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The therapeutic and antineoplastic effects of vitamin B17 against the growth of solid-form Ehrlich tumours and the associated changes in oxidative stress, DNA damage, apoptosis and proliferation in mice

Thanaa A. El-Masry[1,4], Nora H. Al-Shaalan[2], Ehab Tousson[3]*, Manal Buabeid[5] and Anhar M. Alyousef[6]

[1]Pharmaceutical Science Department, College of Pharmacy, Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia
[2]Chemistry Department, College of Science, Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia
[3]Department of Zoology, Faculty of Science, Tanta University, Tanta, Egypt
[4]Departement of Pharmacology and Toxicology, Faculty of Pharmacy, Tanta University, Tanta, Egypt
[5]College of Pharmacy and Health Science, Ajman University, UAE
[6]Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia

Abstract: Many cancer therapies indirectly activate apoptosis by chemical or physical damage of DNA. This study was performed to evaluate protective potential of vitamin B17 (VitB17) against Ehrlich solid tumor (EST) induced changes in the oxidative stress, DNA damage, apoptosis and proliferation in mice. In the experiment, 60 female CD1 mice were randomly and allocated to the following four equal-sized groups [G1, negative control; G2, positive control (VitB17); G3, untreated EST; G4, EST treated with VitB17 (EST+VitB17)]. The untreated EST group displayed major increases in tumor volume, significant increase in the levels of MDA, H_2O_2, NO, PCNA, TNF-α, AFP and dsDNA and notable reductions in the catalase, GSH, P53 and SOD activities. By contrast, reduced levels of TNF-α, AFP, MDA, H_2O_2, NO, PCNA and dsDNA, along with enhanced levels of P53 and the antioxidant indicators catalase, GSH and SOD were observed in the EST+VitB17 group. These results indicate the antineoplastic and antioxidant properties of vitamin B17 with the potential to decrease the oxidative stress associated with ESTs by augmenting the antioxidant defence system.

Keywords: Ehrlich solid tumour, vitamin B17, cytokines, oxidative stress, DNA damage proliferation and apoptotic markers.

INTRODUCTION

Cancer is the most awful disease found among people and the largest single cause of death in human. Most of cancer therapies are chemotherapy and radiation that kill cancer cells by inducing apoptosis however; they can adversely affect the life of patients and represent a direct cause of death (Basuony *et al*., 2015; Al-Rasheed *et al*., 2017, 2018; Tousson *et al*., 2014, 2016, 2018a). Badr *et al*., (2016) and Aldubayan *et al*. (2019) describe Ehrlich carcinoma as an undifferentiated carcinoma typified by non-regression, high transplantable capability, fast proliferation and 100% malignancy along with an extended survival time and the absence of a tumor specific transplantation antigen. According to DeSantis *et al*. (2014), the antineoplastic activity of various chemical compounds in breast cancer (the most prevalent form of cancer in women and the second most prevalent form worldwide) is frequently examined using the Ehrlich solid tumor (EST) as a transplantable tumor model.

The use of complementary and alternative medicine has been a marked increase in recent decades (Tousson *et al*., 2018b; Elmasry *et al*., 2018; Abd Eldaim *et al*., 2019). Many plants products have significant antioxidant activities, which play important roles in cancer treatment. Studies by Milazzo *et al*. (2007) and Santos *et al*. (2014) have focused on vitamin B17 (also known as laetrile or amygdalin), a member of the extensive group of natural cyanide-containing substances (nitrilosides) which was first obtained from apricot kernels by the biochemist Ernst T Krebs Jr. Amygdalin has found use in traditional Chinese medicine for treating a wide range of illnesses, including asthma, bronchitis, colorectal cancer, emphysema and leprosy Milazzo *et al*., 2015). In a study on rats, Firdaws *et al*. (2018) reported that amygdalin is associated with a fatal dose (LD50) of 880 mg/kg body weight when given orally, and 25g/kg when given intravenously, due to the potential for cyanide poisoning. The vitamin B17 or amygdalin presents various biological qualities, being effective against inflammation, antiatherogenic, antioxidant, antitussive and anti-ulcer effects (Lee and Moon, 2016; Qadir and Fatima, 2017; Liczbiński and Bukowska, 2018). The present study was therefore designed to examine the therapeutic and antineoplastic effects of vitamin B17 against the growth of ESTs and the associated changes in oxidative stress, DNA damage, apoptosis and proliferation in mice.

**Corresponding author:* e-mail: ehabtousson@science.tanta.edu.eg

MATERIALS AND METHODS

Chemicals

Vitamin B17 (VitB17) purchased from Sigma Aldrich, Germany was administered in a dose of 175 mg/kg body weight, in accordance with Firdaws *et al*. (2018).

Animals

Sixty female CD1 mice aged 10-12 weeks and weighing 20±2g were supplied by the breeding unit of Egyptian Organization for Biological Products and Vaccines (EOBPV), Abbassia, Cairo. All animals were allowed free access to a standard diet and water and all experiments were conducted according to the guidelines for animal studies published by the Ethical Committee of Faculty of Science, Tanta University with the approval of the Institutional Animal Care and Use Committee (IACUC-SCI-TU-0041).

EST Induction

Ehrlich ascites carcinoma (EAC) cells were sourced from EAC-bearing mice supplied by the Egyptian National Cancer Institute (NCI), Cairo University, Egypt. The EAC cells were counted following aspiration of ascitic fluid (0.2 ml) from the EAC mice and dilution in physiological saline solution. To induce the solid tumour in each of the experimental mice, approximately 2.5–3 million EAC cells were subcutaneously injected into the lower limb left thigh. To ensure the induction of EST a sample of mice were scarified, and the presence of the tumor was confirmed.

Treatment

After two weeks of acclimatisation, the mice were randomly divided among the following four equal-sized groups (n = 15):

G1: Negative control – untreated mice.

G2: VitB17; mice in this group received VitB17 (175 mg/kg body weight/day) by oral administration (stomach tube) for four weeks (Firdaws *et al*., 2018).

G3: EST; mice were injected subcutaneously with 2.5 million cells of EAC per mouse diluted in physiological saline to initiate tumor Ehrlich solid tumor (EST) according to Aldubayan *et al*. (2019).

G4: (EST+VitB17); mice were injected subcutaneously with 2.5million cells of EAC per mouse diluted in physiological saline to initiate EST and left for 2 weeks and then treated with vitamin B17 (175mg/Kg body weight/ day) for another 2 weeks.

Blood Sampling

At the end of the experiment, the mice were fasted for 10 –12 hours, and then anaesthetised intraperitoneal injection of sodium pentobarbital prior to full necropsy. Samples of blood taken from the inferior vena cava of each mouse were allowed to clot at room temperature for 30 mins in non-heparinised glass tubes prior to centrifugation for 10 min at 5000 rpm. The sera were separated into aliquots and held at –80° C until required for assay, at which time they were thawed at room temperature.

Evaluation of alpha fetoprotein tumour marker

Alpha-fetoprotein (AFP) was evaluated via automated using the mini-VIDAS® AFP quantitative enzyme-linked fluorescent assay (ELFA) system (Biomerieux, Marcy-L'Etoile, France).

Evaluating the level of tumour necrosis factor alpha (TNF-α)

TNF-α-specific monoclonal antibodies were used to conduct a quantitative sandwich enzyme immunoassay according to the manufacturer's protocol (R&D systems, Minneapolis, USA).

Evaluating the level of anti-double stranded DNA (anti-dsDNA)

The level of dsDNA autoantibodies was quantified by plasma enzyme immunoassay in accordance with the kit supplier's protocol (Demeditec Diagnostics, Kiel, Germany).

Tissue Sampling and preparation

Solid tumor tissues were removed, cleaned in cold saline, weighed and measured to obtain the mass and volume developed. Tumours were divided into four parts, wrapped in aluminium foil and stored at −80°C until needed for preparing tissue homogenates. Following necropsy, a Potter-Elvehjem-type homogeniser was used to attain 10% w/v homogenisation of the tumour tissues in ice-cold KCl solution (1.15%) and sodium potassium phosphate buffer (0.01mol/l, pH7.4). Following centrifugation of the homogenate for 20 min at 4°C and 10,000g, enzyme assays were performed using the supernatant.

Evaluating biomarkers of Ehrlich tumour oxidative stress

To determine the malondialdehyde (MDA) component, a noxious product of lipid peroxidation in the tumor homogenate was performed as the protocol described by Basuony *et al*. (2015), while the nitric oxide (NO) activity in the tumor homogenate was performed according to Beltagy *et al* (2016). Super oxide dismutase enzyme activity (SOD; EC 1.15.1.1), reduced glutathione (GSH) content and catalase (CAT; EC 1.11.1.6) enzyme activity were measured after the protocols of Saggu *et al*. (2014), while the hydrogen peroxide (H_2O_2) was performed as the protocol described by El-Masry *et al*. (2017).

Histopathological studies

Ehrlich solid tumours removed immediately following necropsy were fixed in 10% neutral buffered formalin. Following the protocol of Tousson (2016), fixed tumour from each groups were stained by standard haematoxylin and eosin counterstain techniques.

Immunohistochemical evaluation of PCNA and P53 immunoreactivites

The avidin Biotin Complex (ABC) approach was used in accordance with Tousson *et al*. (2011) to evaluate the immunoreactivites of PCNA (PCNA-ir) and accordance with Tousson *et al*. (2014) to evaluate P53 (P53-ir) in the sections.

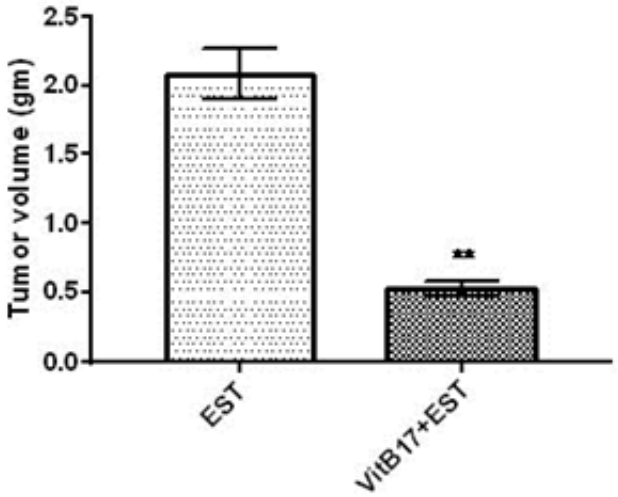

Fig. 1: The effect of VitB17 on the EST volume in mice. Data are presented as the mean ± SEM. **P value = 0.0028 according to the two-tailed test. The level of significance was analysed by the unpaired (two-sample) T-test, with statistical significance indicated by $p<0.01$. The unpaired T-test was significant from the corresponding Ehrlich at $^{NS}P = 0.1234$; *P=0.0332; **P=0.0021; ***P=0.0002; ****P<0.0001.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS) software version 16 was used to analyse the results. The data were stated as the mean ± standard error of mean (SEM) and subjected to statistical analysis via one-way ANOVA (Analysis of Variance). This was followed by the Dunnett test to compare the results between the experimental groups. The significance of these differences was examined by the unpaired T-test, with $p<0.01$ being the condition for statistical significance.

RESULTS

Effect of vitamin B17 on Ehrlich tumour volume

The influence of vitamin B17 treatment on the growth and proliferation of Ehrlich cells is determined by comparing the growth-dependent change in tumour volume for the various experimental groups 14 days after subcutaneous injection of the cells. A significant decrease in tumour volume was detected after the treatment of EST with vitamin B17 as compared with the tumour volume of EST group (fig. 1).

Effect of vitamin B17 on AFP markers

Fig. 2(a) indicates that, while the levels of serum alpha fetoprotein (AFP) are significantly increased in the EST as compared to control and vitamin B17 groups, the AFP levels are significantly reduced in the EST+VitB17 group relative to the untreated EST group.

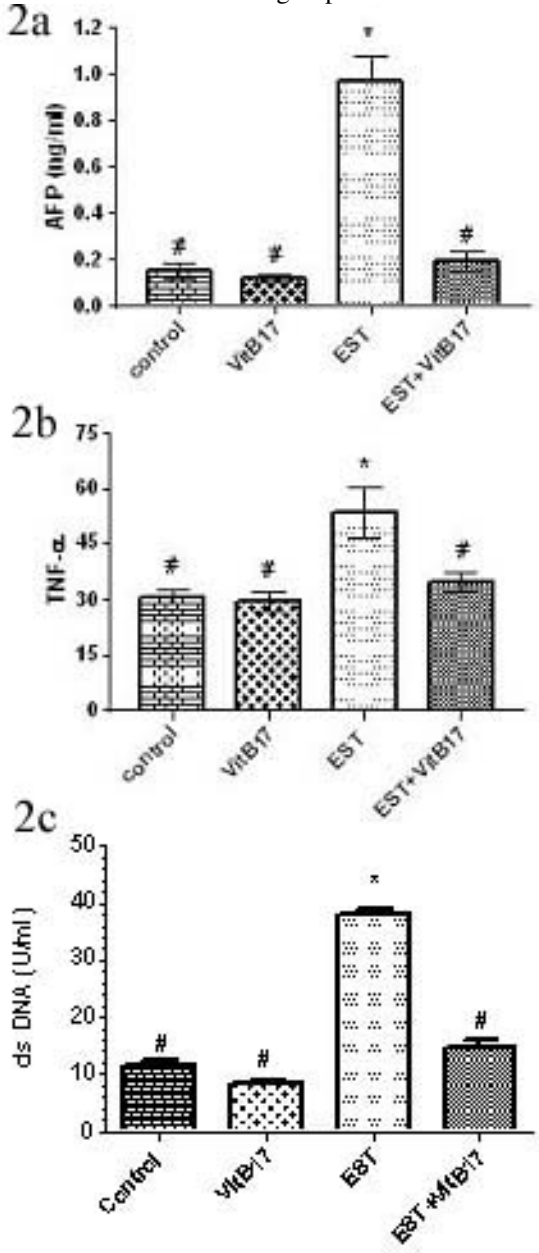

Fig. 2: Variations in the levels of serum AFP, TNF-α and ds DNA in the various experimental groups. 2(a): Serum AFP, 2(b): Serum TNF-α, 2(c): Serum ds DNA. * and $^{\#}$: statistically significant difference relative to the control and untreated EST group, respectively.

Effects of vitamin B17 on TNF-α

Fig. 2b also indicates significantly raised plasma levels of tumour necrosis factor (TNF-α) in the EST as compared to control and vitamin B17 groups, contrasted with

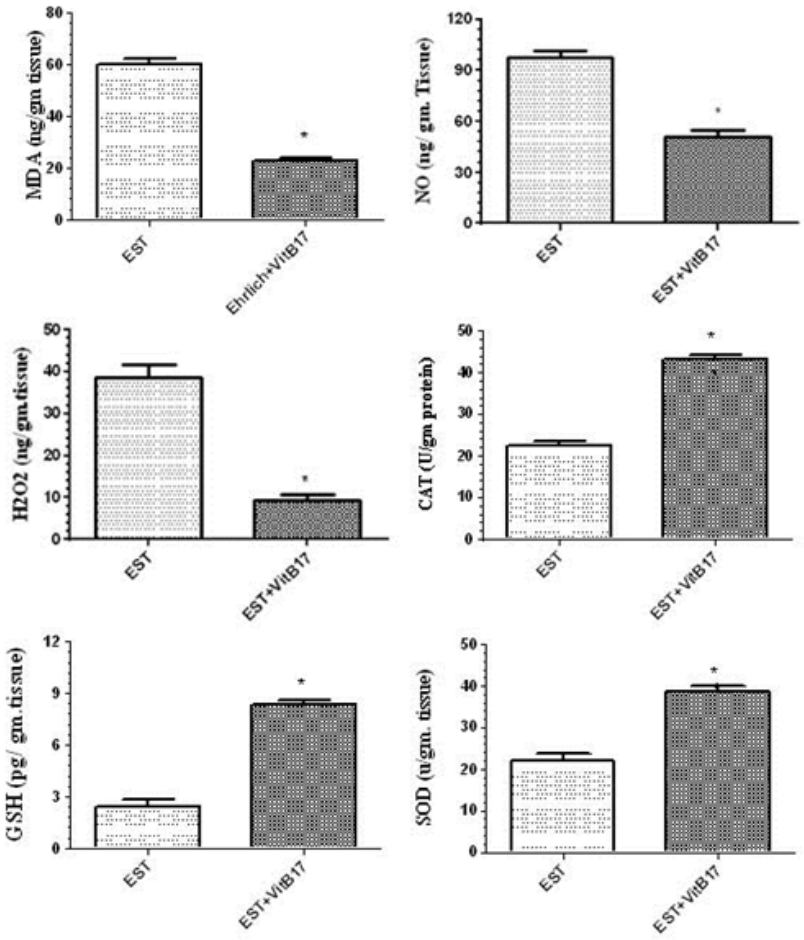

Fig. 3: Variations in the levels of MDA, H_2O_2, NO, GSH, CAT and SOD in the tumour tissues between the untreated EST and the EST+VitB17 groups. The data are presented as the mean ± SE; EST, Ehrlich solid tumour; EST+VitB17, tumour treated with vitamin B17. *Significant difference relative to EST.

significantly reduced levels of TNF-α in the EST+VitB17 group relative to the untreated EST group.

Effects of vitamin B17 on ds-DNA

Fig. 2(c) shows significantly increased in the levels of ds-DNA in the EST group as compared to control and VitB17 groups, but significantly reduced levels of ds-DNA for the EST+VitB17 group relative to the untreated EST group.

Effects of vitamin B17 upon markers of oxidative stress

Fig. 3 indicate significantly increased in the levels of MDA, H_2O_2 and NO, and significantly decreased in the levels of GSH, CAT and SOD in tumour tissue in the untreated EST group, whereas the EST+VitB17 group displayed significantly decreased levels of MDA, H_2O_2 and NO and significantly increased in the levels of GSH, CAT and SOD as compared to the untreated EST group.

Effect of Vitamin B17 on the histopathology of ESTs

Tumor sections in EST group revealed the spread of compact and aggregated tumour tissue cells within the muscle tissues (fig. 4a), the tumor cells are pleomorphic (varying in size and shape and staining characteristics) with large round and polygonal cells along with raised chromatophilic cells indicating proliferation around areas of necrosis and differentiated cells (fig. 4b). Tumor sections in EST+VitB17 group wide zones of apoptotic cells and significant recession of tumour growth (figs. 4c and 4d).

Detection of PCNA protein expression

A fig. 5 indicates the expression of PCNA in the various experimental groups. Tumour section in EST group shows strong positive reaction for PCNA expression (figs. 5A&5B), while a mild positive reactions for PCNA expression were detected in EST+VitB17 (figs. 5C&5D).

Detection of P53 protein expression

A fig. 6 indicates the expression of P53 in the various experimental groups. Tumour section in EST group shows strong positive reaction (figs. 6A & 6B), while a mild positive reactions for P53 expression were detected in EST+VitB17 (figs. 6C & 6D).

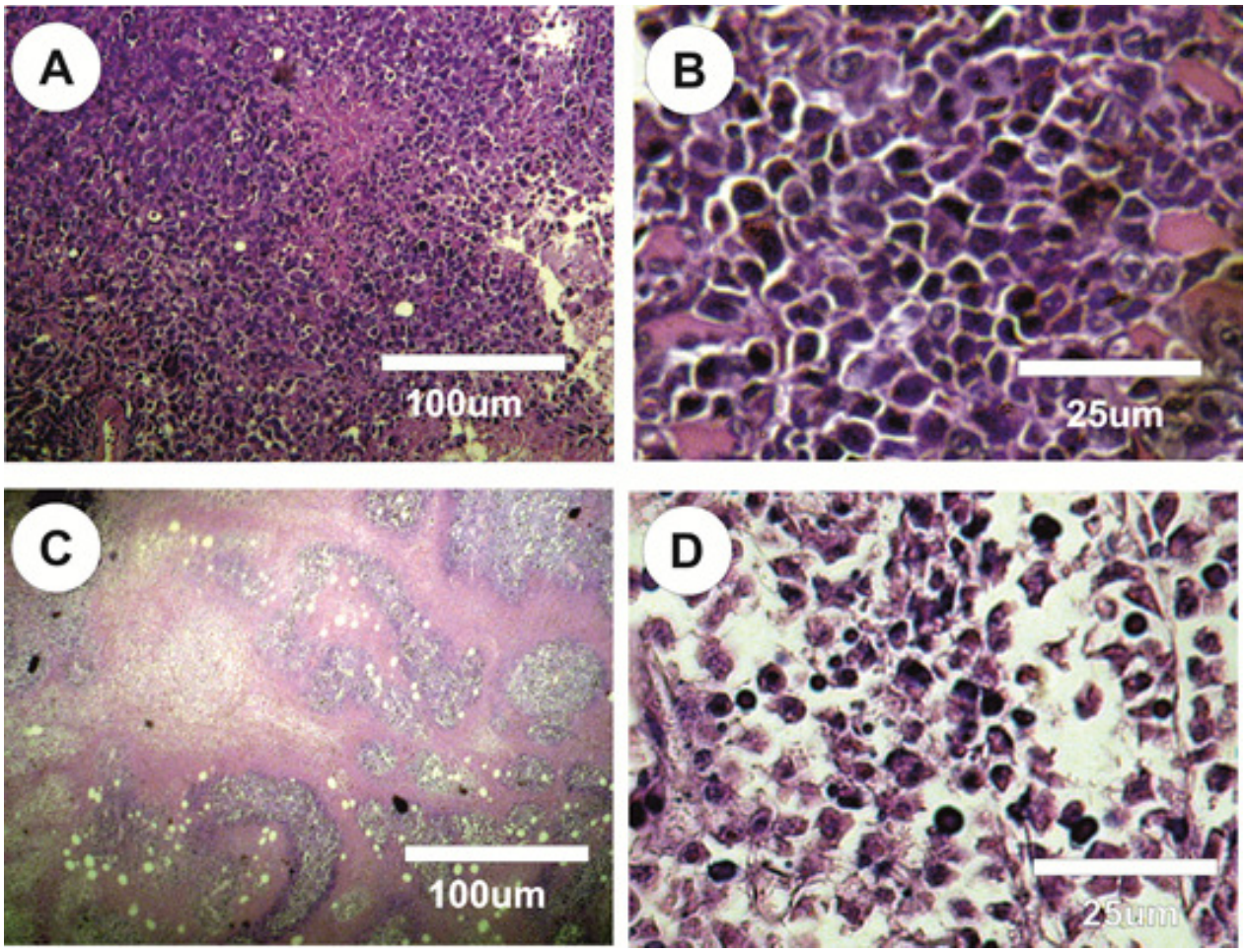

Fig. 4: Photomicrographs of tumour sections stained with haematoxylin and eosin – a) and b) untreated EST; c) and d) EST treated with vitamin B17.

DISCUSSION

Cancer is one of the leading causes of mortality worldwide, according to WHO, more than 10 million new cases of cancer are diagnosed every year, and the statistical trends indicate that this number would double by 2020. According to Aldubayan *et al*. (2019), the Ehrlich tumour first arose spontaneously in the form of breast cancer in mice and has since become the most frequently used transplantable experimental cancer model. In the present study, carcinogenesis was induced in mice by injection of Ehrlich solid tumour (EST) cells in order to examine the possible defensive and curative properties of vitamin B17.

Following treatment of the EST with vitamin B17, a significant decrease in tumour size was noted. Moreover, the slow growing, discontinuous and fragmented appearance of the treated tumour, along with the decrease in tumour weight and volume demonstrated the antitumor activity of vitamin B17 against EST in the mice. These results support the conclusions of Makarević *et al*. (2016) who observed delayed progression of the cell cycle and curtailed *in-vitro* growth of prostate cancer cells in the presence of amygdalin. Similarly, treatment with amygdalin was shown to block invasion by non-small-cell lung carcinoma (NSCLC) in an in-vitro study by Qian *et al*. (2015). Moreover, similar reductions in the growth of bladder, cervical and lung cancer cells have been reported by Makarević *et al*. (2016) and Juengel *et al*. (2016) following VB17 treatment.

According to Gueta *et al*. (2010), cancer patients frequently develop cachexia, a syndrome arising from the effects of cytokines (e.g. TNF-α, IFN-γ) released by macrophages and typified by progressive loss of fat and muscle mass along with symptoms of anaemia, anorexia and extreme weakness. In particular, Warren *et al*. (2009) cite the exceptionally pleiotropic cytokine TNF (tumour necrosis factor) as playing a key part in the host's immune defence, inflammation and homeostasis. The present results revealed notably enhanced levels of plasma TNF-α in the untreated EST group relative to the control group, in complete agreement with a previous study by Aldubayan *et al*. (2019). This result may be ascribed to the enhanced macrophage production of reactive oxygen

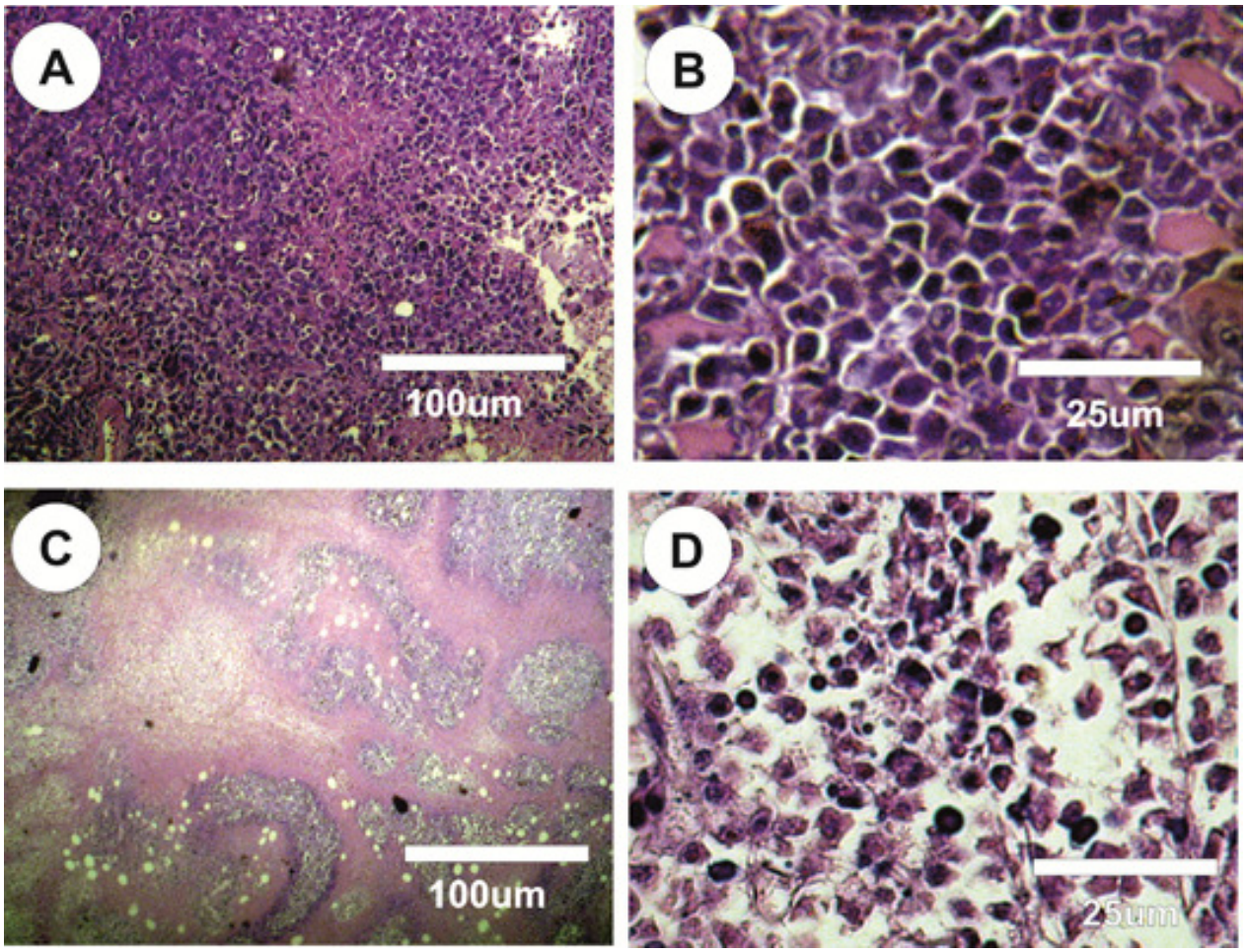

Fig. 4: Photomicrographs of tumour sections stained with haematoxylin and eosin – a) and b) untreated EST; c) and d) EST treated with vitamin B17.

species which, according to Hoek and Pastorino (2002), promote lipid peroxidation and are believed to be important contributors to the pathogenesis of liver damage. Similar increases in the levels of TNF-α have also been previously observed by Abd El-Dayem *et al*. (2012) in female mice with Ehrlich ascites carcinoma. However, the present results do not concur with those of Mansour *et al*. (2010), who reported significantly reduced plasma levels of TNF-α and IL-10 in EST bearing mice.

In the present study, the increase in the levels of TNF-α in EST was reversed following vitamin B17 treatment. In view of previous work by Abboud *et al*. (2018), this may be attributed to the greater effectiveness of vitamin B17 as a free radical scavenger. A previous study by Makarević *et al*. (2014) demonstrated inhibition of the NF-kβ signalling pathways in the presence of amygdalin, which produces an anti-inflammatory effect by decreasing proinflammatory cytokine (e.g. pro-IL-1β) expression.

Tumour markers (α-fetoprotein; AFP), are molecules present in the blood which show enhanced levels in individuals with certain types of cancer. The present study revealed significantly increased in the levels of serum AFP in the EST group relative to the control, this outcome being ameliorated for the vitamin B17 treated EST group. According to Bruce *et al*. (2008), the enhanced levels of serum AFP in mice with EST may be indicative of the inflammatory response. The present results support those of Aldubayan *et al*. (2019), who also observed notably raised levels of AFP in mice with EST relative to a control group.

In the present study, cytotoxicity was evaluated in terms of growth inhibition and dsDNA damage. The extent of dsDNA level was considerably increased in the presence of EST and considerably reduced following treatment of the tumour with vitamin B17. This is supported by Zhu *et al*. (2014), who observed significantly increased in the levels of dsDNA damage in the presence of Ehrlich ascites carcinoma.

According to Rafat *et al*. (2011), oxidative stress has a key influence on the progression of numerous health conditions such as cardiovascular diseases and obesity,

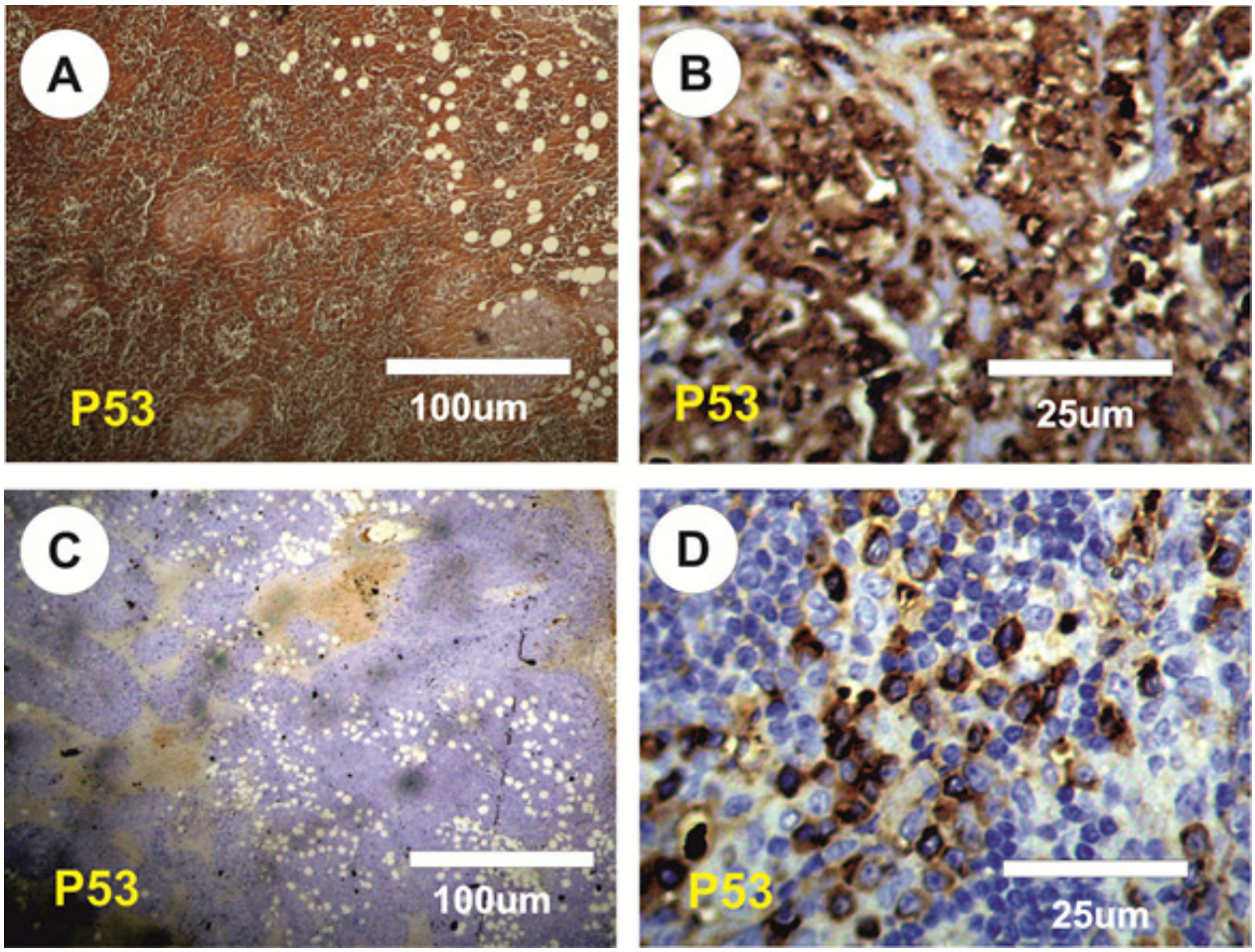

Fig. 6: Photomicrographs of tumour sections stained with apoptotic P53 markers. – a) and b) EST sections showing strong faint positive reaction; c) and d) vitamin B17 treated EST showing mild reaction (brown colour).

and has been identified among the primary factors in the onset of cancer. The current study revealed positive links between the spread of EST cells, apoptosis and alterations in antioxidant mechanisms. In detail, considerably raised levels of MDA, H_2O_2 and NO, along with reduced levels of catalase, GSH and SOD, were noted in the tumour tissues from the EST group. This is in line with a previous study by Abd El-Aziz *et al*. (2014), who also detected significantly raised levels of malondialdehyde (MDA), along with significantly reduced levels of catalase and SOD in the presence of Ehrlich tumour. The decreased GSH levels in the mice with in tumours might be ascribed to an enhanced rate of oxidation and consumption of GSH in the process of hydrogen peroxide removal. Moreover, since Kalaiselvi *et al*. (2013) cite SOD as a prime player in the defence against reactive oxygen species, the reduction in SOD activity in the presence of EST relative to the control group could be linked to the increase in circulating lipid peroxides produced by metabolic activation. The present study demonstrated that; vitamin B17 was effective in controlling antioxidant enzyme activities by raising the levels of catalase GSH and SOD, and decreasing the levels of MDA, H_2O_2 and NO, which suggests that vitamin B17 extract has free-radical-scavenging and antioxidant properties.

Depending upon the cellular context, vitamin B17 extract can trigger a range of phenomena which Wajant (2009) cites as central to the processes of tumour development and progression and as highly significant in monitoring the tumour immune response. These phenomena include angiogenesis, cell migration, differentiation, immune cell activation, apoptosis and necrosis. In particular, cell proliferation and apoptosis are significant aspects of carcinogenesis, with tumour growth depending upon the balance between these two opposing phenomena. Numerous studies, including Eldaim *et al*. (2019), El-Masry *et al*. (2017) and El Barbary *et al*. (2011), have indicated the central role of apoptosis in controlling a range of socially significant diseases. For instance, Eldaim *et al*. (2019) and Tousson *et al*. (2018a) noted the key influence of apoptosis upon tumour progression, citing it as the cause of tumour cell death during chemotherapy, immunotherapy and radiation therapy.

The assessment of cell proliferation is among the primary indicators of prognosis in cancer cases. According to Tousson *et al*. (2011), this can be clinically performed in part by the labelling of cells in S-phase or counting mitotic cells with histopathological grading. The notable increase in PCNA, and the notable reduction in P53, observed in the presence of ESTs in the present study is in line with that of Aldubayan *et al*. (2019) who also noted significantly raised levels of P53, PCNA and KI67 in the presence of Ehrlich tumour. The present results are also supported by Zhou *et al*. (2012), who reported the influence of amygdalin upon the proliferation and apoptosis of HepG2 cells when activated by β-D-glucosidase. Similarly, Chen *et al*. (2013) reported the triggering of human cervical cancer cell line apoptosis by amygdalin. Studies by Makarević *et al*. (2016) and Juengel *et al*. (2016) have indicated reduced development and migration of kidney, liver, lung and prostate carcinoma cell lines, and controlled apoptosis in cervical tumour and triple negative breast cancer cells, in the presence of vitamin B17. The present results also support the work of Lee and Moon (2016), who observed the control of apoptosis and cell adhesion by amygdalin in Hs578T triple-negative breast cancer cells.

CONCLUSION

The present study demonstrated reduced levels of MDA, H_2O_2 and NO, amelioration of AFP, PCNA, TNF-α and DNA damage, and enhanced antioxidant indicators (catalase, GSH and SOD) in the EST group treated with vitamin B17. These results indicate the antineoplastic and antioxidant properties of Vitamin B17 which reduces oxidative stress in the presence of ESTs by augmenting the antioxidant defence pathway.

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