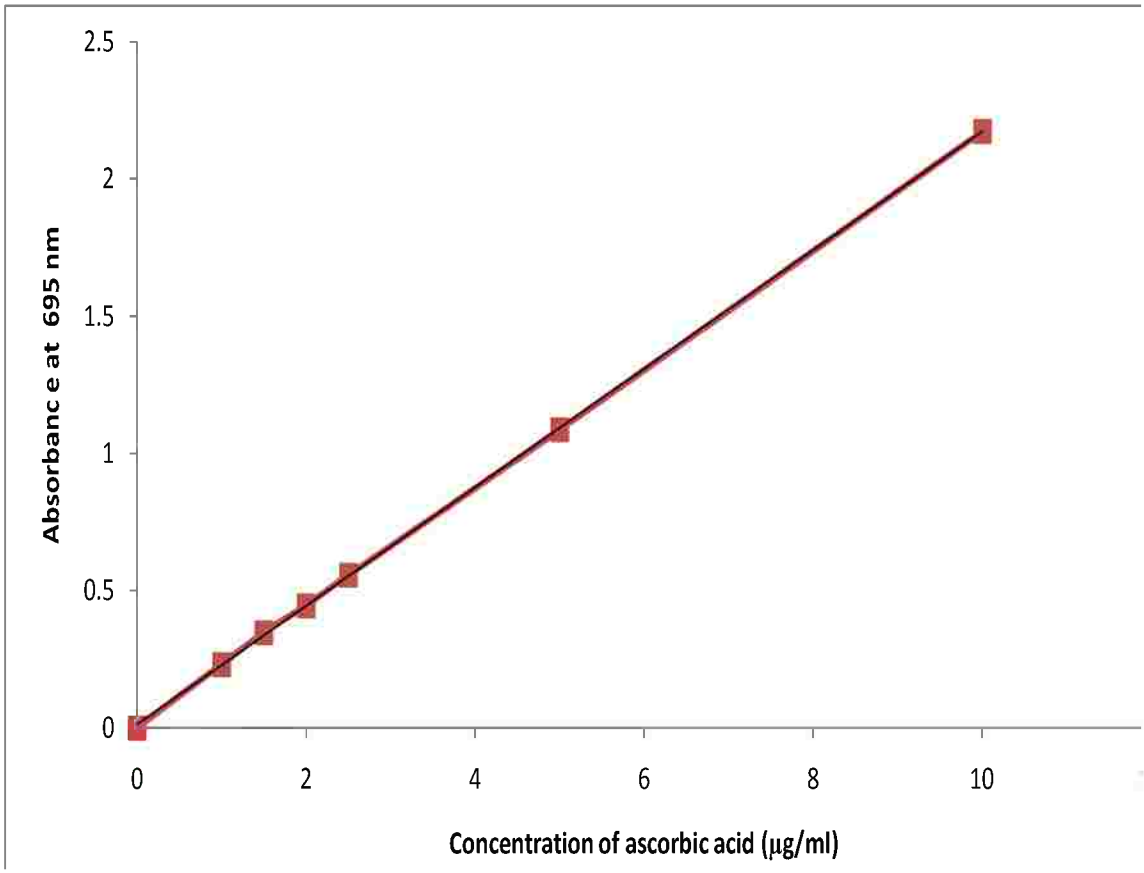


Figure (9): Standard curve of ascorbic acid (µg/ml)

RESULTS

Forty-five breast cancer patients were enrolled in the present study. They were divided into two groups; group (I) which was subjected only to chemotherapy, vitamin supplementation –ve (20 patients) and group (II) which was subjected to chemotherapy plus supplementation with vitamin A and E, vitamin supplementation +ve (25 patients). Demographic and clinico-pathological characteristics are shown in Table (2). Markers of oxidative stress including serum MDA, β-carbonyl protein (β -CP) and total antioxidant capacity (TAOC) are measured in patients of those two groups before and after chemotherapy. Individual data are available in appendices (1 – 3)

Table (2): Demographic and Clinico-pathological Characteristics of Breast Cancer Patients of the Studied Groups

		Group I	Group II
Number of Cases		20	25
Age (years)		55.7 ± 1.5	53.5 ± 1.6
BMI (m^2/Kg)		20.1 ± 0.86	27.0 ± 0.9
Grade	**II**	13	22
	III	7	3
Lymph Nods	**-ve**	5	10
	< 3	4	6
	≥ 3	11	9

1- Serum Malondialdehyde (MDA)

In breast cancer patients of group I, vitamin supplementation –ve, the range of serum MDA level before chemotherapy is 5.12 – 14.49 nmol/ml with a mean value of 10.73 nmol/ml (± 0.57) (± S.E.M). After chemotherapy, the observed range of serum MDA 9.92 – 16.18 nmol/ml with a mean value of 13.10 nmol/ml (± 0.43) (± S.E.M). Thus, a significantly elevation in mean serum MDA concentration level was observed (p_1 = 0.0001) after chemotherapy cycle, Table (3) and Fig. (10).

In breast caner patients of group II, vitamin supplementation +ve, the range of serum MDA level before chemotherapy is 6.71 – 12.90 nmol/ml with a mean value of 10.76 nmol/ml (± 0.33) (± S.E.M). After Chemotherapy, the range of serum MDA level is 3.71 – 12.29 nmol/ml with a mean value 7.39 nmol/ml (± 0.41) (± S.E.M). Thus, in contrast to group I, the mean serum MDA concentration level is significantly reduced after chemotherapy cycle in breast cancer patients of group II (p_1 = 0.0001), Table (3) and Fig. (10).

However, before chemotherapy, the mean concentration level of MDA in patients of group II is not significantly different from that in patients of group I ($p_2 > 0.05$) Table (3). Meanwhile, after chemotherapy, the mean concentration level of MDA in patients of group II is significantly lower than that of group I (p_3 = 0.0001), Table (3) and Fig. (10).

Table (3): Serum Concentration Level of Malondialdehyde (MDA; nmol/ml), Breast Cancer Patients of Group I (Vitamins Supplementation -ve) and Group II (Vitamins Supplementation +ve)

	Group I (n = 20)		Group II (n = 25)	
	Before Chemotherapy	After Chemotherapy	Before Chemotherapy	After Chemotherapy
Serum MDA (nmol/ml)				
Min. - Max.	5.12 – 14.49	9.92 – 16.18	6.71 – 12.90	3.71 - 12.29
X ± S.E.M.	10.73 ± 0.57	13.10 ± 0.43	10.76 ± 0.33	7.39 ± 0.41
p1	0.0001		0.0001	
p2			> 0.05	
p3				0.0001

Mean ± Standard Error of Mean

*p*1: Before Chemotherapy ***vs.*** After Chemotherapy

*p*2: Before Chemotherapy (+ve vitamin) ***vs.*** Before Chemotherapy (-ve vitamin)

*p*3: After Chemotherapy (+ve vitamin) ***vs.*** After Chemotherapy (-ve vitamin)

p-Values < 0.05 were considered significant

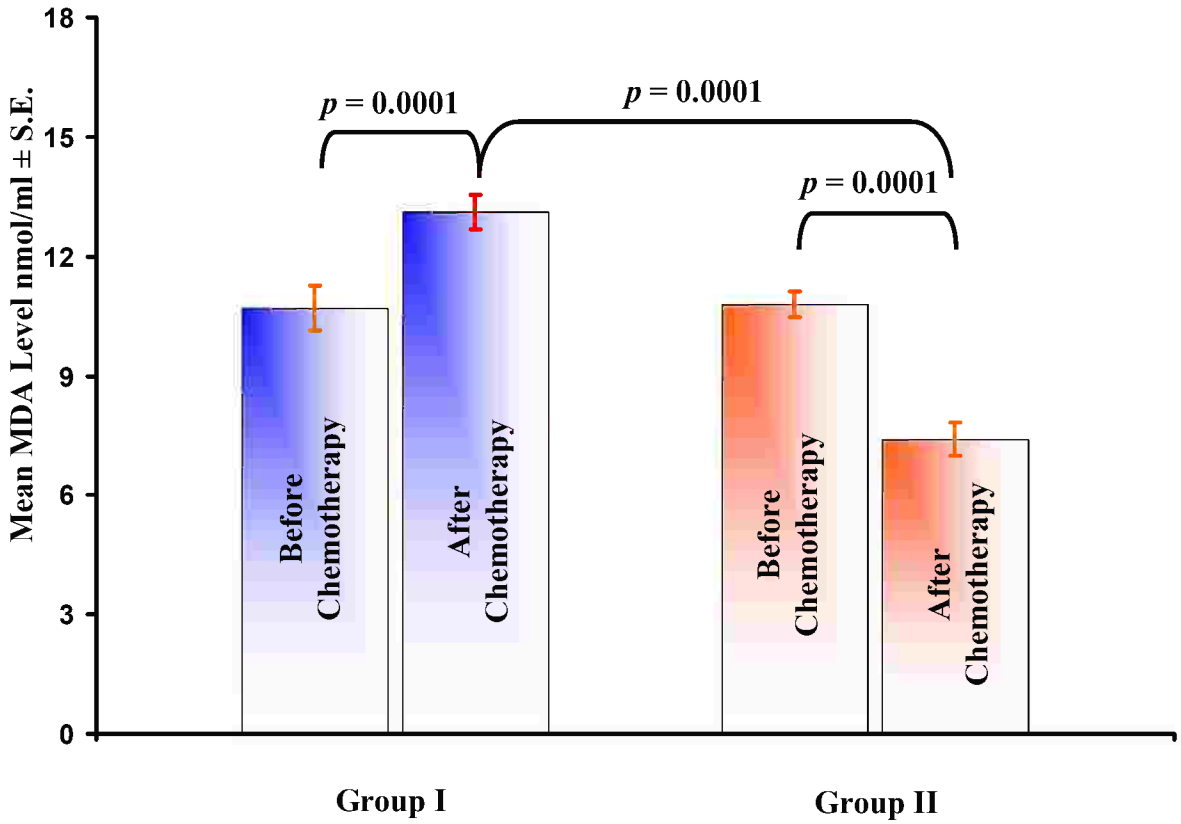


Fig. (10): Mean Serum Concentration Level of MDA nmol/ml

2- Serum β- Carbonyl Protein (β- CP)

In breast cancer patients of group I, vitamin supplementation –ve, the range of serum β- CP level before chemotherapy is 12.02 – 17.55 nmol/ml with a mean value of 15.23 nmol/ml (± 0.36) (± S.E.M). After chemotherapy, the observed range of serum β- CP is 5.84 –34.91 nmol/ml with a mean value of 26.05 nmol/ml (± 1.40) (± S.E.M). Thus, a significant increase in mean serum β- CP concentration level was observed (p_1= 0.0001) after chemotherapy cycle, Table (4) and Fig. (11).

In breast cancer patients of group II, vitamin supplementation +ve, the range of serum β- CP level before chemotherapy is 6.75 – 17.15 nmol/ml with a mean value of 15.37 nmol/ml (± 0.42) (± S.E.M). After Chemotherapy, the range of serum β- CP level is 7.45 – 14.58 nmol/ml with a mean value10.98 nmol/ml (± 0.40) (± S.E.M). Thus, in contrast to group I, the mean serum β- CP concentration level is significantly reduced after chemotherapy cycle in breast cancer patients of group II (p_1= 0.0001), Table (4) and Fig. (11).

However, before chemotherapy, the mean concentration level of β- CP in patients of group II is not significantly different from that in patients of group I (p_2> 0.05), Table (4). Meanwhile, after chemotherapy, the mean concentration level of β- CP in patients of group II is significantly lower than that of group I (p_3 = 0.0001), Table (4) and Fig. (11).

Table (4): Serum Concentration Level β- CP (nmol/ml) in Breast Cancer Patients of Group I (Vitamins Supplementation -ve) and Group II (Vitamins Supplementation +ve)

	Group I (n = 20)		Group II (n = 25)	
	Before Chemotherapy	After Chemotherapy	Before Chemotherapy	After Chemotherapy
Serum β- CP (nmol/ml)				
Min. - Max.	12.02 – 17.55	5.84 – 34.91	6.75 – 17.15	7.45 – 14.58
X ± S.E.M.	15.23 ± 0.36	26.05 ± 1.40	15.37 ± 0.42	10.98 ± 0.40
***p*1**	0.0001		0.0001	
***p*2**			> 0.05	
***p*3**				0.0001

Mean ± Standard Error of Mean

*p*1: Before Chemotherapy ***vs.*** After Chemotherapy

*p*2: Before Chemotherapy (+ve vitamin) ***vs.*** Before Chemotherapy (-ve vitamin)

*p*3: After Chemotherapy (+ve vitamin) ***vs.*** After Chemotherapy (-ve vitamin)

p-Values < 0.05 were considered significant

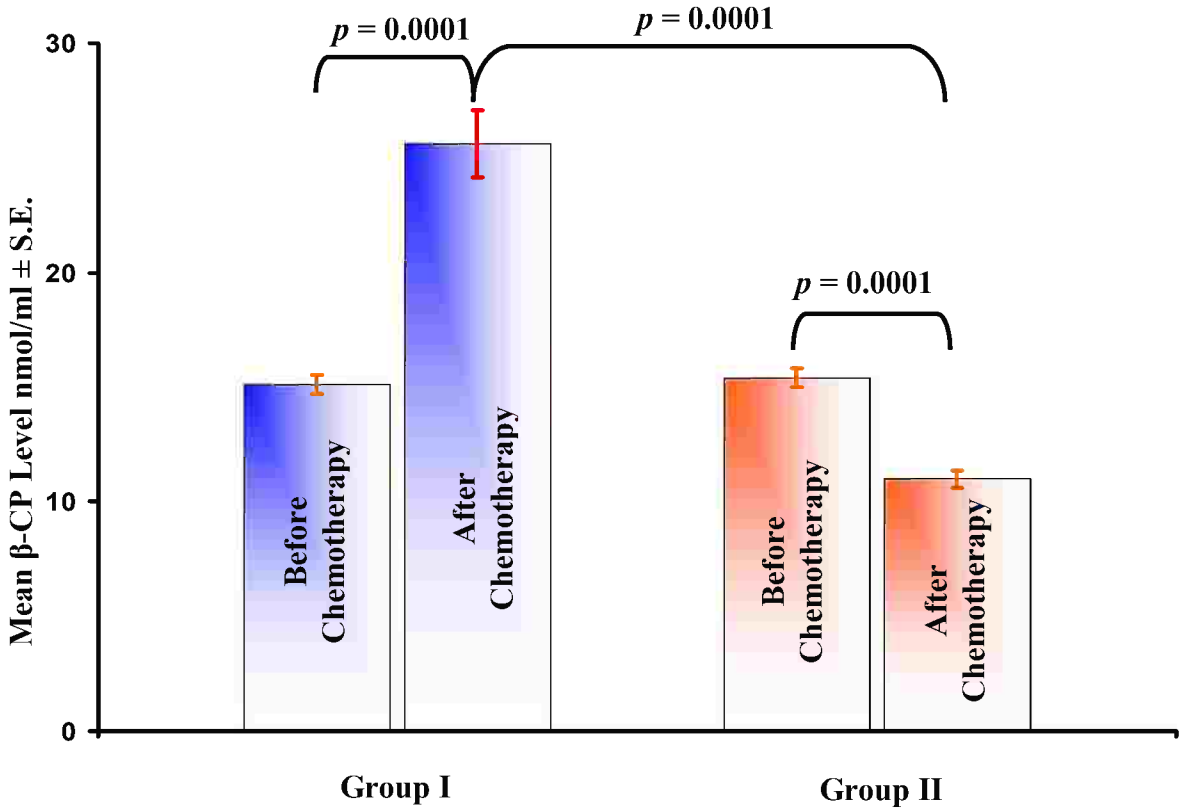


Fig. (11): Mean Serum Concentration Level of β- CP nmol/ml

3- Serum Total Antioxidant Capacity (TAOC)

In breast cancer patients of group I, vitamin supplementation –ve, the range of serum TAOC level before chemotherapy is 0.25 – 1.99 µg/ml with a mean value of 1.16 nmol/ml (± 0.8) (± S.E.M). After chemotherapy, the observed range of serum TAOC is 0.21 –1.00 µg/ml with a mean value of 0.73 µg/ml (± 0.04) (± S.E.M). Thus, a significant decrease in mean serum TAOC concentration level was observed (p_1 = 0.0001) after chemotherapy cycle, Table (5) and Fig. (12).

In breast cancer patients of group II, vitamin supplementation +ve, the range of serum TAOC level before chemotherapy is 0.18 –2.28 µg/ml with a mean value of 1.32 µg/ml (± 0.10) (± S.E.M). After Chemotherapy, the range of serum TAOC level is 1.12 – 3.43 µg/ml with a mean value 2.13 µg/ml (± 0.11) (± S.E.M). Thus, in contrast to group I, the mean serum TAOC concentration level is significantly elevated after chemotherapy cycle in breast cancer patients of group II (p_1 = 0.0001), Table (5) and Fig. (12).

However, before chemotherapy, the mean concentration level of TAOC in patients of group II is not significantly different from that in patients of group I ($p_2 > 0.05$), Table (5). Meanwhile, after chemotherapy, the mean concentration level of TAOC in patients of group II is significantly higher than that of group I (p_3 = 0.0001), Table (5) and Fig. (12).

Table (5): Serum Concentration Levels of TAOC (µg/ml) in Breast Cancer Patients of Group I (Vitamins Supplementation -ve) and Group II (Vitamins Supplementation +ve)

	Group I (n = 20)		Group II (n = 25)	
	Before Chemotherapy	After Chemotherapy	Before Chemotherapy	After Chemotherapy
Serum TAOC (µg/ml)				
Min. - Max.	0.25 -1.99	0.21 – 1.00	0.18 – 2.28	1.12 – 3.43
X ± S.E.M.	1.16 ± 0.08	0.73 ± 0.04	1.32 ± 0.10	2.13 ± 0.11
***p*1**	0.0001		0.0001	
***p*2**			> 0.05	
***p*3**				0.0001

Mean ± **Standard Error of Mean**

*p*1: Before Chemotherapy ***vs.*** After Chemotherapy

*p*2: Before Chemotherapy (+ve vitamin) ***vs.*** Before Chemotherapy (-ve vitamin)

*p*3: After Chemotherapy (+ve vitamin) ***vs.*** After Chemotherapy (-ve vitamin)

p-Values < 0.05 were considered significant

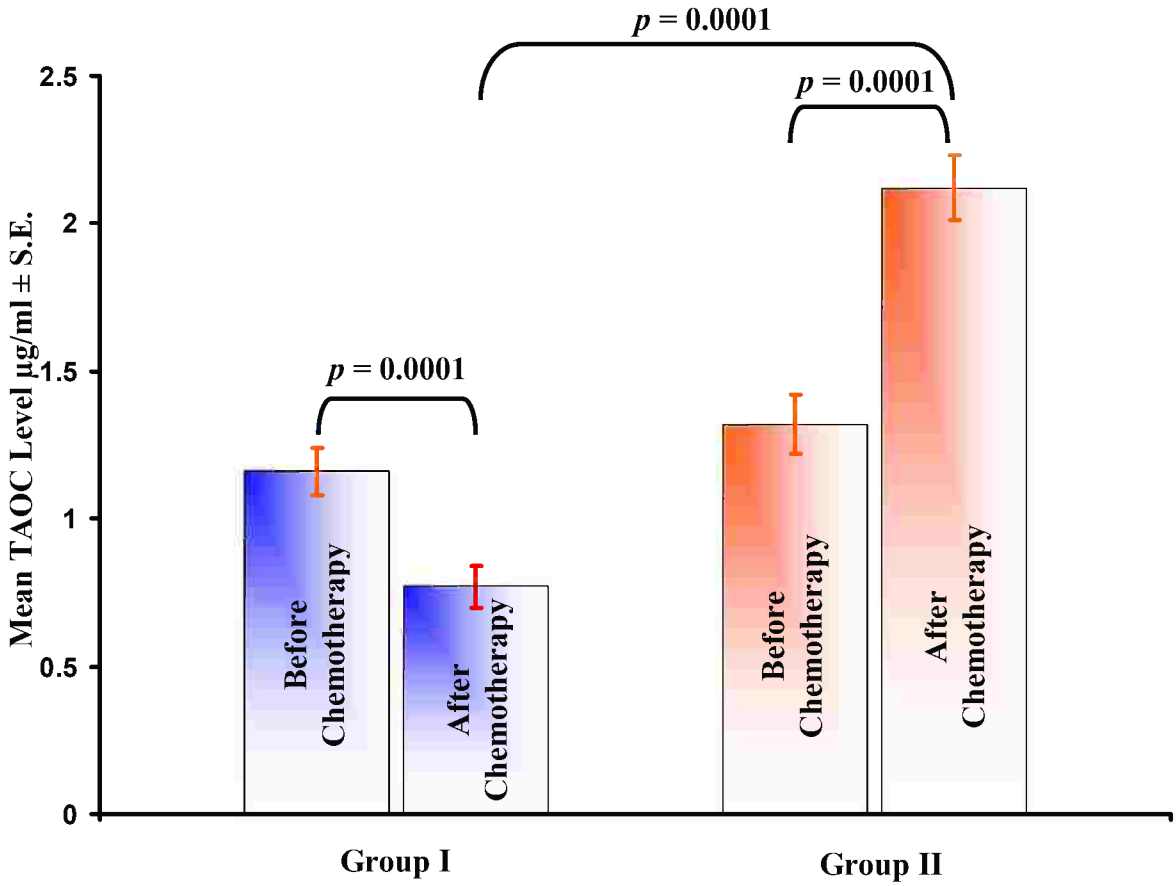


Fig. (12): Mean Serum Concentration Level of TAOC µg/ml

Biostatistical Correlations

The results of the present study showed that the overall correlation between **Serum concentration levels of MDA and β- CP** was a significant positive correlation **(**r = 0.58; *p* = 0.0001), Fig. (13)

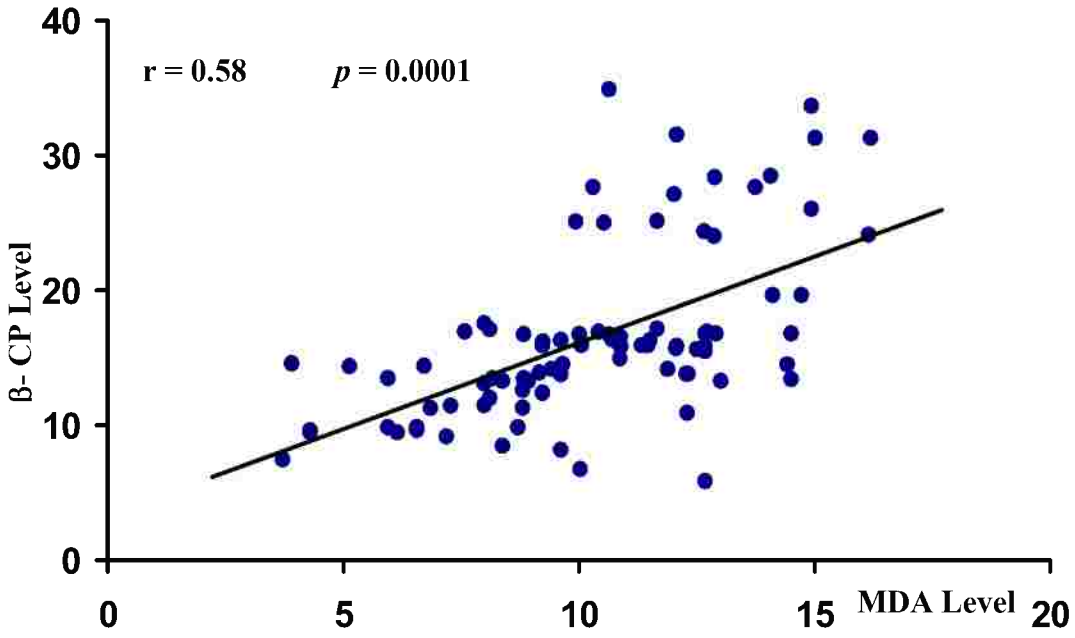


Fig. (13): The overall correlation between Concentration Levels of Serum MDA and β- CP

Also, a significant negative overall correlation has been observed between serum concentration levels of TAOC and each of **Serum MDA (**r = - 0.61; *p* = 0.0001) **and Serum β- CP (**r = - 0.62; *p* = 0.0001), Fig. (14)

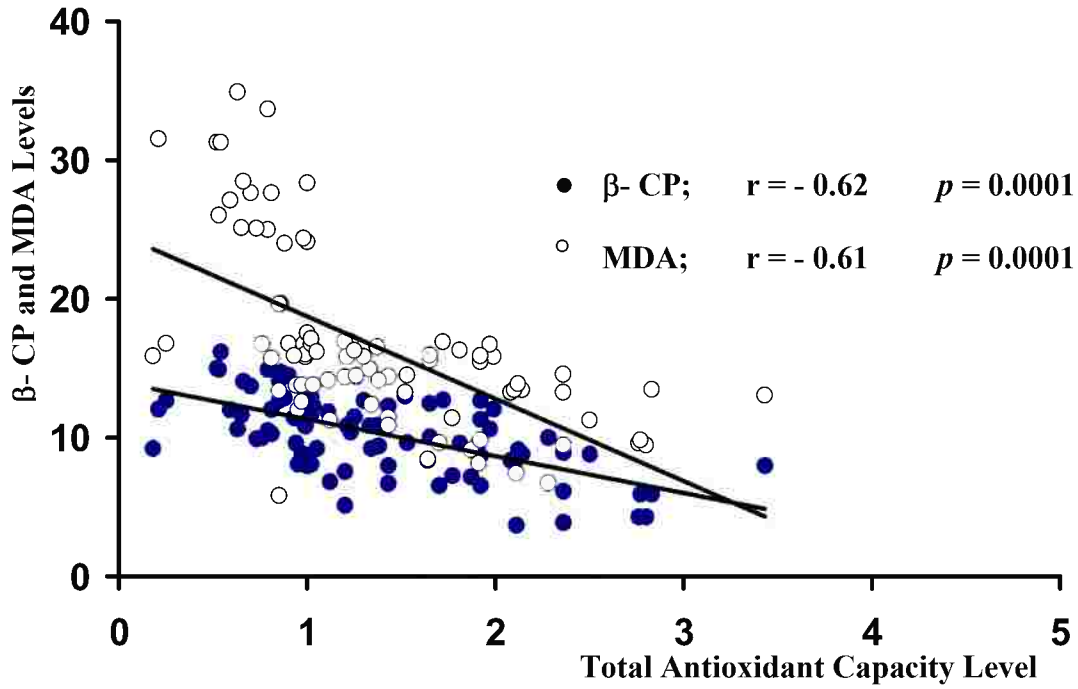


Fig. (14): The overall correlation between TAOC Level and Both of Concentration Levels of Serum β- CP and MDA