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# Potential therapy of vitamin B17 against Ehrlich solid tumor induced changes in Interferon gamma, Nuclear factor kappa B, DNA fragmentation, p53, Bcl2, survivin, VEGF and TNF-α Expressions in mice

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**Abstract**: Breast cancer is the most common cancer in females, and the leading cause of cancer-related mortality in the world. Among the available treatment options for cancer, chemotherapy is the therapy for treating a variety of cancer patients. However, the therapeutic efficacy of current agents is minimal and these drugs do not retard the progression of disease pathology. Lack of appropriate therapy may increase the prevalence of disease in world. Hence, more effective strategies and novel therapies must be pursued for altering the progression of the disease acting through different mechanisms. There is a continuing need for new and improved therapy. Hence, Vitamin B17 is suggested a therapeutic potential for treating breast cancer. This study is to evaluate the potential therapy of vitamin B17 (Vit B17, amygdalin) against Ehrlish solid tumors, bearing mice (EST) induced DNA damage, NF-Kb, TNFα and apoptosis. Sixty female mice were randomly divided into four groups: (I, control group; II, VitB17 group; III, EST group; IV, EST+VitB17 group). EST induced group had elevated in the levels of serum ALT, AST, ALP, creatinine, urea, potassium ions, cholesterol, triglycerides, cytokine IFNγ, NF-kb, DNA damage, tumor TNF-α, VEGF expressions and had an associated reduction in serum albumin, total proteins, sodium ions, tumor NF-kb, Bcl2 and survivin expressions. Treatment of EST with vitamin B17 (EST+VitB17) modulates the changes in liver and kidney functions, electrolytes, cytokines, NF-kb and apoptosis in mice bearing EST. Hence, these findings suggest that vitamin B17 can be a reliable and novel therapy for breast cancer, further validate the neoplastic activity of Vitamin B17 as a potential therapy for other types of cancer is needed.

**Keywords**: Ehrlich solid tumor, amygdalin (vitamin B17), TNF-α; DNA damage, NF-κB and apoptotic markers.

## INTRODUCTION

Breast cancer is the most common female cancer and is the principal cause of cancer-related female mortality in the world (Al-Rikabi and Husain, 2012). There are 1.38 million new diagnoses of breast cancer each year, resulting in 45,800 deaths annually. Breast cancer is not only the leading cause of death by cancer in woman but is also the fifth most common cause of death by cancer worldwide. Epidemiological evidence from the Saudi Cancer Registry suggests that breast cancer cases amongst Saudi women between 2001 and 2008 totalled 6,922 (DeSantis *et al*., 2014). Cases of breast cancer were the highest in the Eastern part of the Kingdom of Saudi Arabia, with 1 out of 100,000 women, suffering from this condition. This level was followed by Riyadh, where figs reached 20.5 out of 100,000 women, and by Makkah, where 19.4 out of 100,000 women experienced breast cancer (Bazarbashi *et al*., 2017).

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Chemotherapy is a cancer treatment regime that is unable to distinguish between the malignant and benign cells, the consequence of which can manifest into multiple side effects (Tousson *et al*., 2016, 2018; Al-Rasheed *et al*., 2017, 2018). Ehrlich carcinoma, undifferentiated carcinomas that are hyperdiploid, demonstrate no spontaneous regression, proliferate rapidly, are highly transplantable, possess 100% malignancy, have a truncated life span, and possess no tumor-specific transplantation antigen. The Ehrlich solid tumor (EST) model is one of several in vivo experimental models employed to investigate breast adenocarcinoma in laboratory animals (Badr *et al*., 2016; Eldaim *et al*., 2019; Aldubayan *et al*., 2019).

The use of complementary and alternative medicine has increased in recent decades (Moustafa *et al*., 2014; Barakat *et al*., 2015; El-Masry *et al*., 2017; Tousson *et al*., 2018b). Phytochemicals are natural compounds possessing anti-oxidant, anti-inflammatory and anti-tumor properties which can prove valuable in the treatment of

several diseases (Salama *et al*., 2015; Oyouni *et al*., 2018, 2019). Vitamin B17, otherwise known as Amygdalin or laetrile, was first obtained from apricot kernels by biochemist Ernst T Krebs Jr. Amygdalin. It is one of several natural nitrilosides from which cyanide can be extracted (Zhou *et al*., 2012; Bolarinwa *et al*., 2014). Amygdalin is known to possess multiple pharmaceutical properties including anti-oxidant, anti-inflammatory, antitussive, anti-asthmatic, and anti-ulcerative effects. Therefore; this study was designed to identify the curative and antineoplastic activity of vitamin B17 against growth of Ehrlish solid tumors bearing mice.

## MATERIALS AND METHODS

***Chemicals***

Vitamin B17 was purchased from the Amazon for Natural Oils Company.

***Induction of Ehrlich Solid Tumor (EST)***

The Egyptian National Cancer Institute (NCI; Cairo University, Egypt) supplied the mice which had been injected with Ehrlich ascites carcinoma (EAC). These were utilized as the source of EAC cells. 0.2 ml of acitic fluid was aspirated from each EAC bearing mice and diluted with physiological saline. Between 2.5 and 3 million EAC cells were injected beneath the skin on the left thigh of each mouse. The presence of the tumor was confirmed by scarfing a select number of mice and the Ehrlich solid tumor was exposed and its size was measured.

***Animals***

Sixty female CDI mice (aged between ten to twelve weeks old and weighing between 23-25 g each) were utilized for the experiments. They had been obtained from the breeding unit at the Egyptian Organization for Biological Products and Vaccines, Abbassia, Cairo. Mice were housed at $25^{o}\pm2^{o}$C, with the humidity maintained at 55%, wood chip bedding, on a 12:12 hour dark/light cycle, with free access to food and water. All animal procedures will be carried out in accordance with Ethical Committee of Faculty of Science at Tanta University guidelines and subject to approval by the Institutional Animal Care and Use Committee (IACUC-SCI-TU-0041).

***Animal Treatments***

Following a two-week acclimatization period, the mice were randomly divided into four groups.

Group 1 (G1): Control group, wherein mice were untreated.

Group 2 (G2): The mice in this group received Vitamin B17 (175 mg/Kg body weight/ day) (Sigma chemical Co, Germany), administered orally via a stomach tube for four weeks according to El-Masry *et al*. (2019).

Group 3 (G3): The group constituted the Ehrlich solid tumor (EST) group in which mice were injected with $2.5x10^{6}$ cells each to stimulate EST described previously in Aldubayan *et al*. (2019).

Group 4 (G4): The group constituted the treated Ehrlich solid tumor (EST) with Vitamin B17 (EST+VitB17). Mice were injected subcutaneously with 2.5 million cells of EAC per mouse and left for 2 weeks. After the development of solid tumor the mice were then treated with vitamin B17 for another 2 weeks.

***Blood Sampling***

By the end of the experiment, mice were euthanized with intraperitoneal injection with sodium pentobarbital and then underwent total necropsy. Blood samples from each mouse were obtained from the vena cava and gathered in non-heparinised glass tubes before being left for thirty minutes to clot at room temperature prior to being subject to a 5000 rpm centrifugal for ten minutes. Sera were separated and stored in aliquots at −80° C until required.

***Liver functions in sera***

Sera was analyzed to determine aspartate transaminase (AST) and alanine transaminase (ALT) activities according to El-Moghazy *et al*. (2014); albumin levels according to Basuony *et al*. (2015); alkaline phosphatase (ALP) activity according to Tousson *et al*. (2014)

***Kidney functions and electrolytes in sera***

Serum was analyzed to determine urea and creatinine concentrations according to the method described by Tousson *et al*. (2011) and Salama *et al*. (2013) respectively. Levels of potassium, sodium, calcium and chloride ions in the sera were assayed using commercial kits (Sensa core electrolyte, India) according to the methods of Abd Eldaim *et al*. (2019).

***Lipid profile in sera***

Serum was analyzed to determine cholesterol and triglyceride levels according to the method described by Salama *et al*. (2012) and Pundir and Narwal (2018) respectively.

***Determination of Interferon gamma (IFNγ) levels***

Interferon gamma (IFNγ) levels were determined according to the method of Farid *et al*. (2015) using an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA).

***Determination of NF-κB levels***

Nuclear factor kappa B (NF-κB) levels in serum were determined described previously in the method of Malaponte *et al*. (2015) using an ELISA kit (Active Motif, Rixensart, Belgium).

***Comet assay (determination of DNA damage)***

A comet assay (single cell gel electrophoresis) was employed to assess and quantify levels of tumor tissue DNA damage as demonstrated by Eldaim *et al*. (2019).

**Table 1**: Effect of vitamin B17 (VitB17) at 1g/ kg tumor volume on body weight EST bearing mice.

| Parameters | Experimental groups | |
|---|---|---|
| | Tumor volume in cm3 | % |
| EST | 1.92 ± 0.31 | ----- |
| EST+VitB17 | 0.57* ± 0.56 | 29.7 |

Values are expressed as means±SE; n=10 for each treatment group; * significantly different from EST.

**Table 2**: Changes in serum liver and kidney functions, electrolytes and lipid profiles in different groups.

| | Control | VitB17 | EST | EST+VitB17 |
|---|---|---|---|---|
| ALT (U/I) | 27.6#±1.05 | 23.2#±0.82 | 68.6*±1.67 | 35.4#±1.03 |
| AST (U/I) | 64.6#±3.60 | 58.5#±2.27 | 154.8*±7.33 | 77.1#±4.12 |
| ALP (U/I) | 150.0#±9.15 | 139.9#±10.25 | 284.1*±12.43 | 189.7*#±9.74 |
| Albumin (mg/dl) | 4.66#±0.56 | 5.13#±0.41 | 3.07*±0.30 | 3.92*#±0.36 |
| T. proteins (g/dl) | 6.29#±0.51 | 6.57#±0.48 | 5.16*±0.55 | 5.92*#±0.40 |
| Creatinine (mg/dl) | 0.43#±0.09 | 0.38#±0.08 | 1.12*±0.14 | 0.49#±0.12 |
| Urea (mg/dl) | 28.8#±1.27 | 25.3#±0.96 | 49.0*±1.72 | 30.1#±1.55 |
| Na ions (mEq/L) | 136.1#±8.5 | 135.7#±9.6 | 127.0*±9.5 | 132.5#*±8.9 |
| K ions (mEq/L) | 3.77#±0.19 | 4.06#±0.31 | 4.98*±0.52 | 4.15#±0.37 |
| Cholesterol (mg/dl) | 82.5#±2.61 | 80.2#±3.55 | 133.8*±5.25 | 101.0#*±5.38 |
| Triglycerides (mg/dl) | 80.3#±3.09 | 72.6#±2.88 | 114.1*±4.00 | 92.5*#±3.52 |

Values are expressed as means±SE; n=6 for each treatment group; (*) & (#) significant difference compared to control and to Ehrlich groups respectively.

**Table 3**: Comet assay parameters obtained by image analysis in cells of tumor in EST (a) and VitB17+EST (b) groups.

| Group | Tailed % | Untailed % | Tails length µm | Tail DNA% | Tail moment |
|---|---|---|---|---|---|
| EST | 18.50 | 71.50 | 5.43±0.17 | 3.36 | 14.88 |
| VitB17+EST | 7.29 | 92.71 | 2.98*±0.09 | 2.21 | 7.18 |

The data are presented as the mean ± SE; EST, Ehrlich solid tumour; EST+VitB17, tumour treated with vitamin B17. * significant difference relative to EST.

***Immunohistochemical investigations***

Expression of p53 proteins and anti-apoptotic Bcl2 proteins (Bcl2) and survivin, apoptotic VEGF and cytokines (NF-κB and TNF-α) in tumor sections were detected using avidin Biotin Complex (ABC) (Elite–ABC, Vector Laboratories, CA, USA). Sections were incubated with anti-rabbit Bcl-2 monoclonal antibody (dilution 1:80 and 1:2000; DAKO Japan Co, Ltd, Tokyo, Japan) according to Elmasry *et al*. (2018) and Tousson *et al*. (2011) surviving protein according to Bache *et al*. (2006), P28 rabbit monoclonal (Thero RM-9028) Vascular Endothelial Growth Factor (VEGF; RB-9031) according to van der Van der Loos *et al*. (2010) or rabbit polyclonal anti-NF-kB/p65 and according to Aldubayan *et al*. (2019).

## STATISTICAL ANALYSIS

Findings analysis was performed using the Statistical Package for the Social Sciences (SPSS software version 16) and data were analyzed using one-way ANOVA (Analysis of Variance) followed by Dunnett test and presented as the mean± standard error of mean (SEM). While Dunnett test comparisons ascertained the differences between groups, unpaired T-tests were employed to compare the significant differences. The standard for statistical significance was fixed at $p<0.05$.

## RESULTS

Effect of vitamin B17 on Ehrlich tumor volume: Following EST treatment with vitamin B17, there was a significant reduction in tumor size as compared to the untreated EST group (table 1).

***Changes in liver function levels***

A significant ($p<0.05$) elevation in levels of serum ALT, AST and ALP and a marked reduction in serum albumin and total proteins were detected in EST group in comparison with the control group (table 2). Conversely, when the Ehrlich solid tumor was treated with vitamin B17 (EST+VitB17), improved liver function was detected along with reductions in serum ALT, AST and ALP levels and increases in serum albumin and total proteins (table 2).

***Changes in kidney function and electrolytes levels***

A significant ($p<0.05$) elevation in the levels of serum creatinine, urea and potassium ions and marked reductions in the levels of serum sodium ions were detected in EST group compared with the control group (table 2). Conversely, reductions in levels of serum creatinine, urea and potassium ions and significant increases ($p<0.05$) in serum sodium ions were detected after the treatment of EST with vitamin B17 (EST+VitB17) (table 2).

***Changes in lipid profiles levels***

Table (2) revealed a significant ($p<0.05$) elevation in levels of serum cholesterol and triglycerides levels were detected in EST group. Significant ($p<0.05$) reductions in serum cholesterol and triglycerides levels after the treatment of EST with vitamin B17 (EST+VitB17) were observed.

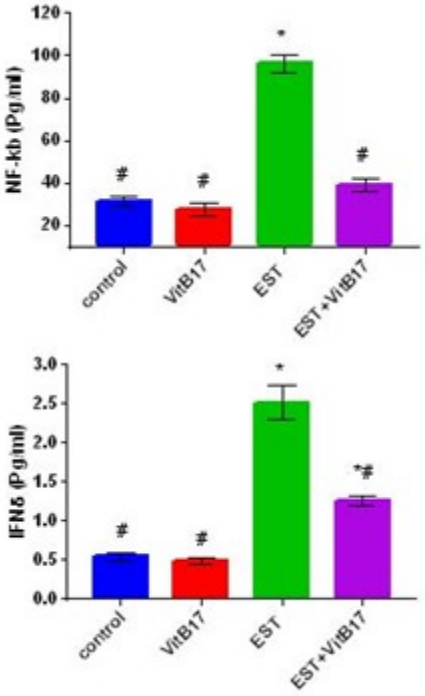


**Fig. 1**: Changes in serum NF-kb and IFNγ in different groups. (*) & (#) had significant differences when compared to the control and to Ehrlich groups respectively.

***Changes in serum NF-kb and IFNγ levels***

Elevation in the levels of serum NF-kb and IFNγ were indicated in EST, while significant ($p<0.05$) reductions in serum NF-kb and IFNγ were detected in EST+VitB17 as compared with EST (fig. 1).

***Changes in tumor DNA damage***

Fig. (2a) and Table (3) revealed that; elevation in DNA damage ($p<0.05$) in EST were detected as manifest in extended tail length, tail DNA and tail moment. In contrast; treatments of EST with vitamin B17 revealed significant ($p<0.05$) reductions in the level of DNA damage (fig. 2b).

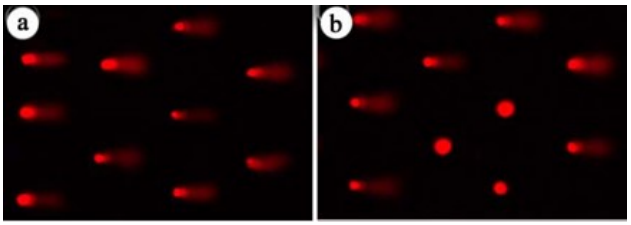


**Fig. 2**: Photomicrographic representation of DNA damage using a comet assay: DNA damage in EST. b: DNA damage in treated EST with VitB17.

***Changes Bcl2 expressions***

Faint to mild positive reactions for anti-apoptotic Bcl2 expressions were detected in tumor sections in EST (fig. 3a). In Contrast; tumor sections in EST+VitB17 revealed strong positive reaction for Bcl2 expressions (fig. 3b).

***Changes survivin expressions***

Fig. (3c) revealed mild positive reactions for survivin expressions in tumor sections in EST while; strong positive reactions for survivin expressions were observed in tumor sections in the EST+VitB17 group (fig. 3d).

***Changes in VEGF expressions***

Fig. (3e) revealed strong positive reactions for VEGF expressions in tumor sections of EST. In Contrast; tumor sections in EST+VitB17 revealed mild positive reaction for VEGF expressions (fig. 3f).

***Changes in nuclear factor-kappa β (NF-κB) expressions***

Tumor sections in EST group reveal mild positive reactions for mild to moderate positive responses for NF-kb expression (fig. 4a). In Contrast; tumor sections in ST+VitB17 revealed negative reactions for NF-kb expression (fig. 4b).

***Changes in TNFα expressions***

Fig. (4c) revealed mild positive reactions for TNFα in tumor sections in EST. In Contrast; tumor sections in EST+VitB17 revealed strong positive responses for TNFα expression (fig. 4d).

## DISCUSSION

Cancer is one of the leading causes of mortality worldwide. This study explores the therapeutic potential and antineoplastic activity of vitamin B17 in relation to ESTs in the mice in relation to liver and kidney functions, electrolytes, DNA damage, NF-Kb, TNFα and apoptosis. Vitamin B17 treatment revealed demonstrable decreases in tumor size and reduced speed of growth and fragmentation within remaining tumors. This indicates the anticancer properties inherent in vitamin B17. The findings support the conclusions already drawn by

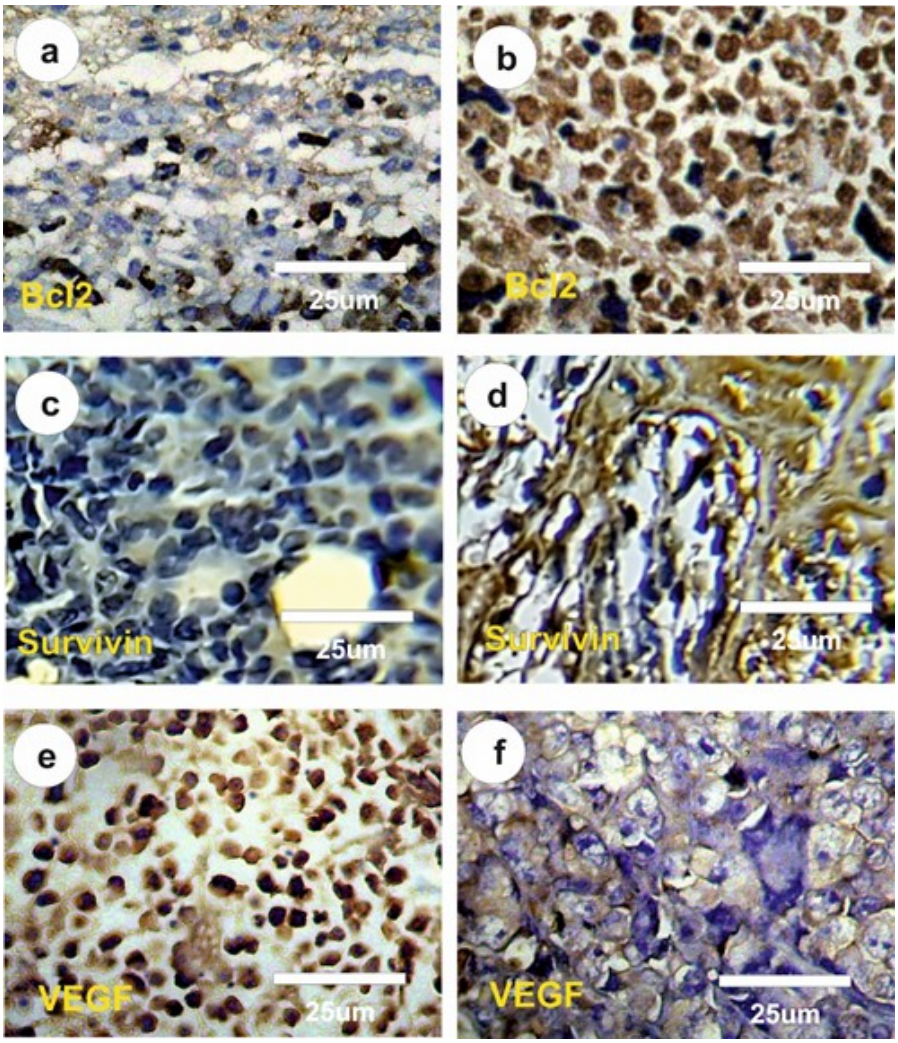


**Fig. 3a-3f**: Photomicrographs of tumor sections in EST and EST+VitB17 groups. a&b: Tumor section in EST and EST+VitB17 groups respectively stained with anti-apoptotic Bcl2. c&d: Tumor section in EST and EST+VitB17 groups respectively stained with anti-apoptotic survivin e&f: Tumor section in EST and EST+VitB17 groups respectively stained with VEGF (brown colour).

Makarević *et al*. (2016) who noted that reduced cell cycle and in-vitro growth in prostate cancer cells coincided with the presence of amygdalin. Likewise, studies by Juengel *et al*. (2016) have both reported a reduction in cells growth in bladder, cervical and lung cancer following treatment with amygdalin.

A significant elevation in the levels of ALT, AST, ALP, urea, creatinine, potassium ions, cholesterol and triglyceride were detected in EST, although there were decreased levels of serum albumin, total proteins and sodium ions in EST. Increased level of AST, ALT and ALP can be interpreted as a result from liver damage or alterations in membrane permeability, thereby indicating severe hepatocellular damage (Bedi *et al*., 2008; Saggu *et al*., 2014). These findings support the study of Gupta *et al*. (2004) who noted that increased liver transaminases in EAC bearing mice was linked to liver dysfunction. Similar results were found by Abou Zaid *et al*. (2011) and Aldubayan *et al*. (2019).

Serum urea and creatinine levels elevation in clinical experiments means renal dysfunction. Similarly, Abou Zaid *et al*. (2011), Eldaim *et al*. (2019) and Aldubayan *et al*. (2019) detected that EST induced mice resulted in an increase in urea and creatinine levels. Also; our results coincide with that of Erian *et al*. (2016) the study reported that EST induced mice saw increased levels in cholesterol and triglyceride. EST+VitB17 caused significant reduction of this elevation in liver enzymes (ALT, AST and ALP) and in kidney parameters (urea, creatinine, potassium ions) close to the normal values indicating the protective effect of vitamin B17 on the hepatic and renal function. The protective effect of vitamin B17 can be correlated to the anti- oxidative defense system as well as its scavenging and/or antioxidant properties (Abdel-

Rahman *et al*., 2011; Makarević *et al*., 2014; El-Masry *et al*., 2019; Mutar *et al*., 2019).

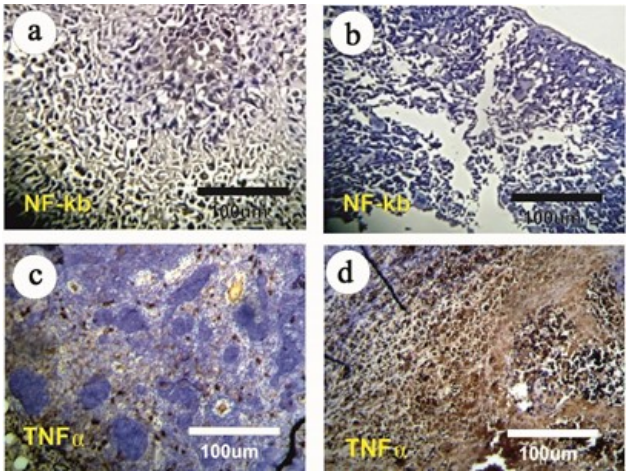


**Fig. 4a-4d**: Photomicrographs of tumour sections in EST and EST+VitB17 groups. a: Tumour sections in EST group stained with nuclear factor-kappa β (NF-κB). b: Tumor sections in EST+VitB17 group stained with nuclear factor-kappa β (NF-κB). c: Tumour sections in EST group stained with TNFα markers. d: Tumour sections in EST+VitB17 group stained with TNFα (brown colour).

Cancer patients frequently experience a syndrome known as cachexia, which results from the action of cytokines such as TNF-α, IFN-γ which are released by macrophages (Bachmann *et al*., 2008; Gueta *et al*. 2010). TNF (tumor necrosis factor) is a highly pleiotropic cytokine which plays a vital role in immune homeostasis, inflammation and host defense (Warren *et al*., 2009). In the current study; a significant elevation in plasma IFN-γ and NF-kb in the untreated EST mice group compared to the control group confirm the previous study of Aldubayan *et al*. (2019) and Abd El-Dayem *et al*. (2019) In addition, an increase in TNF-a expressions in tumor-bearing mice might be a result of increased ROS production by macrophages, which stimulates lipid peroxidation. However, the current study conflicts with the findings of Mansour *et al*. (2010) who noted that EST mice demonstrated reduced TNF-α and IL-10 concentrations compared to control group mice. There are many supporting evidence indicating the effect of vitamin B17. Treatment of EST with vitamin B17, has been reported by Mutar *et al*. (2019) and Abdel-Rahman *et al*., (2011); Makarevic *et al*. (2014) indicated that amygdalin inhibit NLRP3 and NF-kβ signalling pathways, which enhances its anti-inflammatory effect by decreasing proinflammatory cytokine (e.g. pro-IL-1β) expression. In addition, Liczbiński and Bukowska (2018) suggest that amygdalin acts upon the TGFβ/CTGF pathway, follistatin expression, anti-fibrous activity and promotion of muscle cell growth.

Luo *et al*. (2004) promotes tumor cell survival through gene encoding of the NF–kB dependent antiapoptotic molecules. Moreover, Wajant (2009) has stated that vitamin B17 can cause multiple effects which are crucial for the development and progress of tumours and are paramount in tumour immune response measuring.

El-Masry *et al*. (2017) and El Barbary *et al*. (2011) reported that apoptosis is essential to the regulation of numerous diseases. Thus, Tousson *et al*. (2014) contends that apoptosis plays a key role in tumor progression since it causes tumor cell death during chemotherapy, immunotherapy and radiation therapy. The Bcl-2 family comprises of antiapoptotic and pro-apoptotic, two groups which play a key role in cellular death and DNA damage (Raafat *et al*., 2011), thereby suggesting that irregularity amongst anti-apoptotic Bcl-2 family members is a significant identifier of cancer, rather than normal cells. In the current study, Bcl2 and survivin expressions significantly increased whereas VEGF expressions fell in tumor sections in EST and the treatment of EST with VitB17 modulates this result. Weyhenmeyer *et al*. (2012) have stated that Bcl-2 proteins are significant in relation to cancer cell resistance during treatment, thereby suggesting innovative treatment possibilities.

These finding are supported by the work of Zhou *et al*. (2012) who noted the impact of amygdalin on the proliferation and apoptosis of HepG2 cells when activated by β-D-glucosidase. Likewise, Chen *et al*. (2013) notes that cervical cancer cell apoptosis can be prompted by treatment with amygdalin. Amygdalin-B17 can be used therapeutically as an infusion and in natural chemotherapies.

## CONCLUSION

Administration of vitamin B17 in EST bearing mice will kill the cancer cells through apoptosis which thereby regulates the proliferation of cancer cells and inhibits its spread to other organs. Thus, vitamin B17 is a therapy has been adapted to conform to human metabolic regulation; it has the potential to constitute a natural chemotherapy.

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## CONCLUSION

This study discovered the curative role of vitamin B17 (amygdalin) that can be beneficial for treatment of Ehrlish

solid tumors bearing mice induced changes in liver and kidney functions, electrolytes alterations, DNA damage, NF-Kb and TNFα alterations and apoptosis. This study will help the researchers to uncover the critical areas of cancer that many researchers were not able to explore. Thus a new theory on treatments may be concluded.

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