Discussion

Breast cancer is the most common malignancy among women and second most common cause of cancer death after lung. Development of breast cancer is controlled by a balance between cell proliferation and apoptosis [314]. Diverse molecular changes can cause apoptotic dysregulation, including activation of anti-apoptotic factors (Bcl-2), inactivation of pro-apoptotic effectors (p53), and reinforcement of survival signals (Survivin) [315].

The tumor suppressor p53 plays an important role in a variety of cancers including breast cancer. It inhibits the growth of malignant cells either by inducing G1 and G2 arrest, apoptosis or senescence [316]. The innate ability of p53 to regulate the cell cycle and activate apoptosis has led p53 to be extensively studied in the context of tumorigenesis and cell death [317].

High levels of survivin have been identified in a wide range of malignancies, including breast, lung, prostate, pancreatic, and colorectal cancers, but interestingly it is not encountered in normal adult tissue [127]. Some previous studies have revealed a significant relationship with high expression levels of survivin and prognosis in breast cancer, particularly as an independent predictor of shorter duration of survival in patients with poor prognostic features [104, 318].

The release of cytochrome c from mitochrondria in response to proapoptotic signals has been suggested as an initiating event in the apoptotic process [319].

Breakdown of nuclear DNA into its nucleosomal fragments is a key feature of the programmed cell death or apoptosis [320]. cDNA may be either from nuclear or from mitochondrial origin. Increased levels have been detectable in several cancer types, and an association between nuclear cfDNA levels and malignancy as well as tumor size has been described [321, 322].

For years, apoptosis was thought to be the principal mechanism by which chemotherapeutic agents kill cells [323]. Cancer chemoprevention is defined as the use of specific natural or synthetic chemical agents to reverse, suppress or prevent carcinogenic progression to invasive cancer. The goal of cancer chemoprevention is either to prevent or reverse the carcinogenic process in the initiation and promotion phases before the clinical development of cancer, and/or to delete premalignant or latent malignant clones from an organ

by apoptosis or differentiation induction, in order to reduce the incidence of disease (and thus, ultimately, to reduce mortality) [324].

Considerable evidence now supports the view that the various isoforms of vitamin E (and their chemical derivatives) have distinct biochemical properties and distinct abilities to modulate oxidative stress, signal transduction pathways, and pathophysiological processes important in carcinogenesis (e.g., apoptosis and angiogenesis) [325].Vitamin A derivatives have been shown to regulate a variety of metabolic processes boosting cell growth, morphogenesis, cell function and differentiation, proliferation, and apoptosis. They are also immune modulators controlling lymphocyte function, stimulating angiogenesis, and inhibiting carcinogenesis [326].

In the present study, p53 protein showed a significant increase in its level in the control group after chemotherapy (Group Ib) as compared to the corresponding group before chemotherapy (Group Ia). This is may be due to the fact that apoptosis induced by chemotherapeutic agents depends on an intact TP53 pathway, indicating that TP53 could play a pivotal role in defining tumor sensitivity to such agents [327]. In fact, it has been reported that breast cancer patients with TP53mutant-type (TP53mt) tumors showed poorer response to anthracycline-based regimens than do those with TP53 wild-type (TP53wt) tumors [327, 328].

Also, it is well documented that the proliferation of oncogenic cells results in the repression of p53 expression and activation. A multitude of regulators and targets of p53 frames it as a central protein in a complex and divergent network involving numerous cellular pathways [317]. Also decreased p53 levels and mutations in p53 have been linked to high incidences of cancer, thus elevating p53 can act as a mechanism of tumor suppression [329].

The significant increase of p53 in group Ib can be supported with the results of the *in vivo* models that indicated that epirubicin-cyclophophamide treatment induces senescence-like features in TP53 wild-type tumor, probably accounting for cell cycle arrest and subsequent resistance to treatment. Conversely in TP53 mutated tumors, chemotherapy induces mitotic catastrophe and tumor death, accounting for complete response to this association exclusively in patients with TP53 mutated tumors [330]. Also it was demonstrated that multiple stimuli such as ionizing radiatios, DNA lesions, nitric oxide, hypoxia, chemotherapeutic agents, or oncogenic stimuli can activate p53 [331, 332].

The protein p53 is likely the most thoroughly studied tumor suppressor, and it is involved in cellular responses to a wide range of cellular stress [333, 334]. Because p53 is mutated in at least 50% of all human cancers, and cancers with a p53 mutation generally respond poorly to therapeutics, anti-cancer agents working independent of p53 status are studied [335].

Also in our study, the level of serum p53 was significantly increased in vitamins-treated group after chemotherapy (Group IIb) as compared to its crossponding one before chemotherapy (Group IIa). This may be explained by the fact that chemotherapeutic drugs induce apoptosis through p53 pathway [327], in addition to this effect; vitamins supplementation may act synergestically to increase p53 with chemotherapy. These results are supported by the results of Pierpaoli and his associates in 2010, who showed that the expression of p53 and other tumor suppressors expression was increased in breast cancer cells treated with VE [316]. They measured p53, p21 WAF1 and p16 INK4 expression after treatment with α-TOS or tocotrienols (vitamin E derivatives) and found that α -TOS treatment of SKBR3 induced significant dose- and time-dependent up-regulation of p53 [316].

In addition, it is well established that vitamin E demonstrates strong anticarcinogenic activity by modulating the function of oncogenes such as p53. It enhances the expression of wild-type p53 gene product (tumor suppressor gene), and reduces the expression of mutant p53 and other oncogenes [336].

Also, the significant increase of serum p53 in vitamins-treated group may be explained by the the results of Zhang and his coworkers in 2010, they found that treatment of P19 cells with retinoic acid (vitamin A derivative) could induce the expression of p300/CBP associated factor (PCAF), which is concomitant with the increase of acetylated p53 [337]. Acetylation of p53 by PCAF enhances the protein stability of p53 and its transactivation potential via facilitating its interactions with specific promoters and other proteins in the transcriptional machinery [338, 339].

In the present work the level of survivin didn't show any significant difference in group Ib as compared with Group Ia, but it showed a significant decrease in group IIb as compared with both Group IIa and Group Ib.

These results are in accordance with the previous study that showed no stastically significant effect of chemotherapy on serum and urine survivin levels [124].

Survivin showed a significant difference in its expression between malignant and normal adult cells, with very low to absent levels in the normal adult tissue but increased levels in a wide variety of solid tumors [340]. In tumors, the positive expression of survivin correlates with more aggressive behavior and poorer prognosis [341].

Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, regulates two essential cellular processes, i.e., it inhibits apoptosis and promotes cell proliferation. Although expressed at high levels during fetal development, survivin is rarely expressed in normal healthy adult tissues. It is however, upregulated in the majority of cancers [124, 340]. In addition, it is currently known that over expression of survivin is associated with resistance to chemo/hormone therapy and predicts a poor clinical outcome in breast cancer [342].

On the other hand, the significant decrease in serum survivin level in Group IIb as compared to both groups IIa and Ib, can be attributed to the fact that tocotrienols (vitamin E derivative) impede the survival of various tumor cells by inhibiting expression of cell survival proteins such as XIAP (X-linked inhibitor of apoptosis protein), and survivin [343].

Our results are in accordance with Patacsil and his colleagues (2012), who observed the downregulation of XIAP and survivin levels in human pancreatic cancer cells (PANC-1), upon VE treatment at 80 μM concentration. However, the downregulation of survivin expression was more pronounced than that of XIAP [344].

Also, Chi and his team in 2010, documented that the apoptosis induced by ATRA (derivative of vitamin A) may be regulated at least by down-regulated expression of survivin and up-regulated expression of Bax [345]. In addition, our results may be also supported with the results of Kucukzeybek and his coworkers in 2008, who concluded that combination of ARTA with docetaxel results in a significant synergistic cytotoxic activity and apoptosis. They also proved that this combination significantly downregulates survivin and many other genes, which have pivotal roles in regulation of apoptosis and cell cycle progression. Since apoptosis and cell cycle are closely linked processes, there are a number of regulatory molecules that interface between apoptosis and cell cycle progression. In the process of carcinogenesis, altered regulation between

apoptosis and cell cycle, in the favor of aberrant cell proliferation, is the main mechanism. Thus, these regulatory molecules taking role in the induction of apoptosis can also participate in the cell cycle or vice versa [346].

The results of this study revealed that the DNA fragmentation in patients of groups Ib and IIb showed predominatly apoptotic pattern with short DNA fragments (of apoptotic origin) more than the long DNA fragments (of necrotic origin) when compared to their corresponding groups before chemotherapy.

The DNA integrity index didn't show any significant difference when comparing group Ib with Group Ia, and these results can be supported by the findings of Deligezer and his colleagues in 2008, who found that the distribution of the serum DNA integrity did not significantly change during the course of adjuvant therapy, whatever the direction of change of DNA levels during chemotherapy, both apoptotic and nonapoptotic fragments were present in sera of the patients [208].

Also, a previous study reported that combined administration of doxorubicin, cyclophosphamide, and 5-fluorouracil leads to a combination of apoptotic as well as non-apoptotic cell death, this study indicated that necrotic cell death predominates in *in vivo* conditions [347].

The significant decrease in apoptotic index when comparing group IIb with IIa indicated that the apoptotic fragments rather than necrotic fragments were predominant in vitamins-treated group after chemotherapy (group IIb)

This may be attributed to the fact that some forms of vitamin E also mediates apoptosis through DNA fragmentation [348, 349], and upregulation of p53 [350], in certain cells. Also, some evidences suggest that γ -TOC may be a more potent proapoptotic agent than α -TOC. γ-TOC as well as the combination of γ -TOC with δ-TOC induced apoptosis in androgen-sensitive prostate cells but not in androgen-resistant PC3 cells as indicated by DNA fragmentation [351].

Engedal and his team in 2009, reported that retinoic acid regulates Jurkat T cell death in the presence of CH11 and TPA by enhancing apoptosis and not necrosis [352]. Their results indicate that RA is able to partially reactivate several of the proapoptotic caspases. It is believed that it

plays a role in Fas-induced apoptosis, suggesting that RA abrogates mitogen-mediated repression of Fas-induced apoptosis through a reactivation of the caspase cascade [352]. Fas-induced apoptosis is meditated by a caspase cascade. In Jurkat cells, caspase-8 indirectly (via the mitochondria) activates caspase-9, which in turn, directly activates the central executor caspase-3 [353], which can cleave procaspase-6, producing the active form of the effector caspase, caspase-6 [354]. Caspase-3 also cleaves the inhibitor of caspase-activated DNase, DFF45, leading to the release of caspase-activated DNase/DFF40 and thus, apoptotic DNA fragmentation [355].

In the present study there was a significant increase in the release of cytochrome c in Group IIb as compared with IIa, whereas its release didn't show any significant difference when comparing group Ib with Group Ia.

The significant increase of cytochrome c is in accordance with some various studies which revealed that tocotrienols (vitamin E derivatives) can induce apoptosis in a wide variety of tumor cells. These effects are mediated through activation of both extrinsic and intrinsic pathways by the vitamin. The activation of intrinsic pathways by tocotrienols involves mitochondrial depolarization [347] and is mediated through the upregulation of Bax [356, 357] , cleavage of Bid [358] , release of cytochrome c [347, 359, 360], and activation of caspase-9, which in turn leads to activation of caspase-3 [358, 361, 362].

Our results are consistent with the results of Cui and his team in 2007, who showed that carotene supplementation, induces the release of cytochrome c in MCF-7 cancer cells [363]. Also, α-TOS induces dissipation of the mitochondrial inner membrane potential and the release of mitochondrial apoptotic proteins such as AIF, Smac/Diablo and Cytc. In many cases, relocation of Cytc leads to formation of the apoptosome, a complex consisting of Cytc, Apaf-1 and procaspase 9, with the ensuing activation of caspase 9 followed by activation of caspase 3 [364, 365].

Also, Jiang and his colleagues in 2004 found that LNCaP cells (prostate cancer cell line) treated with gamma-tocopherol released cytochrome c [366]. They concluded that LNCaP prostate cancer cell death caused by gamma-tocopherol is linked to the de novo synthesis of sphingolipids [366]. Ceramides are neutral sphingolipids produced from either by the hydrolysis of sphingomyelin or by de novo synthesis from serine and palmitate [366]. Considerable evidence

supports the view that ceramide accumulation in biological membranes promotes the formation of specialized lipid domains called lipid rafts which, in turn, activate several downstream phosphorylation cascades important in promoting apoptosis. It is increasingly apparent that some chemotherapeutic agents are proapoptotic by activating ceramide synthase and increasing membrane pools of ceramide [367, 368]. It is generally accepted that dihydroceramide, the precursor of ceramide, is not proapoptotic [369]. Some researchers [370] have suggested that dihydroceramides inhibit ceramide channel formation in the outer mitochondrial membrane thereby blocking the release of small proteins such as cytochrome c and preventing apoptosis.

In addition, a previous study also found a dose-dependent increase of cleaved form of Bid, which is known to induce the release of cytochrome c by a caspase-dependent mechanism in tumor cell line treated with β-carotene (provitamin A) [371].

In the present study, we found a significant negative correlation between DNA integrity index and serum cytochrome c, and this can be explained by the fact that as the necrotic death of cells predominates over the apoptotic death, the release of cytochrome c decreases.