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Original Article

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Abstract

Breast cancer (BC) is women's leading cause of death, accounting for more than 2 million new cases in 2019. Phytochemicals have been used in traditional medicine for centuries, however, they recently gained attention in cancer research due to their potent antioxidant, anti-inflammatory, and immunomodulatory effects. This study aimed to elucidate the prophylactic and/or therapeutic effect of a phytochemical combination "Breast safeguard" on mammary adenocarcinoma *in vivo* model of Ehrlich cells. Thirty mice were randomized into three groups of Ehrlich group, the Prophylactic group, and the BSG-treated group. A marked enhancement in the mice survival rate and a significant reduction in tumor size was recognized after two weeks of treatment. And TNF-α, and Bax/Bcl2 ratio markers for cancer prognosis and apoptosis, the results showed a significant decrease in tumor volume in the protective group than in the treated one. This phytochemicals combination showed a potent anticancer characteristic by inhibiting Ehrlich tumor proliferation and induction of cell apoptosis indicating its possible use as a promising prophylactic drug in prospective studies.

1. Introduction

According to GLOBOCAN 2020 statistics, breast cancer is currently considered one of the most frequently diagnosed malignancies and the fifth leading cause of cancer-related deaths among women, with an anticipated total of 2,3 million new cases globally **(Sung et al., 2021)**. In transitional nations (Melanesia, Western Africa, Micronesia/Polynesia, and the Caribbean), the incidence rate of breast cancer deaths is 88 percent greater than in transitioned countries (Australia/New Zealand, Western Europe, Northern America, and Northern Europe) **(Łukasiewicz et al., 2021)**.

The disadvantages of conventional cancer treatments, such as surgery, radiotherapy, chemotherapy, or their combined therapy provoked researchers to focus on more selective therapies based on a better understanding of tumor progression's biology and molecular genetics. Currently, 90 percent of chemotherapy failures occur during the invasion and metastasis of malignancies due to drug resistance. The chemotherapy-resistant cancer cells have gained multidrug resistance by blocking cell death (evading apoptosis), altering drug metabolism, undergoing epigenetic modifications, and enhancing DNA repair mechanisms **(Mansoori et al., 2017)**.

Therefore, it is crucial to discover alternative treatments that are safer for the patient and more effective in eradicating cancer. One such alternative is the usage of naturally extracted phytochemicals which are found in vegetables, fruits, spices, and plant roots, such as resveratrol, quercetin, indole-3-carbinol, c-phycocyanin, curcumin, and genistein **(Abdraboh et al., 2020; Ouhtit et al., 2013)**. Phytochemicals may positively affect cancer cell signaling, cell cycle regulation, oxidative stress response and inflammation. They may also modulate non-coding RNAs, upregulate tumor-suppressive miRNAs, and downregulate oncogenic miRNAs synergistically inhibit cancer cell growth and cancer stem cell self-renewal **(Kapinova et al., 2018; Rizeq et al., 2020)**.

One of the first models to study cancer progression is the mammary adenocarcinoma Ehrlich cells, which can be passage from mice to mice in a form of ascites with repeated intraperitoneal injection **(Mandal et al., 2010)**. Due to its better efficacy in generating free neoplastic cells and accuracy in terms of a lifetime, the Ehrlich

ascites model is used as an *in vivo* model for cancer research **(Heimlich et al., 2005; Queiroz et al., 2004)**. The solid form of Ehrlich carcinoma (SEC) developed by subcutaneous injection and serves as a valuable model for assessing anticancer drugs **; Frajacomo et al., 2016; Kabel et al., 2015)**. Previous research has shown that subcutaneous EAC may spread to the lungs, liver, spleen, kidney, bone, diaphragm, blood, and adrenal glands **(Mishra et al., 2018)**

In this study, E. solid tumor model was developed to explore the potential prophylactic and/or therapeutic effects of commercial phytochemicals combination "Breast Safeguard" in diminishing Ehrlich cell growth and survival.

2. Materials and Methods

2.1. Animals:

Healthy adult female Swiss albino mice, weighing approximately 20-25 g, housed and maintained a 12-hour light/dark cycle under a constant temperature of 25ºC with free access to food and drinking water. After an acclimatization period of 1 week, mice were randomly divided into three groups (n=10 per group). All animals were performed in compliance with the Mansoura University guidelines and were approved by animal handling ethical committee (MU-ACUC (SC.MS.23.01.14)).

2.2. Animal grouping:

Group I: Ehrlich cells ($2.5x10^6$ cells) were administered subcuta neously once into mice to develop Ehrlich solid tumor "Ehrlich group" **(E group) (Abdraboh et al., 2021).**

Group II: Ehrlich cells $2.5x10^6$ cells were injected subcutaneously once into mice to elicit Ehrlich solid tumor growth. After two weeks of injection (until the tumor appeared), mice were given an oral dosage of BSG (1 g/kg) daily for two weeks, "Ehrlich group and BSG" **(EB group) (Abdraboh et al., 2021)**.

Group III: Mice were given an oral dosage of BSG (1 g/kg) daily for two weeks as a protective, then mice were injected once subcutaneously with Ehrlich cells $2.5x10^6$ cells to induce Ehrlich solid tumor formation, then mice were given an oral dose of BSG (1 g/kg) daily for the remaining two weeks. "BSG, Ehrlich and BSG group" **(PEB group)**.

2.3. Ehrlich tumor cells:

For solid transplantation (subcutaneous injection), cells were withdrawn from ascites mice then centrifuged for 10 mins at 200g and washed twice by PBS after that cells were counted by trypan blue. The cells were injected subcutaneously by number (2.5×10^6 in 200 µl) into the left flank of each mouse on the 1st day except PEB group injected by cells on the 15th day of treatment **(Calixto-Campos et al., 2013; Frajacomo et al., 2016; Tennanth, n.d.; 1964)**.

2.4. Tumor evaluation:

The upper and lower diameters of tumors were measured using a digital caliper (Sagyma Plus, 0–150mm) every two days to track tumor progression. The tumor volume was determined using the conventional formula for solid tumors, $V=1/2\ (D\ d^2)$ **(Goto et al., 2000)**, where V is the volume, D is the larger diameter of the tumor, and d is the smaller diameter. To reduce bias, all measurements were obtained from the same examiner.

2.5. Biochemical investigation:

After the experimental period (day 30), the animals were fasted for 12 hours before undergoing heart puncture under anesthesia (Ketamine and Xylazine cocktail, 5:1, 0.1 ml/20 g body weight) to collect blood samples. Plasma was separated by cooling centrifugation at 200g for 15 minutes and kept at -80 °C till analysis.

2.6. Histological examination:

Following euthanasia, the solid tumors were dissected and preserved in 10% neutral buffered formalin for 24 hours. The samples were then dehydrated using ethanol solutions, cleaned with xylene, and mounted with paraffin. Tumor was sectioned at five µm and stained with hematoxylin/eosin CSA, from 660 fiber counts per group) and morphological examination as outlined by **(Fonseca et al., 2012)**.

2.7. Biochemical analysis:

The concentration of mouse BAX, Bcl2, and TNF-α were evaluated using the quantitative sandwich ELISA method, which employs a monoclonal antibody specific for each marker by the kit's instructions (CUSABIO, China). Concentrations were determined at a wavelength of 450nm using the Biotech ELX808 microplate reader.

2.8. Statistical analysis

Data were expressed as mean ± SEM, Student's t-test was used to compare the mean differences between samples using GraphPad Prism software version 6.01 (GraphPad Software, CA, USA). For all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. The survival rate of mice after the experiment:

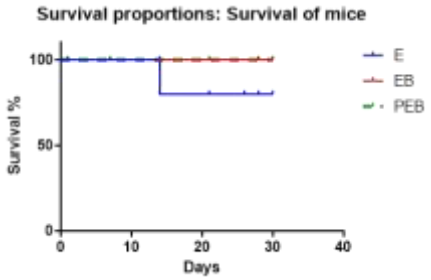

Figure (1): Kaplan-Meier survival curve of mice. According to the log-rank test, there were significant differences among three groups (P= 0.4493).

3.2. Effect of treatment on tumor size and cancer prognosis:

After BSG treatment, the tumor size of EB and PEB groups were significantly reduced to 81.2 and 37.3 mm^3 compared to control group (E) 2823 mm^3, respectively (Fig.2A and 2D).

The TNF-α level was higher in the PEB group than in the EB group, as shown in Fig.2C.

3.3. Histopathological examination of tumor tissue:
The PEB group showed decreased the proliferated neoplastic cells (red arrow) and increased the necrotic cells (black arrow) more than the EB group. In contrast, untreated E. Solid tumors showed massive proliferation of Ehrlich Ascites cancer cells (red arrow), as shown in Fig.2B.

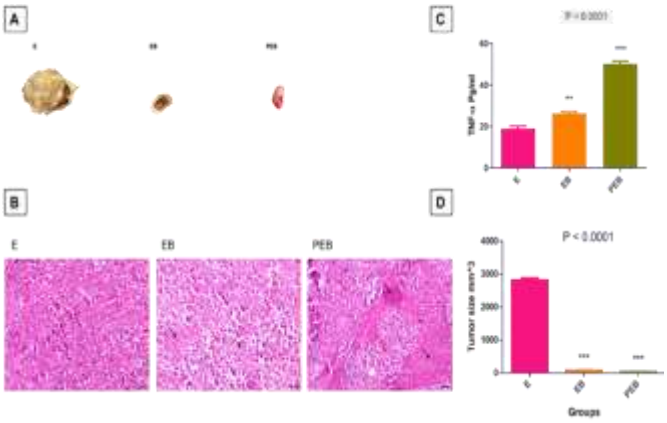

Figure 2: The effect of treatment on tumor size. (A) photograph showed the reduction in tumor size of EB and PEB. (B) H&E of the effect of treatment on Eh. Solid tumor development. E) Ehrlich group. As positive control. EB) Ehrlich tumor subjected to BSG treatment. PEB) Mice were supplemented with a protective dosage of BSG for two weeks before Ehrlich cells injection and continued after tumor development. (Black arrows) point for necrotic areas while (Red Arrows) point for live areas. H&E, X200, bar= 50 µm. (C) The effect of treatment on cancer prognosis (TNF-α) compared to the group untreated. *** at P<0.0001 and ** at P<0.001 to the E group. (D) Statistical analyses illustrated the significant effect of the phytochemicals combination BSG in decreasing the size of tumor in protective group PEB and treated group EB compared to E. group, respectively.

3.4. Effect of treatment on induction of cell apoptosis:
Bax level in peripheral blood was increased significantly in PEB and EB groups compared to the E group, as shown in Fig.3A. Bcl2 level rose slightly in the EB group and significantly decreased in the PEB group compared to the E group, as shown in Fig.3B. Bax/Bcl2 ratio was increased 4-fold in PEB and 2-fold in EB groups which determines cell susceptibility to apoptosis, as shown in Fig.3C.

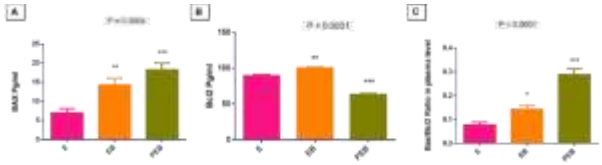

Figure (3): (A) Bax level in peripheral blood increased significantly in EB and PEB groups compared to the E group. Significance was denoted as *** at P<0.0001 for the PEB group, ** at P<0.001 for the EB group compared to the E group. B) Bcl2 level in peripheral blood slightly increased in EB and decreased significantly in the PEB group compared to the E group. Significance was denoted as **, at P<0.001 for the EB group and ***, at P<0.0001 for the PEB group compared to the E group. C) Bax/Bcl2 ratio increased in the PEB group more than in EB. Significance was denoted as *, at P<0.05 for the EB group and ***, at P<0.0001 for the PEB group compared to the E group.

4. Discussion

Cancer is a significant threat to the health of all human communities. Unfortunately, it is a heterogeneous illness at the tissue level, and this heterogeneity poses a formidable obstacle to its accurate diagnosis and therapeutic success **(Fisher et al., 2013; Meacham & Morrison, 2013)**. Crosstalk between invasion and metastasis and therapeutic resistance signaling has been observed for quickly growing solid tumors; however, it is unknown how this idea pertains to non-proliferating, quiescent tumor cells and hematological malignancies **(Weiss et al., 2022)**. In this work, a model for studying breast cancer has been built. It uses the EAC cells' carcinogenic capacity to create subcutaneous tumors in Swiss albino mice. Ehrlich Ascites Carcinoma (Ehrlich cells, EAC), a spontaneous mammary adenocarcinoma in mice, is a well-established tumor biology model. EAC model has been extensively used to research tumor pathogenesis and the creation of anti-tumorigenic drugs **(Mishra et al., 2018)**.

Several recent studies have revealed the anti-cancer effect of naturally derived phytochemicals over the last decade suggesting that oncologists should examine them as a viable alternative treatment **(Choudhari et al., 2020; Ranjan et al., 2019; Zubair et al., 2017)**. Breast safeguard (BSG) is a dietary supplement containing seven phytochemicals: curcumin, indol-3-carbinol, resveratrol, C-phycocyanin, quercetin, gallic acid, and genistein. BSG is the result of our group's research on the anticancer impact of a mixture of six phytochemicals in targeting several signaling pathways to limit the survival, proliferation, and metastasis of primary and metastatic breast cancer cell lines **(Abdraboh et al., 2020; Ouhtit et al., 2013; Rizeq et al., 2020)**.

The results demonstrated a significant decrease in tumor size of the PEB group compared to the EB group **(figure 2A)**, which was illustrated by histopathological examinations which revealed an increase at the necrotic areas in the PEB and EB groups. In the 1970s, TNF-alpha was discovered as the innate immune serum mediator capable of triggering hemorrhagic tumor necrosis. Today, a broad range of biological actions have been linked to this molecule, and its clinical translation has primarily included inhibiting its effects on treating autoimmunity rather than cancer **(Josephs et al., 2018)**. Based on these findings, the inflammatory marker (TNF-α) level of PEB group was

significantly increased after two weeks of therapy compared to the EB and E groups **(fig.2C)**.

It has been revealed that the Bcl-2 family plays a crucial role in inhibiting the intrinsic apoptotic pathway initiated by mitochondrial malfunction. The balance between pro-and anti-apoptotic members of this family may influence the cell's destiny. Bax and Bcl-2 are the most prominent members of the Bcl-2 family. Their putative involvement in tumor growth and prognosis of various human cancers has been the subject of several research over the last decade. Due to the permeabilization of the mitochondrial outer membrane, Bax promotes cell death in response to various cellular stressors. In contrast, Bcl-2 inhibits the action of Bax to avoid apoptosis. The Bax/Bcl-2 ratio may operate as a rheostat to control apoptotic susceptibility **(Khodapasand et al., 2015)**.

BSG has been shown to promote apoptosis on Ehrlich cells which may help to slow or stop the growth of tumors **(Abdraboh et al., 2021)**. Consequently, cell apoptotic capacity was enhanced in treated groups relative to the E group, as measured by the Bax/Bcl2 ratio **(fig.3C)**.

Additionally, BSG may also have a protective effect on healthy breast cells, helping to prevent damage and maintain their normal function. Overall, these findings suggest that BSG may a promising natural supplement for supporting breast health and potentially reducing the risk of breast cancer.

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