

Immunostimulating, proapoptotic and synergistic effects of propolis and its main constituent (chrysin) on the tumor growth and the cell sensitivity to ionizing radiation in mice

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ABSTRACT

The increased interest in new approaches to the immunotherapy of cancer and a considerable demand for therapeutic agents which can modulate the several forms of immunodeficiency have encouraged studies on the immunomodulatory mechanisms of natural and synthetic substances. The present work was an endeavor to evaluate the immunostimulating as well as proapoptotic and synergistic effects of Chrysin (Ch) and Propolis (P) on the tumor growth and the cell sensitivity to ionizing radiation targeting the improvement of cancer therapeutic protocols. Chrysin (20 mg/kg body weight/day) and Propolis (100 mg/kg body weight day) were injected intraperitoneal to mice bearing 1cm³ solid tumor of Ehrlich ascites carcinoma (EAC) for 21 consecutive days. Mice were whole body exposed to 1 Gy of gamma radiation (two fractionated doses at 11 and 25 days post EAC inoculation, respectively). Treatments with Ch or P markedly suppress the proliferation of tumor in mice bearing solid Ehrlich tumor (EAC mice). The activity of interferon-gamma (IFN- γ) was significantly decreased in the treated mice compared to EAC mice. The concentrations of m-RNA for angiogenic factor (tumor necrosis factor alpha -TNF- α), free radicals as well as nitric oxide (NO) concentration were significantly decreased collimated with improvements in apoptotic regulators (Caspase-3 activity) compared to EAC mice.

Moreover, the histopathological investigation confirms the improvement exerted by Ch or P even in EAC mice group or mice bearing solid Ehrlich tumor and subjected to γ -irradiation (EAC+R group). Exposure to gamma radiation sustained the modulatory effect of Ch or P on tumor when compared with EAC mice and those injected with Ch (EAC+Ch) or EAC and injected with P (EAC+P). Hence, Ch or P might represent a potential therapeutic strategy for increasing the radiation response of solid tumor.

Keywords: Chrysin, Propolis, gamma radiation, TNF- α , IFN- γ , apoptosis, Caspase-3, histopathology.

INTRODUCTION

Cancer is considered one of the most common causes of morbidity and mortality worldwide. The target of much research has been on the discovery of natural and synthetic compounds that can be used in the prevention and/or treatment of cancer. Natural products of either plant or animal origin that exhibited antitumor activity have been discovered (Pezzuto, 1997). The increased interest in new approaches to the immunotherapy of cancer, and a considerable demand for therapeutic agents which can modulate the several forms of immunodeficiency have encouraged studies on the immunomodulatory mechanisms of natural

and synthetic substances (Mirandola *et al.*, 2002).

Propolis (P) or bee glue is the generic name for the resinous substance collected by honey bees from various plant sources and used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance against intruders. It is rich in biochemical constituents, including mostly a mixture of polyphenols, flavonoids, phenolic acid and their esters, and phenolicaldehydes and ketones, terpenes, sterols, vitamins, amino acids, etc. It was also demonstrated that P and some of its active substances have a pronounced cytostatic, anticarcinogenic and antitumor effect both

“in vitro” and “in vivo” tumor models (Attia *et al.*, 2007). It has been suggested that the therapeutic activities of P depend mainly on the presence of flavonoids. Flavonoids have also been reported to induce the immune system, and to act as strong oxygen radical scavengers (Wleklik *et al.*, 1997). Chrysin (Ch) is a member of the flavonoid family (5,7-dihydroxyflavone) that is found in honey, propolis and many other plants. Flavonoids have a broad spectrum of biological activities such as antitumor, immunomodulatory, anti-inflammatory, anti-allergy, antioxidant and cardioprotective effects. Chrysin is composed of a large class of polyphenolic compounds present in the diet and many herbal products, which have long been associated with a variety of important biochemical and pharmacological activities in the cancer prevention and health promotion (Meng *et al.*, 2017).

In an attempt to improve cancer therapeutic protocols, this study was undertaken to evaluate the antitumor effect of P and Ch together with γ -irradiation against solid EC tumors in female mice. Different molecular targets were analyzed in order to explore the immune modulation and the suppression effect of P and Ch on the growth of solid tumor. Also, the influence of administration of EAC mice to different polyphenolic compounds deriving from P such as Ch and of P itself has been investigated on Ehrlich ascites tumor growth. Therefore, tumor growth and parameters of immunomodulatory response of Ehrlich solid tumor bearing mice have been analysed.

MATERIALS AND METHODS

Chemicals

Propolis, Chrysin and other chemicals and reagents, used in this study were obtained from Sigma-Aldrich Chemical Co. (Gillingham, UK). Chrysin was dissolved in DMSO and injected intraperitoneal (i.p) at a dose of 20 mg/kg body weight for 21 consecutive days.

Propolis was dissolved in ethanol and injected i.p at a dose of 100 mg/kg body weight for 21 consecutive days.

Radiation Facility

The whole body γ -irradiation of mice was performed with a Canadian gamma cell-40, (^{137}Cs) at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt at a dose rate of 0.46 Gy /min.

Tumor Transplantation

Cell line of Ehrlich Ascites Carcinoma (EAC) was used as a model of solid carcinoma by inoculation in the right thigh of albino mice. The parent line was supplied as gift from Egyptian National Cancer Institute (NCI), Cairo University. Human breast cancer is the source of EAC cells upon modified to grow in female Swiss albino mice. The cell line of EAC was maintained by i.p of 2.5 million cells per animal. Bright line hemocytometer was used to count the EAC before i.p. injection and the dilution was done using physiological sterile saline solution. In order to develop Ehrlich solid tumor (EST) in thigh, 0.2 ml EAC cells (2.5×10^6 cells/mouse) were inoculated subcutaneously (s.c.) in the right thigh of the lower limb of female mouse (Medhat *et al.*, 2017).

Animal Categories

In this study 105 mice (weighing about 25 g) were used. All the experiments were conducted under National Research Centre Guidelines for the use and care for laboratory animals and were approved by an independent ethics committee of the NCRRT.

The animals were categorized into 7 equal groups of 15 mice each as follows:

Group (1): Control (C): Mice neither treated nor irradiated.

Group (2): (EAC): Mice bearing solid Ehrlich tumor.

Group (3): (EAC+R): Mice bearing solid Ehrlich tumor were subjected to 1 Gy whole body γ -irradiation (two fractionated

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doses); at 11 and 25 days post EAC inoculation, respectively.

Group (4): (EAC+Ch): Mice bearing solid Ehrlich tumor were injected i.p. with chrysin for 21 consecutive days at the 11 days after EAC inoculation.

Group (5): (EAC+Ch+R): Mice bearing solid Ehrlich tumor were injected with Chrysin as in group 8 along with a first whole body γ -irradiation at a dose level of 0.5 Gy 30 min after the first injection of Ch and the second dose of irradiation as in group 3.

Group (6): (EAC+P): Mice bearing solid Ehrlich tumor were injected i.p. with propolis for 21 consecutive days at the 11 days after EAC inoculation.

Group (7): (EAC+P+R): Mice bearing solid Ehrlich tumor were injected with Propolis as in group 10 along with a first whole body γ -irradiation at a dose level of 0.5 Gy 30 min after the first injection of propolis and the second dose of irradiation as in group 3.

Tumor Volume Monitoring

Tumor volume was measured at different time intervals during the experimental period using a vernier caliper on the 7th, 15th, 21th days from the tumor has been reached 1cm³ during the experimental period. The volume of solid tumor was calculated using formula $[A * B^2 * 0.52]$, where A and B are the longest and the shortest diameter of tumor, respectively (Papadopoulos *et al.*, 1989).

Mice were sacrificed at the end of experiment. The skeletal muscle (normal control), tumor tissues and liver were collected for biochemical investigations.

Quantitative Real-time PCR

RNA isolation and reverse transcription:

RNA was extracted from the tumor tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. Genomic DNA was

eliminated by a DNase-on-column treatment supplied with the kit. The RNA concentration was determined spectrophotometrically at 260 nm using the Nano Drop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels. RNA (1 μ g) were used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with MgCl₂ (25mM), RTase buffer (10X), dNTP mixture (10mM), oligo(dT) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/ μ l). This mixture was incubated at 42°C for 1 h.

Quantitative real time PCR:

qRT-PCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions of 40 cycles of 15 s at 95°C and 60 s at 60°C after an initial denaturation step at 95°C for 10 min. Each 10 μ l reaction contained 5 μ l SYBR Green Master Mix (Applied Biosystems), 0.3 μ l gene-specific forward and reverse primers (10 μ M), 2.5 μ l cDNA and 1.9 μ l nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table (1). Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the endogenous control GAPDH (Livak and Schmittgen, 2001).

Table 1: Primers used for QRT-PCR

Primer	Sequence
TNF-α	Forward: 5'-ACGGCATGG ATCTCAAAGAC-3' Reverse: 5'-CGGACTCCGCAAAGTCTAAG-3'
GAPDH	Forward: 5'-CTCCATTCTTCCACCTTTG-3' Reverse: 5'-CTTGCTCTCAGTATCCTTGC-3'

ELISA Detection

Enzyme-linked immune sorbent assay (ELISA) for levels of IFN- γ and Caspase-3 were determined by using ELISA Kit (R & D systems) according to the manufacturer's instructions on the supernatants of sample tissue homogenates. In brief, microplates were coated with 100 μ l/well of capture antibody, and then they were incubated overnight at 4°C. After washes, the plates were blocked with assay diluent at room temperature for 1 h. One hundred microliters of a serum sample was added to each well of the plate, followed by incubation for 2 h at room temperature. Working detector was added into each well, and the plate was incubated for an additional 1h at room temperature before the addition of substrate solution. The reaction was stopped by adding stop solution. The absorbance was read using ELISA reader. The concentrations were calculated from standard curve according to the instructions in the protocol.

Nitric Oxide Determination

Nitric oxide (NO) level in the liver tissues was determined colorimetrically as nitrite by Griess reaction (Miranda *et al.*, 2001).

Measurement of radical-scavenging ability in hepatic tissue using electron spin resonance spectroscopy

Liver tissues were quickly removed from mice and were gently lyophilized and evaporated to dryness under vacuum. All samples were dissolved in a small volume of toluene, and were transferred to a round ESR cell. Cells were capped with a rubber septum and were thoroughly deoxygenated

by nitrogen bubbling before ESR spectroscopy was performed. The ESR spectra were recorded at room temperature using a Bruker EPR ER-200D spectrometer, and spectral accumulation was done by using a Bruker ER-140 (ASPECT 2000) data systems. The microwave power was 2 mW, the modulation amplitude was 1 G and 1 E4 receiver gain. The response time constant was 10 ms, with a field-sweeping rate of 100 G/ 42 s. The height of powder sample inside the quartz tube was about 10 mm. ESR spectral analyses were performed through the use of a computer simulation program (Oehle and Janzen, 1982).

Histopathological Study

Autopsy samples were taken from the thigh muscle of mice in different groups and fixed in 10% formalin solution for 24 h at room temperature. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by Slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for routine examination through the light electric microscope (Banchroft *et al.*, 1996).

Statistics:

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test by using statistical package of social science (SPSS) version 20.0 for windows.

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$P \leq 0.05$ were considered as a level of significance.

RESULTS

Impact of P, Ch administration and/or γ -irradiation exposure on tumor volume in different mice groups

Table (2) demonstrated that the size of solid EAC tumor reached 6500.40 mm³ 7th days from the tumor has been reached 1cm³ during the experimental period, and enlarged to 38700.46 mm³ at

the end of the experiment. A gradual significant decrease in the tumor size during treatment of EAC-bearing mice with Ch, or P was demonstrated, compared to untreated EAC-bearing mice. On the other hand, exposure of EAC-bearing mice to γ -irradiation produced a marked significant reduction in the tumor size, and a radio-sensitizing effect was observed in decreasing the tumor size of γ -irradiated-EAC-bearing mice co administered with Ch, or P, respectively.

Table 2: Statistical significance of the tumor volume in different groups.

Groups	Tumor volume (mm ³)		
	7 days	15 days	21 days
EAC	65.40±10.05	197.51±17.76	387.46±35.33
EAC+R	47.40±4.76 ^b	132.00±17.5 ^b	320.60±21.50
EAC+Ch	39.24±5.89 ^b	107.46±15.92 ^b	181.36±19.2 ^b
EAC+Ch+R	100.85±1.85 ^b	68.26±6.72 ^b	121.43±30.9 ^b
EAC+P	45.91±4.75 ^b	129.00±26.19 ^b	177.80±7.08 ^b
EAC+P+R	28.60±3.07 ^b	38.80±4.16 ^b	134.00±4.04 ^b

EAC: Ehrlich solid tumor, R: radiation, Ch: chrysin, P: propolis.

Each value represents the mean \pm SE (n=6).

a: significantly different from EAC.

Impact of Ch, P administration and/or γ -irradiation exposure on angiogenic parameters in different mice groups

Treatment of mice bearing solid tumor with Ch and P (E+ Ch and E+P groups) induced significant decreases ($P < 0.05$) in TNF- α concentration. Also the

NO contents of tumor tissue were significantly ameliorated. More pronounced decreases in TNF- α and NO concentrations were observed in E+Ch+R, E+P+R group than that observed E+ Ch and E+P or E+R mice groups (Table 3).

Table 3: mRNA expression of tumor necrosis factor alpha (TNF- α) and NO concentration in the right thigh muscle (control) or tumor tissue in different groups.

Groups	TNF- α Mean \pm SE	NO Mean \pm SE	Groups	TNF- α Mean \pm SE	NO Mean \pm SE
C	1.00±0.001 ^b	3.05±0.41 ^b	EAC+Ch+R	46.00±2.16 ^{ab}	5.67±0.24 ^b
EAC	96.20±4.37 ^a	14.09±0.35 ^a	EAC+P	82.18±1.27 ^{ab}	8.95±0.18 ^{ab}
EAC+R	77.40±1.63 ^{ab}	10.67±0.29 ^{ab}	EAC+P+R	62.34±1.49 ^{ab}	6.87±0.49 ^{ab}
EAC+Ch	56.80±1.35 ^{ab}	7.94±0.28 ^{ab}			

C: control, EAC: Ehrlich solid tumor, R: radiation, Ch: chrysin, P: propolis.

Each value represents the mean \pm SE (n=6).

a: significantly different from control. b: significantly different from EAC.

Impact of Ch, P administration and/or γ -irradiation exposure to mice on Caspase-3, IFN- γ

Table (4) showed that mice bearing solid EAC tumors manifested that IFN- γ and caspase-3 levels were significantly decreased, respectively, compared to non

EAC-bearing mice. Treatment of EAC-bearing mice with Ch, or P resulted in a pronounced elevation in IFN- γ and caspase-3 respectively, compared to untreated EAC-bearing mice. On the other hand, exposure of EAC-bearing mice to γ -irradiation either alone or in combination

with Ch, or P treatment produced a significant sharp increase in IFN- γ and caspase-3 levels, respectively, compared to untreated EAC-bearing mice.

Table 4: Interferon gamma concentration (IFN- γ), caspase-3 concentration in the right thigh muscle or tumor tissue in different groups.

Groups	IFN- γ Mean \pm SE	Caspase-3 Mean \pm SE	Groups	IFN- γ Mean \pm SE	Caspase-3 Mean \pm SE
C	23.07 \pm .83 ^b	15.80 \pm 1.42 ^b	EAC+Ch+R	65.78 \pm 0.50 ^{ab}	6.70 \pm 1.68 ^{ab}
EAC	106.56 \pm 2.3 ^a	1.26 \pm .015 ^a	EAC+P	85.40 \pm 1.4 ^{ab}	6.58 \pm .24576 ^{ab}
EAC+R	91.132 \pm 0.73 ^a	5.16 \pm 0.85 ^{ab}	EAC+P+R	74.50 \pm 2.4 ^{ab}	9.53 \pm 0.19 ^b
EAC+Ch	81.71 \pm 0.22 ^{ab}	4.75 \pm 0.34 ^{ab}			

C: control, EAC:Ehrlich solid tumor, R: radiation, Ch: chrysin, P: propolis.

Each value represents the mean \pm SE (n=6).

a: significantly different from control. b: significantly different from EAC.

The effect of liver tissue ESR free radical in different mice groups

Table (5) showed that mice bearing solid EAC tumors manifested high significant increase for the free radical peak intensity, compared to non EAC-bearing mice. Treatment of EAC-bearing mice with Ch, or P resulted in a

pronounced decline in free radical peak intensity, compared to untreated EAC-bearing mice. On the other hand, exposure of EAC-bearing mice to γ -irradiation either alone or in combination with Ch, or P treatment produced a significant sharp decrease in free radical peak intensity, compared to untreated EAC-bearing mice.

Table 5: Statistical analysis for the peak intensity for liver tissue ESR free radical in different mice groups.

Groups	Intensity (AU) Mean \pm SE	Groups	Intensity (AU) Mean \pm SE
C	17052.06 \pm 10.90	EAC+Ch+R	45716 \pm 6.99
EAC	89707.71 \pm 9.08	EAC+P	71523.48 \pm 3.97
EAC+R	47573.38 \pm 5.98	EAC+P+R	65084.86 \pm 2.94
EAC+Ch	58411.96 \pm 8.99		

C: control, EAC:Ehrlich solid tumor, R: radiation, Ch: chrysin, P: propolis.

Each value represents the mean \pm SE (n=6).

Histopathological study:

The Impact of Ch and P treatment on histopathology of EAC bearing mice irradiated or not irradiated was investigated.

Photomicrographic examinations of control mice thigh muscles tissue demonstrated normal histological structure of striated bundles (Fig. 1A). However, photomicrograph of EAC mice displayed compactness of the tumor cells scattered within the muscular tissues and aggregated in focal manner infiltrating and penetrating the muscle

bundles. Groups of large round and polygonal cells with pleomorphic shapes, hyperchromatic nuclei and binucleation, several degrees of cellular and nuclear pleomorphism were observed (Fig. 1B).). Further, in mice exposed to whole body gamma radiation, a focal area of aggregated intact Ehrlich tumor cells was appeared rather than musculature necrosis in other sides (Fig. 1D). On the other hand, the histopathological investigation performed showed a great destruction of tumor tissue represented by the appearance

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of dead and necrotic cells after Ch, or P administration.

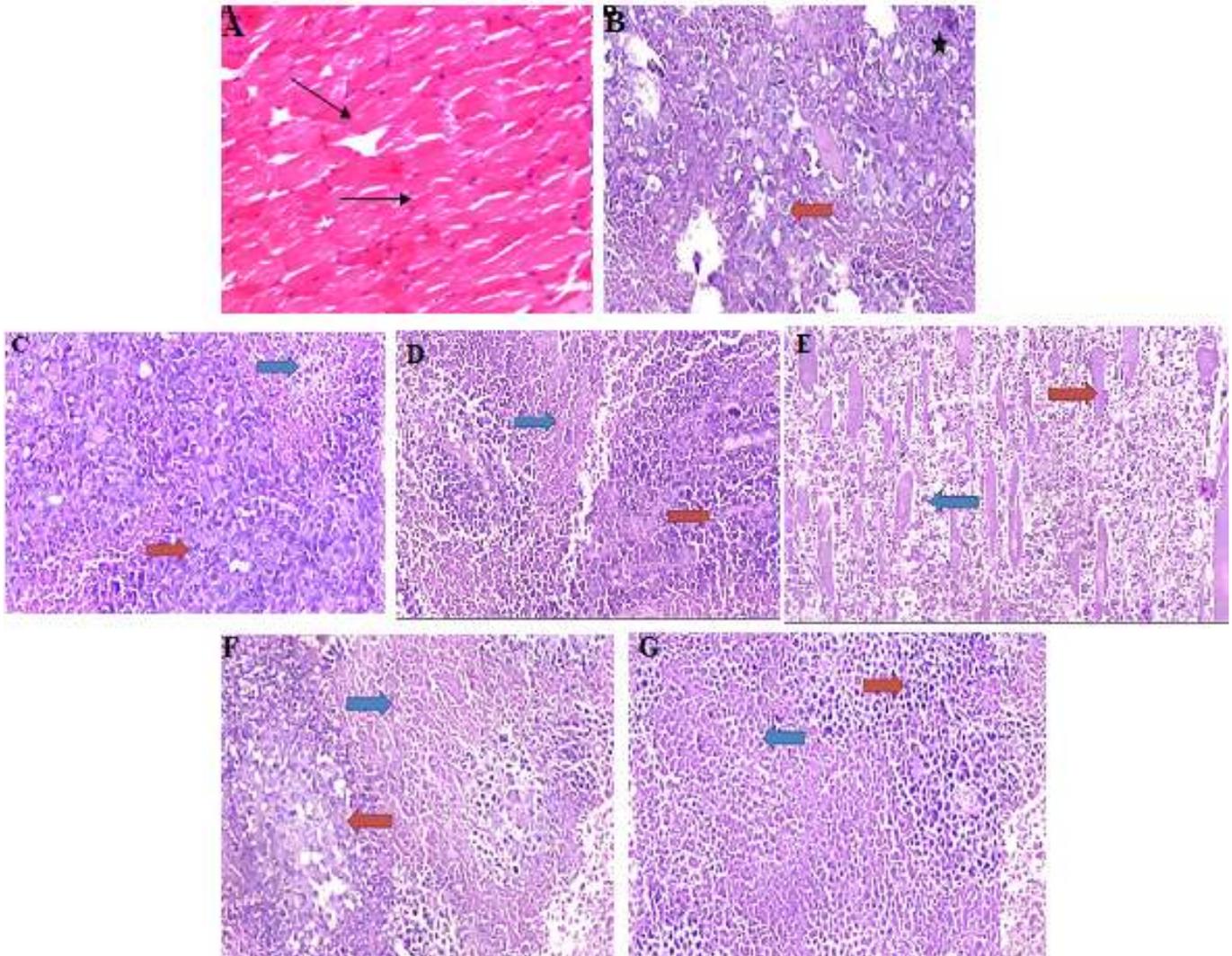


Fig. 1: Photomicrographs of sections of right thigh muscles, stained with Hx-E, X40 .

A: Normal control; **C** displayed normal histological structure of striated bundles. (→). **B:** Mice bearing solid tumor; **E** Ehrlich tumor cells exhibiting pleomorphism, hyperchromatisa (*), infiltrating and penetrating the muscle bundles and aggregated in focal manner in between (→). **C:** propolis showed malignant cells (red arrow) surrounded by 10% of tumor necrosis (blue arrow), **D:** irradiated mice bearing solid tumor; **E+R.** showed malignant cells (blue arrow) surrounded by 20% of tumor necrosis (red arrow).

DISCUSSION

It is expected that cancer will be the most important reason of death and the leading obstacle to rise in life expectancy; over 18 million new cancer cases with over 50 % mortality has been mentioned in 2018 estimates. Due to the great importance of the immune system in the development of cancer, there has been an attempt to develop immunotherapies directed against tumors with the aim of increasing the antitumor immune response and consequently the eradication of the neoplastic in progress (Celińska-Janowicz *et al.*, 2018).

In this study, a significant upregulation was recorded in IFN- γ activity and TNF- α messenger RNA (mRNA) expression in solid EC tumors, compared to non EC-bearing mice. TNF- α is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. Its potential as a protumor factor also has a long history (Zaidi and Merlino, 2011). One common theme that seems to emerge from the studies that show protumorigenic effects of IFN γ is that tumors that are exposed to IFN γ show greater immunoevasive capabilities. For example, several IFN γ pathway target genes are known to be involved in immunosuppressive and immunoevasive mechanisms that are geared toward suppression of cytotoxic lymphocyte (CTL) – and natural killer cells (NK) cell-mediated antitumor immune responses (RazaZaid, 2019). TNF- α is a major inflammatory mediator that induces multiple changes in endothelial cell gene expression, including induction of adhesion molecules, integrins, and matrix metalloproteinases (MMPs), therefore acting as an autocrine growth factor for tumor angiogenesis (Song *et al.*, 2012).

Further, the significant increase in NO concentration of EAC bearing mice might be due to TNF- α over expression. It

has been demonstrated that TNF- α is a mediator of NO synthesis (Liu *et al.*, 2010). Nitric oxide is produced from L-arginine by a family of NO synthase (NOS) isoenzymes. These enzymes comprise three distinct isoforms, encoded by three different genes and include neuronal (nNOS codified by NOS-1), inducible (iNOS/NOS-2), and endothelial (eNOS/NOS-3) forms. Although, at baseline, the main source of plasma NO is related to eNOS, during several clinical conditions, such as inflammation, iNOS is activated (Assmann *et al.*, 2016). The inducible form of NO synthase is expressed mainly through TNF- α activated pathway (Medhat *et al.*, 2017).

The disturbance in the angiogenic and apoptotic regulators leads to tumor proliferation and growth, which was clearly demonstrated by the increase in EC tumor volume. Neovascularization enhances the ability of the tumor to grow and increases its invasiveness and metastatic ability (Dhankhar *et al.*, 2010). Apoptosis is a programmed cell death that maintains the stability of the internal environment through removing genetic mutations and unstable cells. However, this process is inhibited in cancer which leads to the accumulation of various genetically unstable cells.

The present results demonstrated a significant decline in the level of apoptotic molecule (caspase-3) in the solid EC tumors, compared to non EC-bearing mice. Caspase-3 mediated apoptosis is a major focus in the field of cancer growth inhibition, because activation of proteolytic caspase cascade is a critical component in the execution in apoptotic cell death (Choi and Kim, 2009). The increase in TNF- α expression is accompanied with decrease in caspase -3 activities. These findings are online with Zhang *et al.* (2018) who found that knockdown of trans membrane TNF- α expression enhances the therapeutic efficacy of Doxorubicin in a xenograft

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mouse model where the combination of tmTNF- α inhibition and DOX treatment resulted in much more pronounced suppression of tumor growth. The current data revealed that the upregulation of TNF- α genes and induction of IFN- γ activity and NO concentration along with apoptosis suppression in solid EC-bearing mice collectively enhance tumor cell proliferation and neovascularization, which ultimately resulted in the acceleration of tumor growth, invasiveness, and metastatic ability of tumor cells.

The current modalities of cancer treatment are mainly comprised of surgery, radiation based therapy, chemotherapy, gene therapy and/or hormonal therapy. Another approach that has gained importance is the use of bio toxins or api-toxin such as animal venoms as cancer therapeutic agents. These bio toxins are produced by living organisms as a defense mechanism against predators and are known to have both toxicological as well as pharmacological effects (Zhang, 2015).

The result of this study revealed that treatment of EC-bearing mice with P or Ch significantly reduced the growth of solid tumors, and a synergistic effect was demonstrated following combination of both treatments with γ -irradiation. As a key player in tumor proliferation, angiogenesis, and inflammatory cascade induction, the down regulation of TNF- α expression following treatment of irradiated and non-irradiated EC-bearing mice with P or Ch reduced tumor growth via multiple mechanisms, including suppression of tumor cell proliferation (reduction in tumor volume), angiogenesis (down regulation of tumor TNF- α expression and NO concentration), and enhancing apoptosis (induction of tumor caspase-3). Propolis evokes several therapeutic properties, including anticancer activity. These activities are attributed to the action of polyphenols. Previously, it has been demonstrated, that one of the most

abundant polyphenolic compounds in ethanolic extracts of propolis are chrysin (Celińska-Janowicz *et al.*, 2018). Chrysin, Inhibit Tumor Necrosis Factor expression level -Related Apoptosis—Inducing Ligand Receptor-1 (TRAIL-R1) on Activated RAW264.7 Macrophages (Warat *et al.*, 2014).

Propolis had good antitumor effects on different cancer cells such as the human breast cancer cells (MCF-7 and MDA-MB-231), lung cancer cells (A549), human cervical carcinoma cell (HeLa) and the antitumor bioactive constituents are flavonoids and esters. In addition, it exhibits excellent anti-inflammatory activities in macrophages (Raw 264.7), ox-LDL stimulated HUVECs and intestinal epithelial Caco-2 cells by modulating key inflammatory mediators of mRNA transcription, inhibiting the production of specific inflammatory cytokines, blocking the activation of nuclear factor NF- κ B, TNF- α and activating AMPK and ERK signaling pathway (Chang *et al.*, 2017).

Where the earliest stages of angiogenesis are defined by vasodilatation mediated by NO and an increased vascular permeability of pre-existing capillaries or post-capillary venules in response to overexpressed VEGF (Kumar and Kuttan, 2011), and the inducible form of NO synthase is expressed mainly through TNF- α activated pathway (Azab *et al.*, 2011), so TNF- α is the maestro for controlling angiogenesis process.

Propolis and Ch has demonstrated potential efficacy in inhibiting TNF- α and NO concentration (Chang *et al.*, 2017). Nitric oxide, a physiological signaling molecule, is involved in many cellular functions, including cell proliferation, survival and death. A recent study suggested that LPS/TLR4-induced signaling cascades leads to inducible nitric oxide synthase (iNOS) induction, and inhibition of iNOS might be as a novel effective target therapy against triple negative breast cancer. It was found that

Chinese P and CAPE obviously inhibited the production of NO, which might inhibit MDA-MB-231 cells survival (Granados-Principal *et al.*, 2015).

Apoptosis was another major cause for Chinese P and CAPE to inhibit LPS-stimulated MDA-MB-231 cells survival. It was reported that P could induce cancer cells apoptosis. It was also found that Chinese P and CAPE activated caspase 3- the executor of apoptosis in LPS-stimulated breast cancer cells, which might be induced by activating autophagy and depressing TLR4 signaling pathway (Frezza *et al.*, 2014).

It was revealed that Ch possess moderate cytotoxic effect on CT26 cells in a concentration dependent manner. In addition, the findings from fluorescence microscopy, Annexin-PI and caspase assays showed that the cytotoxicity of Ch was related to the recruitment of the intrinsic pathway of apoptosis cell death (Bahadori *et al.*, 2016). The proapoptotic effect of Ch has been reported in breast carcinoma, cervical cancer, leukemia, lung cancer (NSCLC), and colon cancer in vitro. A study by Zhang *et al.* (2004) has demonstrated that Ch and tetraethyl bis-phosphoric ester (one of chrysin derivatives) exhibited potential anti-cancer effects in human cervical carcinoma cells. In conclusion, treatment of E, E+R groups with P or Ch exerted a marked effect in the retardation of tumor growth as compared to tumor bearing mice group. These observations could be attributed to the immune stimulation, antiapoptotic and antioxidant capacity of P or Ch.

REFERENCES

- Assmann, T.S.; Brondani, L.A.; Bouças, A.P.; Rheinheimer, J.; Souza, B.M.; Canani, L.H.; Bauer, A.C. and Crispim D. (2016). Nitric oxide levels in patients with diabetes mellitus: A systematic review and meta-analysis. *Nitric Oxide*, 61: 1-9.
- Attia, W.Y.; El-Shaikh, K.A.; Gabry, M.S.; Othman, G.A. (2007). Prophylactic effect of propolis against tumor growth in mice through stimulation of the immune system. *Egypt. J. Exp. Biol. (Zool.)*, 3: 219 – 228.
- Azab, K.S.; Osman, S.A. and El-Fatih, N.M. (2011). Implication of caspase-3 and granzyme B expression and activity in spleenocytes of Ehrlich ascites carcinoma mice subjected to immunotherapy. *North Am. J. Med. Sci.*, 3: 371-377.
- Bahadori, M.; Baharara, J. and Amini, E. (2016). Anticancer properties of chrysin on colon cancer cells, in vitro and in vivo with modulation of Caspase-3, -9, Bax and Sall4. *Iran J. Biotech.*, 14(3): e1374.
- Bancroft, J.D.; Stevens, A. and Dawson, M.P. (1996). *Theory and Practice of Histological Techniques*. 4th ed. Churchill Livingstone; 273-292
- Celińska-Janowicz, K.; Zaręba, I.; Lazarek, U.; Teul, J.; Tomczyk, M.; Pałka, J. and Milytk, W. (2018). Constituents of propolis: chrysin, caffeic acid, p-coumaric acid, and ferulic acid induce PRODH/POX-dependent apoptosis in human tongue squamous cell. *Carcinoma Cell (CAL-27)*, 9: 336.
- Chang, H.; Wang, Y.; Yin, X.; Liu, X. and Xuan, H. (2017). Ethanol extract of propolis and its constituent caffeic acid phenethyl ester inhibit breast cancer cells proliferation in inflammatory microenvironment by inhibiting TLR4 signal pathway and inducing apoptosis and autophagy. *BMC Complementary and Alternative Medicine*, 17: 471.
- Choi, E.J. and Kim G.H. (2009). Apigenin induces apoptosis through a mitochondria/caspase-pathway in human breast cancer MDA-MB-453 cells. *J. Clin. Biochem. Nutr.*, 44:260- 265.

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- Dhankhar, R.; Dahiya, K.; Singh, V.; Sangwan, L. and Kaushal, V. (2010). Nitric oxide and cancer. *J. Clin. Diag. Res.*, 4: 2550-2559.
- Frozza, C.O.; Ribeiro, Tda S.; Gambato, G.; Menti, C.; Moura, S.; Pinto, P.M.; Staats, C.C.; Padilha, F.F.; Begnini, K.R. and de Leon, P.M. (2014). Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells. *Food Chem. Toxicol.*, 63: 195–204.
- Granados-Principal, S.; Liu, Y.; Guevara, M.L.; Blanco, E.; Choi, D.S.; Qian, W.; Patel, T.; Rodriguez, A.A.; Cusimano, J. and Weiss, H.L. (2015). Inhibition of iNOS as a novel effective targeted therapy against triple-negative breast cancer. *Breast Cancer Res.*, 17: 25.
- Kumar, P.P. and Kuttan, G. (2011). Nomilin inhibits tumor-specific angiogenesis by downregulating VEGF, NO and proinflammatory cytokine profile and also by inhibiting the activation of MMP-2 and MMP-9. *Eur. J. Pharmacol.*, 668: 450-8.
- Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, 25: 402–408.
- Liu, J.G.; Zhao, H.J.; Yan-Juan, L. and Wang, X.L. (2010). Effect of selenium-enriched malt on VEGF and several relevant angiogenic cytokines in diethylnitrosamine-induced hepato carcinoma rats. *J. Trace Elements in Med. Biol.*, 24: 52-57.
- Medhat, A.M.; AzabKh, Sh.; Said, M.M.; El Fatih, N.M. and El Bakary, N.M. (2017). Antitumor and radiosensitizing synergistic effects of apigenin and cryptotanshinone against solid Ehrlich carcinoma in female mice. *Tumor Biol.*, 39910): 1-13.
- Meng, X.; Fang, S.; Zhang, Z.; Wang, Y.; You, C.; Zhang, J. and Yan, H. (2017). Preventive effect of chrysin on experimental autoimmune uveitis triggered by injection of human IRBP peptide 1-20 in mice. *Cell Mol. Immunol.*, 14(8):702-711.
- Miranda, K.M.; Espey, M.G. and Wink D.A. (2001). A rapid simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, 5(1): 62-71.
- Mirandola, L.; Justo, G.Z. and Queiroz, M.L.S. (2002). Modulation by *Acanthospermum austral* extracts of the tumor induced haematopoietic changes in mice. *Immunopharmacol. Immunotoxicol.*, 24: 275-288.
- Oehler, U.M. and Janzen, E.G. (1982). Simulation of isotropic electron spin resonance spectra: a transportable basic program. *Can. J. Chem.*, 60:1542-1548.
- Papadopoulos, D.; Kimler, B.F. and Estes, N.C. (1989). Growth delay effect of combined interstitial hyperthermia and brachytherapy in a rat solid tumor model. *Anticancer Res.*, 9:45-47.
- Pezzuto, J.M. (1997). Plant derived anticancer agents. *Biochem. Pharmacol.*, 53:121.
- RazaZaid, M. (2019). The Interferon-Gamma Paradox in Cancer. *J Interferon Cytokine Res.*, 39(1): 30–38.
- Song, K.; Zhu, F.; Zhang, H. and Shang Z. (2012). Tumor necrosis factor- α enhanced fusions between oral squamous cell carcinoma cells and endothelial cells via VCAM-1/VLA-4 pathway. *Exp. Cell Res.*, 318: 1707–1715.
- Warat, M.; Szliszka, E.; Korzonek-Szlacheta, I.; Król, W. and Zenon,

- P. (2014). Czuba, chrysin, apigenin and acacetin inhibit tumor necrosis factor-related apoptosis—inducing ligand receptor-1 (TRAIL-R1) on Activated RAW264.7 macrophages. *Int. J. Mol. Sci.*, 15(7): 11510–11522.
- Wleklik, M.; Zahorska, R. and Luczak, V. (1997). Interferon-inducing activity of flavonoids. *ActaMicrobiol. Pol.*, 32: 1141-1148.
- Zaidi, M.R. and Merlino, G. (2011). The two faces of interferon-gamma in cancer. *Clin. Cancer Res.*, 17(19):6118–6124.
- Zhang, T.; Chen, X.; Qu, L.; Wu, J.; Cui, R. and Zhao, Y. (2004). Chrysin and its phosphate ester inhibit cell proliferation and induce 999apoptosis in Hela cells. *Bioorg. Med. Chem.*, 12(23): 6097-6105. DOI: 10.1016/j.bmc.2004.09.013
- Zhang, Y. (2015). Why do we study animal toxins? *DongwuxueYanjiu*, 36: 183-222.99
- Zhang, Z.; Lin, G.; Yan, Y.; Li, X.; Hu, Y.; Wang, J.; Yin, B.; Wu, Y.; Li, Z. and Yang, X.P. (2018). Transmembrane TNF-alpha promotes chemoresistance in breast cancer cells, *Oncogene.*; 37(25): 3456–3470.

التحفيز المناعي والتأثيرات الاستباقية والتأزيرية للبروبوليس ومكوّنه الرئيسي (كركنين) على نمو الورم وحساسية الخلية للإشعاع المؤين في الفئران

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مستخلص

شجع الإهتمام المتزايد بالنهج الجديدة للعلاج المناعي للسرطان، والطلب الكبير على العوامل العلاجية التي يمكن أن تعدل الأشكال العديدة من نقص المناعة، على إجراء دراسات حول آليات التعديل المناعي للمواد الطبيعية والإصطناعية. تم إجراء الدراسة الحالية ك محاولة لتقييم التأثير المحفز مناعياً والتأثير الإستباقي وكذلك التأثير التأزيري للكركنين والبروبوليس على نمو الورم وحساسية الخلية السرطانية للإشعاع المؤين الذي يستهدف تحسين البروتوكولات العلاجية للسرطان. تم حقن الكركنين (20 مجم / كجم من وزن الجسم/ يوم) والبروبوليس (100 مجم/ كجم / يوم) داخل الصفاق للفئران التي تحمل ورماً صلباً بحجم 1 سم³ من سرطان استسقاء إيرليش (EAC) لمدة 21 يوماً متتالياً. تعرضت الفئران لجرعة مقدارها 1.0 جراى من أشعة جاما (على جرعتين مجزأتين: الأولى عند اليوم 11 بعد تحميل الورم، والثانية عند اليوم 25 بعد التحميل). أوضحت النتائج أن العلاج بالكركنين أو البروبوليس يمنع بشكل ملحوظ انتشار الورم في الفئران . وانخفض نشاط انترفيرون جاما (INF- γ) بشكل ملحوظ مقارنة بفئران المقارنة الحاملة للورم . كما تم انخفاض تركيزات m-RNA للعوامل المولدة للورم ألفا (TNF- α) والجذور الحرة وكذلك تركيز أكسيد النيتريك (NO) بشكل كبير بالتزامن مع التحسينات في منظمات موت الخلايا المبرمج (Caspase-3 activity) مقارنة بالفئران الحاملة للورم. علاوة على ذلك، يؤكد الفحص النسيجي التحسن الذي حدث بواسطة الكركنين أو البروبوليس في مجموعة الفئران الحاملة للورم (EAC) أو مجموعة الفئران التي تحمل ورم إرليخ الصلب وتعرض للإشعاع (EAC + R). أدى التعرض لإشعاع جاما إلى استمرار التأثير المعدل للكركنين أو البروبوليس على الورم عند مقارنته بالفئران الحاملة للورم والمحقونة بالكركنين (EAC + Ch) أو بالفئران الحاملة للورم والمحقونة بالبروبوليس (EAC + P). وبالتالي، قد يمثل الكركنين أو البروبوليس استراتيجية علاجية محتملة لزيادة الاستجابة الإشعاعية للورم الصلب.