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Fagonia Arabica Extract Exerts Antitumor Effect on Mice Bearing Ehrlich Carcinoma

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Abstract

Background: To date, no studies have investigated the anticancer potential of *Fagonia arabica*. we aimed to investigate the antitumor potentiality and radiosensitizing effect of *Fagonia arabica* ethanolic extract (FAEE) on mice bearing solid ehrlich carcinoma (EC).

Method: This experimental animal study included 80 Balb-c mice and divided them into four groups: Group I: 10 EC-bearing mice as untreated controls; Group II: 10 EC-bearing mice exposed to a single dose of ionizing radiation (IR) at tumor localization (6Gy); Group III: 30 EC-bearing mice, each 10 mice received different dose of FAEE (250, 500, and 1000 mg/kg/day); Group IV: 30 EC-bearing mice, each 10 mice received different dose of FAEE (250, 500, and 1000 mg/kg/day) plus a single dose of IR (6Gy). P-FOXO3a and p-AKT levels were measured in tumor tissue homogenate via ELISA technique. BCL-2 gene expression was assessed with real-time polymerase chain reaction. Tumor tissues were stained with haematoxylin and eosin stain and examined.

Results: FAEE has antiproliferative effect on EC-bearing mice reflected by the decrease in tumor volume and tumor growth rate in a dose-dependent manner. Combination of FAEE with IR significantly increased radiation-induced tumor damage in comparison with IR alone. We observed a significant decrease in the concentration of p-AKT and p-FOXO3a and down-regulation of BCL-2 gene in the EC-bearing mice treated with FAEE only or in combination with IR.

Conclusion: FAEE may be an effective antitumor agent against breast cancer. FAEE exerts radio-sensitizing effect, especially at a dose of 500 mg/kg. FAEE interferes with the apoptosis process via decreasing p-AKT and p-FOXO3a and down-regulation of BCL-2 gene.

Keywords: *Fagonia arabica*, Ionizing, Radiation, Ehrlich tumor, p-FOXO3a, p-AKT

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Introduction

Breast cancer is an important public health problem worldwide,[1] and is the second most common cancer, impacting 2.1 million women each year. It also accounts for the greatest number of cancer-related deaths among women.[2,3]

The most conventional strategies for cancer therapy include surgery, radiotherapy, and chemotherapy.[4] Radiotherapy is the art of using ionizing radiation to destroy malignant tumors while minimizing the damage to normal tissues. Although higher doses of radiation can produce better tumor control, the dosage that can be given is limited by the possibility of normal tissue damage, that severely affects the quality of life.[5] Hence, seeking new therapeutic agents and effective therapies for preventing or controlling the complications and side-effects of routine drugs is of great importance.[6] Radiosensitizers are agents that enhance the sensitivity of cancer cells towards radiotherapy. The enchantment of radio-responsiveness of tumors by using radiosensitizers is suggested to be a promising strategy to improve radiotherapy efficiency.[7]

During development and carcinogenesis, the protein kinase B (AKT) pathway plays a prominent role in cell growth and cell survival. AKT is activated by phosphatidylinositol 3-kinase (PI3K).[8] Activation of AKT disturbs the balance of cell survival and apoptosis by promoting pro-survival transcription factors and inhibiting the fork head box transcription factor O class 3a (FOXO3a) pro-apoptotic transcription factor.[9]

FOXO3a belongs to a family of fork head box class O (FOXO) transcription factors, which are involved in diverse physiopathological processes. FOXO is one subfamily of the fork head transcription factor family with essential roles in cell fate decisions and has a crucial function as a tumor suppressor in a variety of cancers.[10] FOXOs encourage apoptosis signaling by either inducing expression of pro-apoptotic members of the BCL-2 family, enhancing expression of death receptor ligands, such as Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), or increasing the levels of different cyclin-

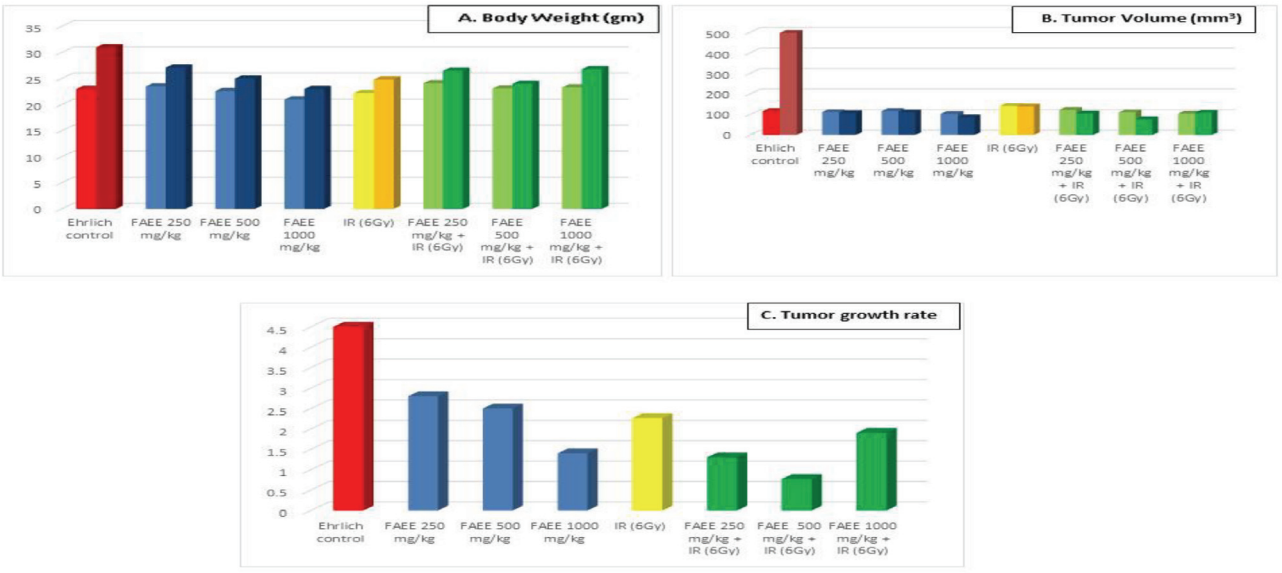

Figure 1. A) The bar chart represents the mean values of body weight at the beginning of the experiment and those after 14 days in all the studied groups; B) The bar chart represents the mean values of tumor volume at the beginning of the experiment and those after 14 days in all the studied groups; C) The bar chart depicts the mean values of tumor growth rate at the beginning of the experiment and those after 14 days in all the studied groups.

Ehrlich Control: Untreated Ehrlich Carcinoma (EC)-bearing mice
FAEE: EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg)
IR (6Gy): EC-bearing mice treated with 6 Gray single-dose of ionizing radiation
FAEE+IR (6Gy): EC-bearing mice supplemented with different doses of FAEE plus 6 Gray single-dose of ionizing radiation

Table 1. Statistical analyses of p-AKT and p-FOXO3a (ng/L) in all the studied groups

Tissue p-AKT	Untreated EC-bearing mice	Irradiated mice	FAEE supplemented mice			Irradiated+ FAEE treated mice		
			250	500	1000	250+6Gy	500+6Gy	1000+6Gy
Mean ± SE	**713.5 ± 7.8**	**487.1 ± 9.7**	**603.2 ± 10.4**	**562.4 ± 15**	**376.5 ± 8.3**	**452.1 ± 6.9**	**369.25 ± 2.1**	**482.3 ± 10.4**
P1	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	
P2			0.000*	0.000*	0.000*	0.31	0.000*	1.000
Tissue p-FOXO3a								
Mean ± SE	**705.7 ± 27**	**473.2 ± 25**	**596.9 ± 16.**	**438.2 ± 15.5**	**363.5 ± 16**	**352 ± 14**	**227 ± 19.4**	**497 ± 2.8**
P1		0.000*	0.002*	0.000*	0.000*	0.000*	0.000*	0.000*
P2			0.000*	0.818	0.002*	0.001*	0.000*	0.984

P1: P value comparing between the untreated EC-bearing mice and each studied group; *P2: P* value comparing between the groups treated with IR only and each studied group; *: Statistically significant at $P \leq 0.05$; **Untreated EC-bearing mice**: Untreated Ehrlich Carcinoma (EC)-bearing mice; **Irradiated mice**: EC-bearing mice treated with 6 Gray single-dose of ionizing radiation; **FAEE supplemented mice**: EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg); **Irradiated+ FAEE treated mice**: EC-bearing mice supplemented with different doses of FAEE plus 6 Gray single-dose ionizing radiation; SE: Standard error

dependent kinase inhibitors.[11] In different cancers, loss of FOXO3a has been detected and its cellular localization and phosphorylation status are known to have prognostic significance in breast[12] and ovarian cancers.[13]

Desert plants are unique adaptation from environmental conditions. Fagonia is a genus of plant in the family of zygophyllaceae with about 20 species around the Mediterranean to India, southern Africa, California, and Chili. The local name of Fagonia is dhamasa.[14] Phytochemical investigation of Fagonia species has yielded a

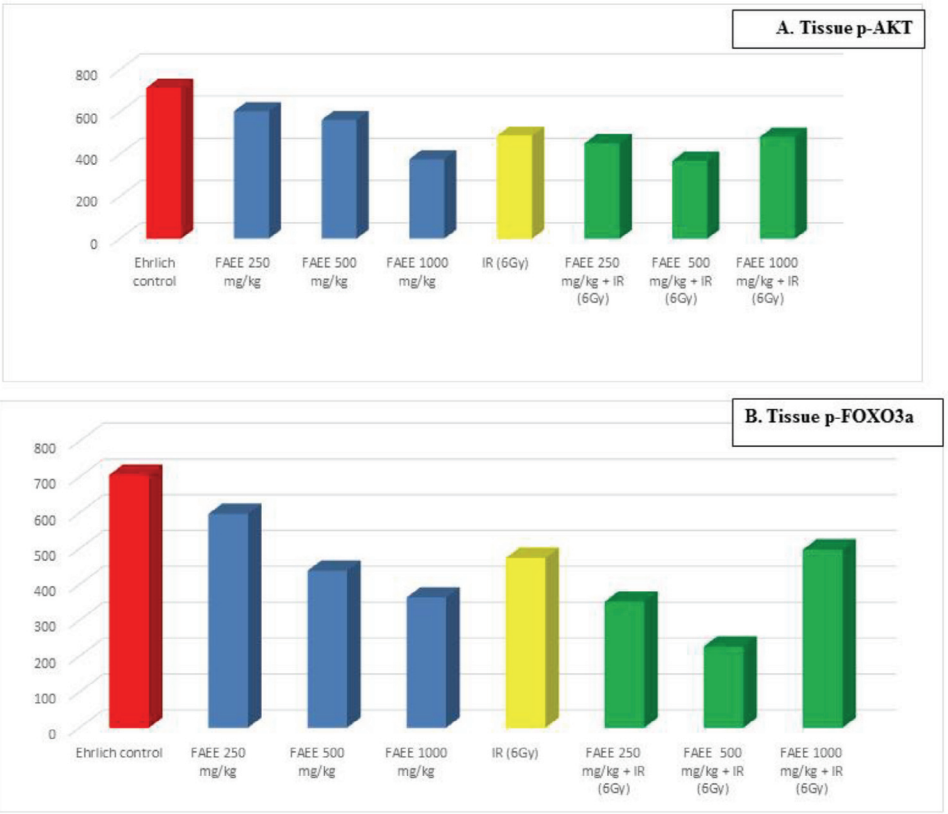

Figure 2. A) The bar chart illustrates the mean values of tissue p-AKT in all the studied groups; B) The bar chart represents the mean values of tissue p-FOXO3a in all the studied groups.
Ehrlich control: Untreated Ehrlich carcinoma (EC)-bearing mice
FAEE: EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg)
IR (6Gy): EC-bearing mice treated with 6 Gray single-dose of ionizing radiation
FAEE+IR (6Gy): EC-bearing mice supplemented with different doses of FAEE plus 6 Gray single-dose of ionizing radiation

Table 2. Statistical analyses of BCL-2 gene expression in all the studied groups

Bcl-2 gene expression	Untreated EC-bearing mice	Irradiated mice	FAEE supplemented mice			Irradiated+ FAEE treated mice		
			250	500	1000	250+6Gy	500+6Gy	1000+6Gy
Mean ± SE	**4.1 ± 0.1**	**1.1±0.02**	**0.37 ± 0.009**	**0.24 ± 0.01**	**0.06 ± 0.002**	**0.26 ± 0.03**	**0.08 ± 0.07**	**0.36 ± 0.02**
P1		0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
P2			0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

P1: P value comparing between the untreated EC-bearing mice and each studied group; *P2: P* value comparing between the groups treated with IR only and each studied group; *: Statistically significant at $P \leq 0.05$; **Untreated EC-bearing mice**: Untreated Ehrlich carcinoma (EC)-bearing mice; **Irradiated mice**: EC-bearing mice treated with 6 Gray single-dose of ionizing radiation; **FAEE supplemented mice**: EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg); **Irradiated+ FAEE treated mice**: EC-bearing mice supplemented with different doses of FAEE plus 6 Gray single-dose of ionizing radiation; SE: Standard error

variety of chemical constituents, including flavonoids, fatty acids, alkaloids, proteins, and amino acids.[15] Antimicrobial, antioxidant, and antihaemorrhagic activities have been documented for Fagonia species.[16] Not enough is known about its anticancer activity. Thus, we aimed to investigate the antitumor potential and radiosensitizing effect of *Fagonia arabica* ethanolic extract (FAEE) on the mice bearing solid Ehrlich carcinoma (EC).

Materials and Methods

This experimental animal study was conducted on 80 solid Ehrlich carcinoma-bearing mice that were divided into four groups: Group I: 10 EC-bearing mice as untreated controls; Group II: 10 EC-bearing mice exposed to a single dose of ionizing radiation (IR) at tumor localization (6Gy at a dose rate 300 Mu/min); Group III: 30 EC-bearing mice, each 10 mice received different doses of FAEE (250, 500, and 1000 mg/kg/day) once daily via oral intubation for a period of 14 days; Group IV: 30 EC-bearing mice, each 10 mice received different doses of FAEE (250, 500, and 1000 mg/kg/day) 2 hours before exposure to a single dose of IR at tumor localization (6Gy at a dose rate 300 Mu/min), FAEE administration was then continued once daily via oral intubation

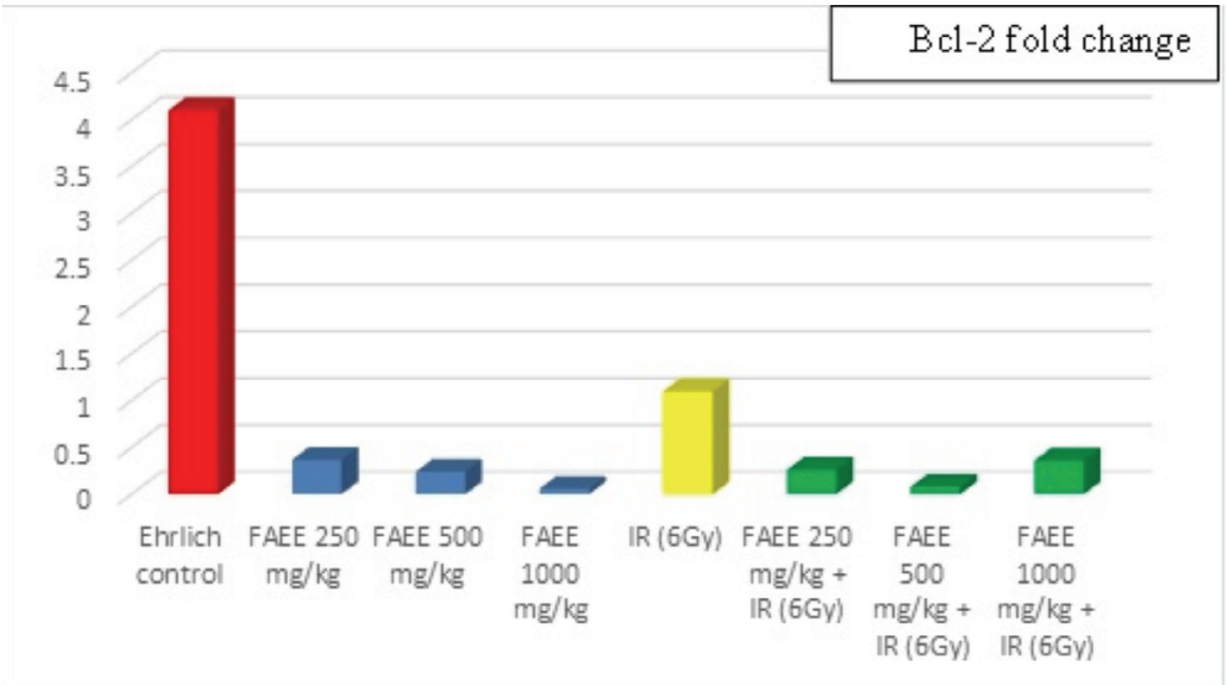

Figure 3. The bar chart represents fold changes in BCL-2 gene expression in all the studied groups.
Ehrlich control: Untreated Ehrlich carcinoma (EC)-bearing mice
FAEE: EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg)
IR (6Gy): EC-bearing mice treated with 6 Gray single-dose of ionizing radiation
FAEE+IR (6Gy): EC-bearing mice supplemented with different dose of FAEE plus 6 Gray single-dose of ionizing radiation

Table 3. Statistical analyses of ALT and AST activity in all the studied groups

ALT	Untreated EC-bearing mice	Irradiated mice	FAEE supplemented mice			Irradiated+ FAEE treated mice		
			250	500	1000	250+6Gy	500+6Gy	1000+6Gy
Mean ± SE	**96.6 ± 3**	**107 ± 3.4**	**39.2 ± 1.4**	**32.6 ± 0.8**	**26.5 ± 2.6**	**33.7 ± 1.34**	**28.7 ± 3.06**	**27 ± 1.5**
P1		0.03*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
P2			0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
AST								
Mean±SE	**104.2 ± 1.06**	**109 ± 2.2**	**41.3 ± 1.7**	**30 ± 0.46**	**23 ± 0.46**	**34.7 ± 0.8**	**26.1 ± 1.09**	**24.2 ± 0.7**
P1		0.356	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
P2			0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

P1: *P* value comparing between the untreated EC-bearing mice and each studied group; *P2*: *P* value comparing between the groups treated with IR only and each studied group; *: Statistically significant at $P \leq 0.05$; **Untreated EC-bearing mice:** Untreated Ehrlich carcinoma (EC)-bearing mice; **Irradiated mice**: EC-bearing mice treated with 6 Gray single-dose of ionizing radiation; **FAEE supplemented mice:** EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg); **Irradiated+ FAEE treated mice:** EC-bearing mice supplemented with different dose of FAEE plus 6 Gray single-dose of ionizing radiation; SE: Standard error

for 14 consecutive days.

Animals

The animals were purchased from the animal house of Medical Technology Center, Medical Research Institute, Alexandria University. The mice used in this study were adults with an average age of about 10-12 weeks old and weighting 20-25 g. They were housed 10 per cage throughout the experiment and were maintained on a standard laboratory diet and water. All procedures were performed by the Institutional Animal Care and Use Committee (IACUC)-Alexandria University (Ethics code: AU01221122412). The research also adheres to the ARRIVE guidelines and the National Research Council's guide for the care and use of laboratory animals.

Collection and preparation of Fagonia arabica plant extract

Fagonia arabica was collected from wadi habitat at El-Dabaa region (180 Km west of Alexandria city). The plant materials were dried in shade, in a relatively dark area. The whole plant was powdered using Wiley mill, considering smaller particles to be suitable for efficient solvent extraction. Ethanolic extract was prepared using a standard plant extract preparation (maceration) protocol. Specifically, 400 g of the plant material was suspended in 2000 ml solvent (1:5 w/v) for three days with constant agitation. Thereafter, the solution was filtered, and the solvents were

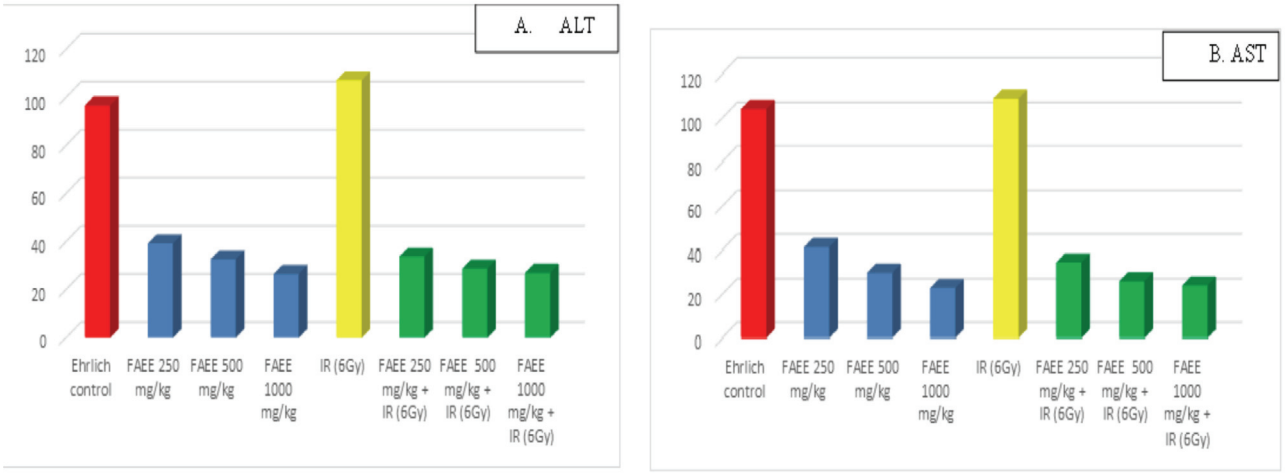

Figure 4. A) The bar chart demonstrates the mean values of ALT activity in all the studied groups; B) The bar chart shows the mean values of AST activity in all the studied groups.
Ehrlich control: Untreated Ehrlich carcinoma (EC)-bearing mice
FAEE: EC-bearing mice supplemented with different doses of *Fagonia arabica* ethanolic extract (FAEE) (250, 500, and 1000 mg/kg)
IR (6Gy): EC-bearing mice treated with 6 Gray single-dose of ionizing radiation
FAEE+IR (6Gy): EC-bearing mice supplemented with different doses of FAEE plus 6 Gray single-dose of ionizing radiation

evaporated using a rotary evaporator under reduced pressure. The dried extract was stored at -20°C until use.[17]

Acute toxicity test for plant extract

The oral acute toxicity study of the extract was carried out in the Balb-C mice using up and down procedure as per OECD, 2001. The mice received the extract at various doses (300, 600, 1000, 2000, 3000, and 4,000 mg/Kg) orally by gavage. They were followed for toxic symptoms continuously for the first 48 h after dosing.[18] The acute toxicity test was crucial for the determination of plant extract, test dose, and safety. All the tested doses of the plant extract proved safe, showing no signs of behavioral changes and morbidity.

Transplantation of Ehrlich solid tumors in the mice

A line of Ehrlich ascites carcinoma (EAC) cells (obtained from the National Cancer Institute, Cairo University) was maintained intraperitoneally in the female Balb-C mice. The intraperitoneal fluid containing Ehrlich ascites cells was collected. RBCs in the tumor fluid were disrupted by giving a hypotonic shock with 0.4% NaCL for 15 min and centrifuged. The pellet was washed in normal NaCL (0.9%) and resuspended in phosphate buffered saline (PBS, 130 mM NaCL, 2 mM KCL, 7.8 mM $Na_2HPO_4.2H_2O$, 1.4 mM $KH_2PO_4.2H_2O$ made in H_2O, PH7.4). Approximately 2×10^6 (diluted in PBS) in 0.2 ml were subcutaneously injected into the right

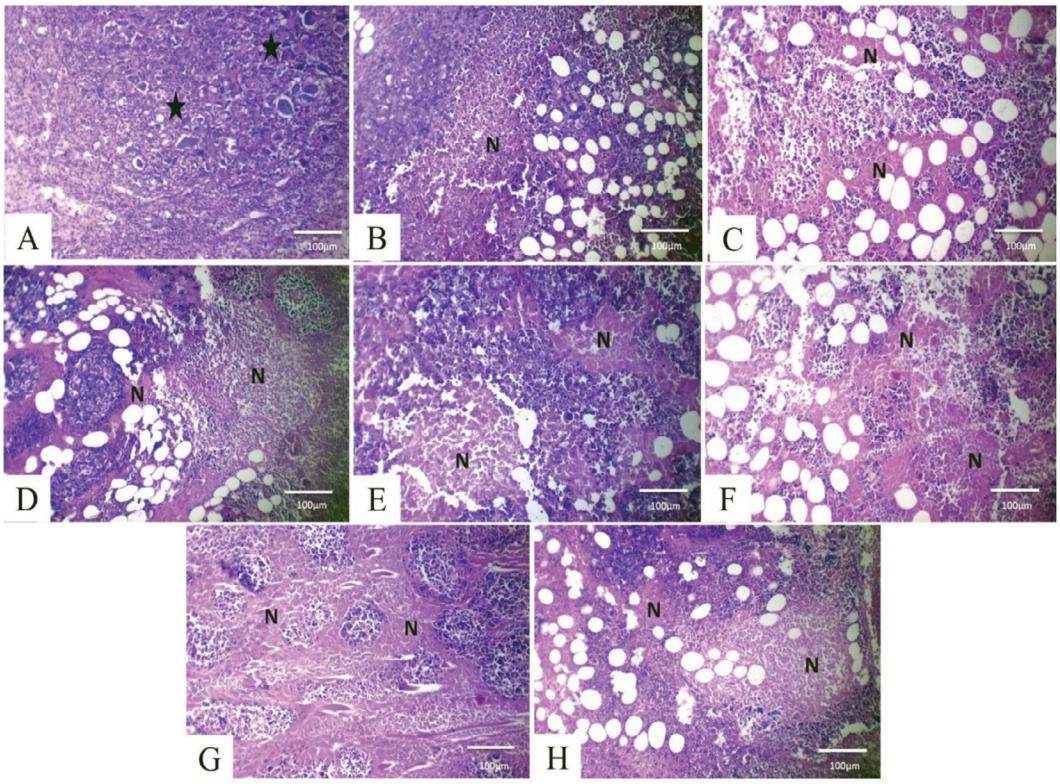

Figure 5. A) The untreated solid EC tumor tissue shows large polygonal and polymorphic hyperchromatic malignant cells present in the subcutaneous tissue (stars); B) Solid tumor of the 250 mg/kg FAEE-supplemented group shows apoptotic tumor cells and focal areas of mild necrosis (N); C) Solid tumor of the 500 mg/kg FAEE-supplemented group illustrates degenerated areas of tumor with highly pyknotic nuclei and focal areas of moderate necrosis (N); D) Solid tumor of the 1000 mg/kg FAEE-supplemented group shows wide areas of regression of tumor cell invasion, and more apoptotic tumor cells and moderate diffuse necrosis (N); E) Solid tumor of the X-irradiated group demonstrates tumor cell remnants and areas of moderate diffuse necrosis (N); F) Solid tumor of the X -irradiated group supplemented with 250 mg/kg FAEE shows degenerated areas of apoptotic EC tumor cells with pyknotic nuclei, inflammation, and severe diffuse necrosis (N); G) Solid tumor of the X -irradiated group supplemented with 500 mg/kg FAEE depicts degenerated areas of apoptotic EC tumor cells with pyknotic nuclei and severe diffuse necrosis (N); H) Solid tumor of the X -irradiated group supplemented with 1000 mg/kg FAEE shows degenerated areas of apoptotic EC tumor cells with pyknotic nuclei and severe diffuse necrosis (N).
EC: Ehrlich control; FAEE: Fagonia arabica ethanolic extract

thigh of the lower limb of the mice and allowed to develop into a solid tumor for 10 days. The tumor volume was measured with a Vernier caliper and the mice with a palpable solid tumor mass of approximately 100 mm^3 (developed after 10 days from EC-inoculation) were subsequently used in the study.[19]

Tumor volume (mm^3) = 1 / 2ab2 (where a is the tumor diameter and b is the tumor minor diameter).

Tumor growth rate = $\frac{\text{measurement of tumor size-starting tumor size}}{\text{starting tumor size}}$

Radiation exposure

Irradiation with x-ray was carried out in a linear accelerator (PRIMUS, Mid-Energy, Toshiba Medical systems, Tokyo, Japan) with x-ray energy outputs of dose rates of 300 Mu/min. The mice were secured in plastic holders with an opening above the tumor region. The collimated x-ray beam irradiated an area of 24 × 24 mm^2 at the tumor site, which was large enough to cover the entire area of the largest tumor.

Blood sample collection

At the end of the experiment, 2 ml of blood samples were collected from the heart of each mouse with a syringe. The blood sample was left to coagulate at 25ºC for 15 min and serum was separated by centrifugation. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities, malondialdehyde (MDA), and total antioxidant capacity (TAC) were determined colorimetrically according to the manufacturer's instructions (Biodiagnostic, Egypt).

Preparation of tissue homogenate

Immediately after collecting the blood, solid tumors were collected, washed in ice cold saline, blotted dry, and weighted. They were then homogenized in 0.2 M phosphate buffer PH 7.4 (1:9 w/v). Homogenization was done using a glass homogenizer with a loose filling Teflon pestle in an ice bath (4ºC). Centrifugation of the homogenates was carried out at 4º C for 10 minutes at 6000 × g to obtain the supernatant for determination of p-AKT and p-FOXO3a with ELISA according to the manufacturer's instructions (Shanghai Coon Koon Biotech, China).

Preparation of tumor tissue for histopathology study

Small pieces of the transplanted tumor were fixed in 10% neutral buffered formalin for 24 h. They were washed under running tap water for 24 h, dehydrated in ascending series of ethyl alcohol, cleared in xylene, and embedded in paraffin wax. Sections of 5 microns thick were cut using rotary microtome and stained with Mayer's haematoxylin and eosin (H&E) stain. That paraffin sections were brought down to distilled water, stained with haematoxylin for 7 minutes, then counterstained with eosin for 3 minutes. Afterwards, they were dehydrated in ascending series of alcohol, cleared in xylene, and mounted in Canada balsam.[20]

Determination of p-AKT in tumor tissue homogenate using ELISA

A double-antibody sandwich ELISA was used to assay the level of p-AKT in the mice samples. p-AKT was added to monoclonal antibody enzyme well, which was pre-coated with mouse p-AKT monoclonal antibody. Primarily, it was incubated; p-AKT antibody was then labeled with biotin and combined with Streptavidin-HRP to form immune complex. It was incubated and washed again so that the uncombined enzyme is removed. At this stage, chromogen solutions A and B were added, the color of the liquid changed into blue, and affected by the acid, the color finally became yellow. The chroma of color and the concentration of p-AKT in the sample were positively correlated.

Determination of p-FOXO3a in the tumor tissue homogenate using ELISA

A double-antibody sandwich ELISA was used to assay the level of p-FOXO3a in the mice samples. p-FOXO3a was added to monoclonal antibody enzyme well, which was pre-coated with p-FOXO3a monoclonal antibody. Following incubation, p-FOXO3a antibody labeled with biotin was added and combined with Streptavidin-HRP to form immune complex. Subsequently, incubation and washing were carried out again so that the uncombined enzyme is removed. Chromogen solutions A and B were added; the color of the liquid then changed into blue, and under the effect of the acid, the color finally became yellow. The chroma of color and the

concentration of the mouse p-FOXO3a in the sample were positively correlated.

BCL-2 gene expression analysis

Total RNA was extracted from Ehrlich tumor tissues using RNeasy mini kit (Qiagen, Germany) and the expressions of BCL-2 was determined via real-time polymerase chain reaction (RT-PCR) using Rotor Gene SYBR Green PCR Kit (Qiagen, Germany) according to the manufacturer's protocols.

Statistical analyses

Data were supplied to the computer and analyzed using IBM SPSS software package version 20 (Armonk, New York: IBM Corp). The data were expressed as mean values ± SE and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test to assess the significant differences among the treatment groups. The criterion for statistical significance was set at $P < 0.05$ for the biochemical data. The significance of the obtained results was assessed at the level of 5%.

Results

Effect of FAEE and IR treatment on body weight, tumor volume, and tumor growth rate

The average body weight, tumor volume, and tumor growth rate in all studied groups are illustrated in figures 1a-c. The body weight was found to be significantly increased in the untreated EC-bearing mice after 14 days due to the increase in the tumor size. Administration of FAEE without or with IR significantly decreased the body weight gain in a dose-dependent manner when compared with the untreated control mice. At the end of the experiment, the size of the solid tumors in the Ehrlich control mice augmented from 100 to 500 mm^3. A gradual significant decrease was observed in the tumor volume following administration of FAEE and / or IR, showing a synergistic suppressive effect. The tumor growth rate was found to be significantly increased in the untreated EC-bearing mice. Administration of FAEE at the doses of 250, 500, and 1000 mg/kg significantly decreased the tumor growth rate when compared with the untreated mice. IR only and co-treatment of FAEE plus IR significantly decreased tumor growth rate.

Effect of FAEE and radiation treatment on tissue p-AKT and p-FOXO3a

Statistical analyses of tissue p-AKT and p-FOXO3a (ng/l) in all the studied groups are listed in table 1 and figures 2a, b.

As presented in table 1, the concentration of tissue p-AKT in the EC-bearing mice treated with IR only ($P1 < 0.001$), those supplemented with different doses of FAEE (250, 500, and 1000 mg/kg) ($P1 < 0.001$), and the mice supplemented with different doses of FAEE in combination with IR (250mg/kg + 6Gy, 500mg/kg + 6Gy and 1000 mg/kg + 6Gy) ($P1 < 0.001$) were statistically significantly lower than that in the untreated EC control mice.

Compared with the EC-bearing mice treated with IR only, the level of tissue p-AKT in the mice supplemented with either 250 mg/kg or 500 mg/kg FAEE was significantly higher ($P2 < 0.001$), whereas this level in those supplemented with 1000 mg/kg FAEE was significantly lower ($P2 < 0.001$). With respect to the group of the mice supplemented with FAEE in combination with IR (500mg/kg + 6Gy), the level of this parameter was significantly lower than that in the group treated with IR only ($P2 < 0.001$). On the other hand, the mice supplemented with either 250 or 1000 mg/kg FAEE in combination with radiation (6Gy) showed insignificant differences when compared with those treated with IR (6Gy) alone ($P2 = 0.31$, $P2 = 1.000$, respectively).

With respect to tissue p-FOXO3a, the treatment with IR only ($P1 < 0.001$), different doses of FAEE ($P1 < 0.001$), and combination of different doses of FAEE with IR ($P1 < 0.001$) significantly decreased this parameter in comparison with untreated control mice. Compared with the EC-bearing mice treated with IR only, the level of this parameter in the mice supplemented with 250 mg/kg FAEE was significantly higher ($P2 < 0.001$), whereas this level in those supplemented with 1000 mg/kg FAEE was significantly lower ($P2 = 0.002$). An insignificant difference was observed between the groups of the mice supplemented with 500 mg/kg FAEE and those treated with IR only ($P2 = 0.818$).

Effect of FAEE and radiation treatment on BCL-2 gene expression

A statistical analysis of BCL-2 gene expression in all the studied groups is illustrated in table 2 and figure 3.

As can be seen in table 2, the treatment with IR only ($P1 < 0.001$), different doses of FAEE ($P1 < 0.001$), and the combination of different doses of FAEE with IR ($P1 < 0.001$) significantly decreased BCL-2 gene expression compared with its expression in the untreated EC-bearing control mice. The treatment with different doses of FAEE (250, 500, and 1000 mg/kg) significantly decreased BCL-2 gene expression compared with its expression in the mice treated with IR alone ($P2 < 0.001$). Likewise, BCL-2 gene expression in the EC-bearing mice supplemented with different doses of FAEE in combination with IR (250mg/kg + 6Gy, and 500mg/kg + 6Gy) was statistically significantly lower than that in the mice treated with IR alone ($P2 < 0.001$).

Effect of FAEE and radiation treatment on ALT and AST activity

Statistical analysis of serum ALT and AST activity in all the studied groups are illustrated in table 3 and figures 4a, b.

It was observed that the untreated EC-bearing mice had higher levels of ALT and AST. Moreover, exposure to IR significantly increased ALT and AST. Supplementation of the EC-bearing mice with different doses of FAEE alone or in combination with IR significantly decreased both ALT and AST levels.

Histopathological results

Examination of the tumor tissue of the untreated solid EC that present at the site of Ehrlich tumor cells inoculation in the subcutaneous tissue revealed protruded masses of rapid progression. Light microscopic examination of these masses exhibited entirely viable tumors formed of sheets of large polygonal and polymorphic hyperchromatic malignant cells with bizarre giant forms; no evidence of necrosis was identified (Figure 5a).

Solid tumors of the EC-bearing mice supplemented with 250 mg/kg FAEE showed polygonal, polymorphic hyperchromatic malignant cells, along with apoptotic tumor cells and focal areas of mild necrosis (Figure 5b). Similarly, solid tumors of the EC-bearing mice supplemented with 500 mg/kg FAEE showed degenerated areas of tumor with highly pyknotic nuclei and focal areas of moderate necrosis (Figure 5c). Moreover, solid tumors of the EC-bearing mice supplemented with 1000 mg/kg FAEE showed regression of tumor cell invasion, more apoptotic tumor cells, and moderate diffuse necrosis (Figure 5d).

Solid tumor of the X-irradiated EC-bearing mice indicated tumor cell remnants and areas of moderate diffuse necrosis (Figure 5e). Following supplementation of the X-irradiated EC-bearing mice with 250 (Figure 5f), 500 (Figure 5g), and 1000 mg/kg (Figure 5h) FAEE, respectively, degenerated areas of apoptotic EC tumor cells with pyknotic nuclei and severe diffuse necrosis were observed. In addition, a more pronounced effect on tumor regression was noticed in the EC-bearing mice supplemented with 500 mg/kg FAEE.

Discussion

The present study revealed that FAEE and radiation alone or in combination had a potent antiproliferative and cytotoxic effect against the EC-bearing mice in a dose-dependent manner. This anticancer effect was confirmed by a significant decrease in tumor volume, tumor growth rate, and weight gain as compared with the untreated EC-bearing mice. The maximum antiproliferative effect was produced at a dose of 1000 mg/Kg of FAEE. These results were further confirmed by the histopathological examination of solid tumor of the EC-bearing mice stained with H&E, where the EC-bearing mice supplemented with 1000 mg/kg FAEE showed regression of tumor cell invasion and more apoptotic tumor cells and moderate diffuse necrosis. This suggests that FAEE may be an effective antitumor agent against breast cancer. Several naturally occurring substances exert anticancer effects by induction of apoptotic signaling. Cancer is a pathological state involving uncontrolled proliferation of tumor cells. Reduced tumor volume and body weight indicated a

reduction in tumor proliferation and induction of apoptosis.[21] To the best of our knowledge, our study is the first to explore the anticancer effect of *Fagonia arabica* extract on an in vivo model.

Radiotherapy is currently estimated to be used on around 50% of cancer patients and contributes to about 40% of curative treatment for cancers.[22] However, like chemotherapeutic agents, ionizing radiation does not affect all the target cells, which can lead to severe side-effects in the surrounding tissues. Moreover, a substantial number of human malignant tumor cells have a bad response to ionizing radiation. Accordingly, the effect of radiation on tumor tissues can be optimized by adding radio-sensitizing agents, in order to achieve a greater degree of tumor damage than expected from radiation only, thereby minimizing large doses of radiation and also sparing normal tissues.[23] *Fagonia arabica* is a tropical herb belonging to the family of Zygophyllaceae distributed with a high concentration in the horn of the African region.[14] Despite the small flow of data on the promising potential of *Fagonia arabica*, no studies have to date investigated its anticancer potential.

Notably, Lam et al.[24] reported (in vitro) that Fagonia cretica extract induces cell cycle arrest and apoptosis in two phenotypically distinct breast cancer cell lines. Mainly, extract activity involves DNA damage and p53-induction, but is not fully dependent on p53 functionality. The antiproliferative, cytotoxic and antitumor effect of Zygophyllaceae family member Tribulus terrestris against breast cancer cells was documented. Recently, Patel et al.[25] analyzed the impact of the methanolic leaf and seed extracts as well as saponin from the leaves and seeds of Tribulus terrestris on the expression of selected group of genes from the apoptotic pathway in MCF-7 cells and concluded that Tribulus terrestris extracts may exert their anticancer effect by triggering more than one apoptotic pathway. As well, Peganum harmala (another family member) seed extract was exposed on an MDA-MB-231 cancer cell line and its growth inhibition was followed through both morphological changes observation and following genes involved programmed cell death. Markedly, it induced cell death and decreased the cell growth in the breast cancer cell line. The cell death was caused by apoptosis, which was triggered by both intrinsic and extrinsic pathways which suggest that herb might be useful for preventing the development of tumors.[26]

The current study revealed that the combination of different doses of FAEE with IR significantly increases radiation-induced tumor damage reflected by the decrease in the tumor volume and tumor growth rate in comparison with the mice treated with IR alone. Our histopathological results demonstrated that the X-irradiated EC-bearing mice showed tumor cell remnants and areas of moderate diffuse necrosis. Following supplementation of the X -irradiated mice with either 250, 500, or 1000 mg/kg FAEE, degenerated areas of apoptotic EC-tumor cells with pyknotic nuclei and severe diffuse necrosis were observed. The most pronounced effect on tumor regression was noticed by administration of 500 mg/kg FAEE. This suggested that FAEE exerts radiosensitizing effect, especially at a dose of 500 mg/kg. Radiosensitizers are compounds that when combined with radiation, achieve greater tumor inactivation than expected from the additive effect of each modality.[27] It was noticed that saponins, structurally similar to diosgenin existing in *Fagonia arabica*, may block cell cycle, suppress proliferation, and induce apoptosis in human sarcoma cell lines.[28]

PI3K/AKT pathway is a cell survival pathway important for normal cell growth and proliferation. A great deal of research on breast cancer has shown that deregulation of this pathway is implicated in tumorigenesis; hence, this has become an important target for breast cancer treatment. For note, phosphorylated AKT is an attractive molecular target since it contributes to the development of breast cancer and confers resistance to conventional therapies.[29] FOXO3a is an important downstream effector of the PI3K/AKT pathway. AKT controls transcription of FOXO3a target genes through modulation of FOXO3a activity by phosphorylating three conserved threonine/ serine residues, leading to deactivation and release of FOXO3a from DNA

and translocation to the cytoplasm. Cytosolic retention of FOXO3a prevents the transactivation of downstream target genes, such as Bim and p27Kip1, which induce tumor cell apoptosis.[30]

In this study, we observed significant elevation of p-AKT and p-FOXO3a in the untreated EC-bearing mice. On the other hand, we noticed a significant decrease in the concentration of p-AKT and p-FOXO3a in the IR treated mice or after FAEE administration. The highest inhibition was observed at 1000 mg/kg, which significantly decreased p-AKT and p-FOXO3a compared with the mice treated with IR alone. We hypothesized that inhibition of AKT phosphorylation by FAEE would lead to nuclear accumulation of FOXO3a, increased transcription of downstream responsive genes, and apoptosis of tumor cells.

There are a few studies on the reduction of p-AKT caused by Fagonia extract. Lam et al.[24] showed that an aqueous extract of Fagonia cretica is able to induce cell cycle arrest and apoptosis in wild type p53 MCF-7 and mutant p53 MDA-MB-231 cells. In addition, extract treatment induces FOXO3a expression, which may be attributed to direct DNA damage or induction of DNA repair pathways. Another study demonstrated that harmine (the active constituent in Peganum harmala) induced cell death and growth inhibition in human colorectal carcinoma SW620 cells. Importantly, the study recorded that harmine decreased the levels of p-AKT and p-ERK, and this inhibition of the PI3K/AKT and ERK signaling pathways may be involved in harmine-induced cell cycle arrest and apoptosis in SW620 cells.[31]

Regarding the effect of FAEE on BCL-2 gene expression, our results showed that FAEE alone or in combination with IR significantly lowered the gene expression of BCL-2 compared with that in the untreated EC-bearing mice. This result suggested that FAEE induces apoptosis through inhibition of the anti-apoptotic gene BCL-2. Preliminary phytochemical screening of Fagonia extract showed the presence of saponins, steroid, tannin, triterpenes, alkaloids, phenols compound, and flavonoids. Flavonoids have been found to possess antimutagenic and antimalignant effects. Moreover, they have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis. The antitumor properties of the extracts may be owing to these compounds.[31]

A decrease in endogenous antioxidant enzymes with enhanced free radical generation and MDA is well documented in carcinogenesis.[32] Several reports have documented that MDA, the end product of lipid peroxidation, is higher in cancer tissues than in normal tissues.[33] Similarly, in the present work, the increase in MDA and decrease in the level of TAC were observed in the untreated EC-bearing mice. However, FAEE administration significantly decreased MDA levels and increased TAC levels in comparison with those in the untreated EC-bearing mice. This result demonstrated the potential of FAEE treatment in attenuation of oxidative stress via free radical scavenging activity in EC- bearing mice. *Fagonia arabica* has pronounced effects on the cancer cells, improving the native antioxidative defense system and the antioxidant property by decreasing lipid peroxidation and scavenging free radicals.

For the past several years, serum enzymes have been considered to play an important role as diagnostic markers in neoplasia and in determining the disease condition.[34] Several reports have revealed that EC causes liver damage and disturbances in hepatic cell metabolism, which lead to changes in serum enzymes activities.[35-37] In the present study, the elevated levels of AST and ALT in the untreated EC-bearing mice were observed. Such increase may be assigned to the hepatocellular damage by Ehrlich carcinoma. FAEE administration at all the doses restored the elevated biochemical parameters to the normal range, indicating the protective effect of FAEE against tumor cell-induced hepatotoxicity.

These results are in agreement with those reported by other studies, indicating a significant recovery of hepatic damage after treatment with Fagonia indica, which was evident from the decreased plasma levels of hepatic enzymes and recovered hepatic architecture. These results represented the hepatoprotective activity of Fagonia extract.[18,38]

Further investigation is needed to explore clinical applications of *Fagonia arabica* alone or in combination with available anticancer drugs in breast cancer and other cancers. Moreover, further research could be recommended aiming to identify the active phytochemical components of *Fagonia arabica* and their roles.

Conclusion

FAEE exhibits antiproliferative properties against EC-bearing mice, suggesting that FAEE may be an effective antitumor agent against breast cancer. The combination of different doses of FAEE with ionizing radiation significantly increased the radiation-induced tumor damage in comparison with that in the EC-mice treated with radiation only. This suggested that FAEE exerts radiosensitizing effect, especially at a dose of 500 mg/kg. FAEE alone or in combination with IR significantly decreased the concentration of p-AKT and p-FOXO3a and downregulated the gene expression of BCL-2 compared with those in the untreated EC-bearing mice. These effects may be involved in growth inhibition and apoptosis of EC. FAEE exerted an anti-oxidant activity and demonstrated a hepatoprotective effect against tumor cell-induced hepatotoxicity.

Conflict of Interest

None declared.

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