

# Phenolic Profile and *In Vivo* Cytotoxic Activity of Rice Straw Extract

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## ABSTRACT

**Background:** Previous work of our team exhibited that rice straw (RS) has antitumor activity *in vitro* and inhibit proliferation of liver, lung, prostate, and breast cancer human cell lines. In this work, we extended our research to screen the antitumor activity of RS ethanol extract as a single treatment and in the presence of combined radiotherapy with a low dose of gamma radiation against murine Ehrlich solid carcinoma (EAC) model. **Objective:** To evaluate the most common waste in Egypt RS to screen out its *in vivo* cytotoxic activity and as combined therapy with radiotherapy. **Method:** Tested sample RS was investigated for its content of phenolics by LC/MS/MS, in addition, ethanolic extracts of the tested sample were investigated as antitumor on female mice inoculated with EAC cells as a single treatment and in the presence of combined radiotherapy with a low dose of gamma radiation (LDR). **Results:** LC/MS/MS revealed that rice straw was rich in phenolic acids (vanillic, p-coumaric, ferulic, and sinapic acid) along with catechin and flavonoids aglycones (quercetin, apigenin, and kaempferol). Rice straw and/or exposure to a low dose of  $\gamma$ -radiation caused a marked suppression of tumor growth and induced significant reduction in VEGF level & in IL-6 level with significant elevation in IL-10 serum level. Rice straw caused a significant down regulation in the gene transcription level of MCL1 and b-catenin, and a significant up-regulation of Caspase-3 and Bax gene expression. RS extract and LDR (EC + RS + R group) revealed that there was a mild form of necrosis with severe apoptosis in the tumor cells. **Conclusion:** From the aforementioned results, it can be concluded that RS/LDR effectively and synergistically work towards inhibition of cancer cell proliferation. These findings were well supported with histopathological studies suggesting that RS/low dose gamma radiation can serve as a good therapeutic agent against cancer but still need further clinical studies.

**Key words:** Rice straw, Cytotoxic, LC/MS/MS, Phenolics, Histopathology

## INTRODUCTION

Rice is the major staple foods in half of the world. The milling of rice results in a 70% yield while unconsumed portions of the rice include rice straw (20%), rice bran (8%), and rice germ (2%). A major portion of this residue is disposed of by burning which causes environmental problems. Several secondary metabolites detected in unconsumed portions of the rice and residues include phenolics<sup>1</sup> and flavones.<sup>2</sup>

Plant extracts known for their antitumor activity are a useful tool in complementary medicine. As conventional medicine gained resistance, complementary natural products become of great interest recently. Rice straw is a byproduct of rice plantation and considered a problem in Egypt. Its medicinal properties are not fully identified, taking into consideration that it is an inexpensive plant source and one of the sources of environmental pollution in Egypt resulted from burning it. Rice straw extract contains phytochemicals known for their antioxidant and plays a role as anticancer agents by acting as reducing agents<sup>3</sup> and hydrogen donors, suppress the naturally produced free radicals and delays oxidative reactions such as lipid oxidation.<sup>4</sup> Combining radiotherapy with

chemotherapy enhance cancer treatment results for a more comprehensive attack. Chemoradiotherapy is able to enhance the efficacy of chemotherapy and radiotherapy. Guided treatments with plant natural products and radiotherapy are a potential approach in cancer therapy.<sup>5</sup>

Ehrlich ascites carcinoma is a type of spontaneous murine mammary adenocarcinoma adapted to ascites form and carried in outbred mice by serial intraperitoneal (i/p) passages. They are characterized by rapid proliferation in the mouse host for their lacking H-2 histocompatibility antigens. It is used as a preliminary level in chemotherapeutic studies to evaluate the antitumor efficacy of any compound due to the fact that it resembles human tumors..

Radiotherapy is considered an important therapeutic model for the treatment of cancer. Ionizing radiation either from environmental, natural, or occupational exposures enhanced different cellular responses depending upon rate and dose of exposure.<sup>6</sup> Various animal experiments demonstrated that low-dose irradiation is of beneficial and stimulatory effects, including anti-inflammatory autoimmune disease<sup>7</sup> declining inflammation markers C-reactive protein and matrix metalloproteinase-3,<sup>8</sup> activation of the innate and adaptive immune response,<sup>9</sup> inhibition in tumor growth or metastasis.<sup>8,10</sup>

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Previous work of our team<sup>11</sup> exhibited that Rice straw (RS) is of antitumor activity *in vitro*, inhibiting proliferation of liver, lung, prostate, and breast cancer human cell lines. In this work, we extended our research to screen the antitumor activity of RS ethanol extract against murine Ehrlich solid carcinoma model with the combined effect of low dose of gamma radiation.

## MATERIALS AND METHODS

### Plant material

The main materials used are rice straw from rice (*Oryza sativa*) L. Family: Poaceae, which is harvested from "private farm" at Zagazig, in EL-Sharkya, Egypt. Rice straw is the stalks left over after the grains of rice have all been removed in the milling process. Plant material was air-dried, reduced to coarse powder and kept in tightly closed amber colored glass containers. Voucher specimens are kept in the Department of Pharmacognosy, Faculty of Pharmacy Cairo University with a voucher number OR/SA/104. The authentication of all samples was kindly confirmed by Prof. Dr. Mohamed Nabil Attia, Head of Cultivated Plants Department, Faculty of Agriculture, Zagazig University.

### Method of extraction for LC/MS/MS

Dried powdered sample (1 g) was placed in a fit conical flask with 20 ml 2 M NaOH, shaken for 4 hrs, pH adjusted to 2 with 6 M HCl, centrifuge at 5000 rpm for 10 min, the supernatant collected was extracted twice with diethyl ether and ethylacetate 1:1. The organic phase was separated and evaporated at 45°C then re-dissolved in 2 ml methanol.<sup>12</sup>

### Method of extraction for biological activities

Dried plant sample of Rice straw (700 g) was repeatedly extracted with ethanol (70%) to exhaustion and complete extraction to yield 3.4 gm dry residue. The extract was filtered, concentrated and dried. The residue yielded was stored for further studies.

### Chemical characterization method

ESI-MS positive and negative ion acquisition mode was carried out on a XEVO TQD triple quadrupole instrument and Waters Corporation, Milford, MA01757 U.S.A, mass spectrometer

The sample (100 µg/mL) solution was prepared using high-performance liquid chromatography (HPLC) analytical grade solvent, filtered using a membrane disc filter (0.2 µm) then subjected to LC-ESI-MS analysis. Samples injection volume (10 µL) were injected into the UPLC instrument equipped with reverse phase ACQUITY UPLC - BEH C18 column (1.7 µm - 2.1 × 50 mm). Sample mobile phase was prepared by filtering using 0.2 µm disc membrane filter and degassed by sonication before injection. Mobile phase elution was made with the flow rate of 0.2 mL/min using gradient mobile phase comprising two eluents: eluent A is H<sub>2</sub>O acidified with 0.1% formic acid and eluent B is Me OH acidified with 0.1% formic acid. Elution was performed using gradient program: 90% A (2-5 min), 70% A (5-15 min), 30% A (15-22 min), 90% B (22-25 min), 100% B (26-32 min). The parameters for analysis were carried out using negative ion mode as follows: source temperature 150°C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440°C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h. Mass spectra were detected in the ESI negative ion mode between m/z 100–1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data.

### Experimental animals

Adult female Swiss albino mice weighing 25 ± 5 g were purchased from the Animal House Colony, National Research Center, Giza, Egypt. Mice were housed in the animal house of The National Centre for Radiation Research and Technology (NCRRT) in standard plastic cages. Mice were kept in the laboratory under controlled conditions of temperature (27 ± 20 °C) and humidity (60 ± 5%) with 12 h light/12 h dark cycles in well-ventilated cages with free access to standard laboratory pellet chow and water ad libitum. All the experimental procedures were carried out according to the principles and guidelines of the Ethics Committee of The National Centre for Radiation Research and Technology and Faculty of Pharmacy Cairo University under approval number (MP 2272).

### Cell line

The Ehrlich Ascites Carcinoma<sup>13</sup> cell line. The EAC cell line used in this study was supplied by The Egyptian National Cancer Institute (NCI), Cairo University (Giza, Egypt). The cell line was maintained by weekly intraperitoneal injection of 2.5 million cells in a volume of 0.2 mL per mouse in female Swiss albino mice. The EAC cells were diluted using physiological sterile saline solution, and cells were counted using the Bright-Line TM hemacytometer.

### Radiation facility

Mice whole-body gamma irradiation was performed at The National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority (Cairo, Egypt) using the 137 Cesium biological irradiator source (gamma-cell-40), Atomic Energy of Canada Limited (Chalk River, ON, Canada). Mice were exposed to a single dose of 0.25 Gy at a dose rate of 0.423 Gy/min at the 8<sup>th</sup> day after EAC inoculation.

### Investigation of the antitumor activity

#### Determination of LD50 using experimental animals

Determination of the LD50 of RS extract is an essential initial screening experiment performed with all new compounds in the evaluation of the toxic characteristics of the compound *in vivo* and provides information on health hazards likely to arise from short-term exposure to the drugs.<sup>14</sup> A total of 40 female Swiss albino mice were divided into four groups and administered with elevated doses of RS extract (100, 200, 300, 500, and 1000 mg/kg body weight) through intraperitoneal injection. Mice were observed for 1-7 days after administration for signs of toxicity, and LD50 was determined for the dose killed 50% of mice count number.<sup>15</sup> The optimum selected dose for evaluating the *in vivo* antitumor activity of the tested compounds was calculated approximately as (1/10) of its LD<sub>50</sub> value.

#### Tumor transplantation

To produce Ehrlich solid tumor in female Swiss albino mice, 0.1 mL of EAC cells (2-3 × 10<sup>6</sup> cells/mouse) was inoculated intramuscularly in the right thigh of the lower limb of a female mouse.

#### Experimental design

Eighty female Swiss albino mice were randomly divided into eight equal groups each of 10 mice as follows:

**Group 1 (Control):** mice were intraperitoneal (i.p.) injected with 0.2 ml of sterile saline.

**Group 2 (Ehrlich solid carcinoma (EC):** Mice were subcutaneously injected at the right thigh of the lower limb with 0.1 ml (2 × 10<sup>6</sup> cells of Ehrlich ascites carcinoma cells).

**Group 3 (RS):** Mice were i.p. injected with 0.2 ml (100 mg/Kg b.w.) of rice straw extract started from the 7<sup>th</sup> day after EAC inoculation for 21 days.

**Group 4 (R):** Mice the whole body were exposed to a single dose of (0.25 Gy) gamma irradiation at the 8<sup>th</sup> day after EAC inoculation.

**Group 5 (RS + R):** Mice were i.p. injected with rice straw extract as described in group 3 and exposed to gamma irradiation as mentioned in group 4.

**Group 6 (EC + RS):** Ehrlich solid tumor-bearing mice were i.p. injected with rice straw extract as described in group 3.

**Group 7 (EC + R):** EC bearing mice were exposed to gamma radiation as described in group 4.

**Group 8 (EC + RS + R):** EC bearing mice were injected i.p. with rice straw extract as described in group 3 and exposed to gamma radiation as described in group 4.

At the end of four weeks, all animals were sacrificed 24 hours after the last treatment under urethane anesthesia. Blood samples were collected, sera were separated and tumors were dissected.

### Tumor volume monitoring

The volume of solid tumor was measured by the Vernier caliper after inoculation with EAC cells. The tumor volume was calculated by the following equation: Tumor volume =  $\frac{1}{2}$  (length  $\times$  width<sup>2</sup>), where length is the greatest longitudinal diameter and the width is the greatest transverse diameter. The percent tumor growth inhibition was calculated on day 15 and 20 by comparing the average values of treated groups with that of the tumor-bearing control group (EC tumor volume - treated group tumor volume / EC tumor volume  $\times$  100). Tumor growth in saline-treated control animals was taken to be 100%.

### Biochemical analysis

The serum level of inflammatory cytokine IL-6 and IL-10 were measured by ELIZA kite following the instruction of the manufacturer (R&D system Inc. and My Biosource, USA). VEGF level was determined in tumor tissue homogenate using ELIZA kit produced by (Ray Biotech, Inc.)

### Gene expression of MCL-1, Caspase-3, $\beta$ catenin, and Bax

#### RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

To investigate the changes in mRNA expression for MCL-1, Caspase-3,  $\beta$  catenin, and Bax, total RNA was isolated from 50 mg of Ehrlich solid tumor tissue using TRIZOL1 reagent (Invitrogen) according

to Chomczynski.<sup>16</sup> Total RNA was isolated in accordance with the manufacturer's instructions and its integrity confirmed by agarose gel electrophoresis. Total RNA was converted to cDNA by using reverse transcriptase (Invitrogen) using template 1  $\mu$ g RNA. The PCR primers used in this study were purchased from Bioneer (Daejeon, Korea).

#### Quantitative real-time polymerase chain reaction (qPCR)

RT-PCR was performed in a thermal cycler step one plus (Applied Biosystems, USA) using Sequence Detection Software (PE Biosystems, CA). The oligonucleotides utilized in these experiments are listed in Table 1. A reaction mixture of total volume 25  $\mu$ l consisting of 2xSYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2  $\mu$ l of cDNA, PCR thermal-cycling conditions included an initial step at 95°C for 5 min; 40 cycles at 95°C for 20s, 60°C for 30 s, and 72 or 20 s. Relative expression of MCL-1, Caspase-3,  $\beta$  catenin, and Bax mRNA was calculated using the comparative Ct method according to Pfaffl.<sup>17</sup> Calculations were performed by calculating the values of  $\Delta$  cycle threshold ( $\Delta$ Ct) by normalizing the average Ct value of each treatment compared to the endogenous control  $\beta$ -actin.

#### Histopathological examination

All histological analyses were performed in routinely processed formalin-fixed, paraffin embedded tissue sections of 5 mm thickness. They were stained with hematoxylin-eosin stain and the slides were examined with a light microscope. Randomly selected fields were evaluated for cellular and tubular structures.

#### Statistical analysis

Data are reported as the mean  $\pm$  SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by LSD as a post-hoc test. The level of significance between mean values was set at  $P \leq 0.05$ . All statistical analyses were performed using SPSS software (Version 20.0).

## RESULTS

### Identification of phenolic compounds in rice straw by LC/MS/MS

The identification of the compounds (Table 2) was based on comparing the molecular ion peak in the negative [M-H]<sup>-</sup> and positive[M + H]<sup>+</sup> mode and the product ion [Ms-Ms ions] with the literature<sup>18-20</sup> and previously identified compounds.<sup>11</sup>

Rice straw was found to be rich in phenolic acids, vanillic, p-coumaric, ferulic, and sinapic acid along with catechin and flavonoids aglycones (quercetin, apigenin, and kaempferol). Ferulic acid and P-coumaric acid were the major identified phenolic acids followed by sinapic acid and vanillic acid contributing 19% and 15%, 2.4% and 2% of the area of identified compounds; respectively. On the other hand, catechin and flavonoidal aglycones (quercetin, apigenin, and kaempferol) recorded 4%, 7%, 3%, and 5%; respectively.

**Table 1: Sequence of the primers used for real-time PCR.**

Gene Symbol	Primer sequence	Gen bank accession number IDs
MCL-1	5'-TGTAAGGACGAAACGGGACT-3' 5'-AAAGCCAGCAGCACATTTCT-3'	NM_021846.2
Caspas-3	5'- TACCCTGAAATGGGCTTGTGT-3' 5'- GTTAACACGAGTGAGGATGTG -3'	XM_006253130.3
$\beta$ -catenin	5'- TGGACCCTATGATGGAGCATG-3' 5'- GGTCAGTATCAAACCAGGCCAG-3'	XM_008766691.2
Bax	5'- CGGCGAATTGGAGATGAACTGG-3' 5'- CTAGCAAAGTAGAAGAGGGCAACC-3'	NM_017059.2
$\beta$ -actin	5'-TGTTGTCCCTGTATGCCTCT-3' 3'-TAATGTACGCACGATTTCC-5'	NM_031144.3

### Acute toxicity study

The LD50 of RS extract was found to be 1000 mg/kg body weight. Accordingly, the safe dose was calculated by dividing LD50 by 10 that was nontoxic to be used in the *in vivo* study against solid Ehrlich tumor-bearing mice

### Tumor volume monitoring and percentage growth inhibition

Monitoring Ehrlich solid tumor size cleared that RS extract has an antitumor effect on Ehrlich solid tumor (Figure 1). The inoculation of EC cells in the thigh region of female mice produced a tumor with a mean size of 830 mm<sup>3</sup> on the 10<sup>th</sup> day after EC cells inoculation. Tumor size increase with time reaching 2646 and 2945 mm<sup>3</sup> at the 20<sup>th</sup> and 25<sup>th</sup> day of inoculation. RS and/or exposure to a low dose of  $\gamma$ -radiation (at 2<sup>nd</sup> day) caused a marked suppression of tumor growth compared to the corresponding EC group. Percentage of growth inhibition

was markedly increase in 15<sup>th</sup> and 20<sup>th</sup> day in all treated groups but RS combined with LDR recorded the highest inhibitory percentages (57.46% and 66.89%; respectively)

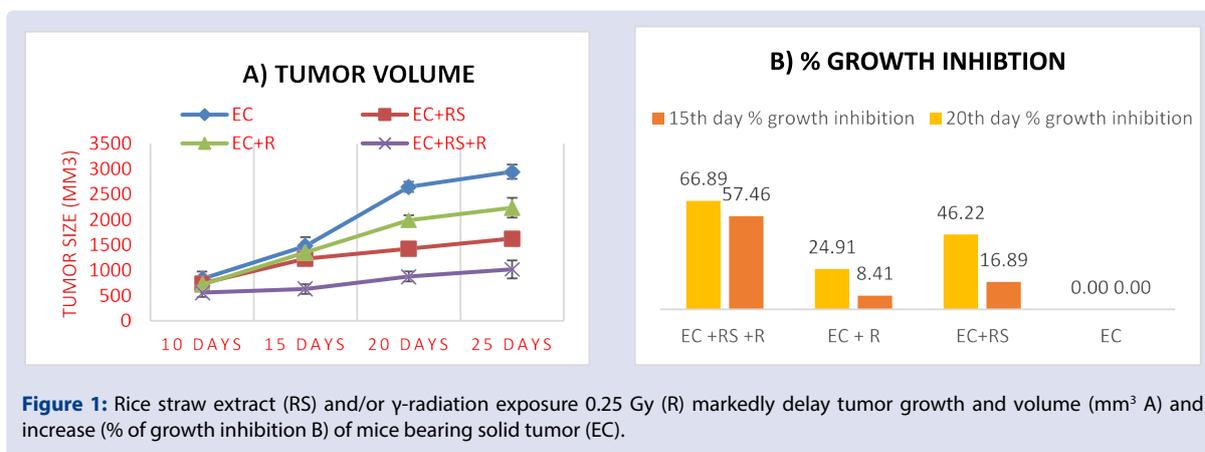
### Biochemical analysis

Comparing to Control, EC-bearing mice (EC group) showed a significant elevation of serum IL-6 and reduction in IL-10 (Figure 2A). Administration of RS extract and/or exposure to single low dose of gamma radiation (0.25Gy; R) to EC-bearing mice (EC + RS, EC + R, and EC + RS + R groups) induced significant reduction ( $p < 0.05$ ) in IL-6 level with significant elevation ( $p < 0.05$ ) in IL-10 serum level. While, VEGF (Figure 2B) was significantly elevated in the EC group while the level was reduced in the treated tumor-bearing groups (EC + RS, EC + R, and EC + RS + R groups).

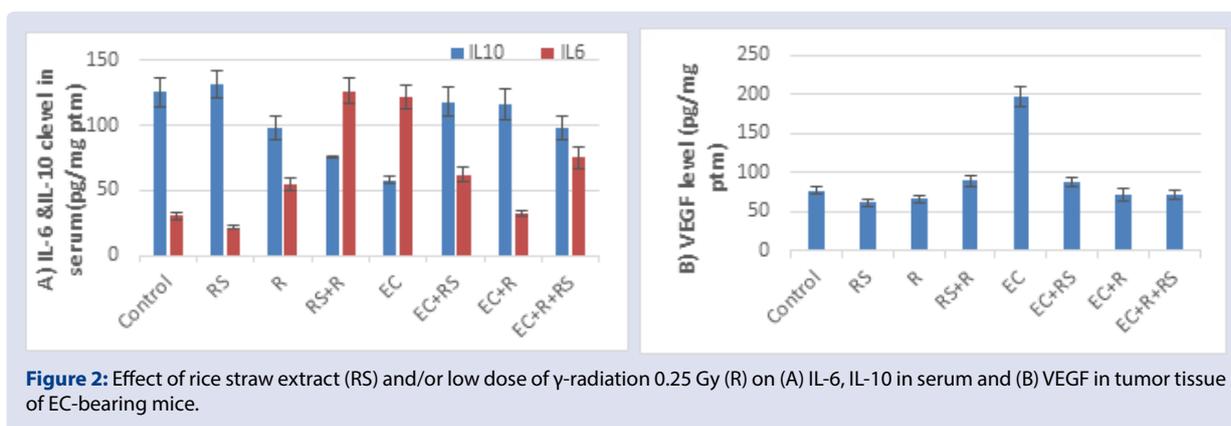
In further work we examined the role of RS and/or exposure to single low dose of gamma radiation (0.25 Gy) (Figure 3) on gene expression

**Table 2: LC/ms/ms of phenolic compounds identified in rice straw extract.**

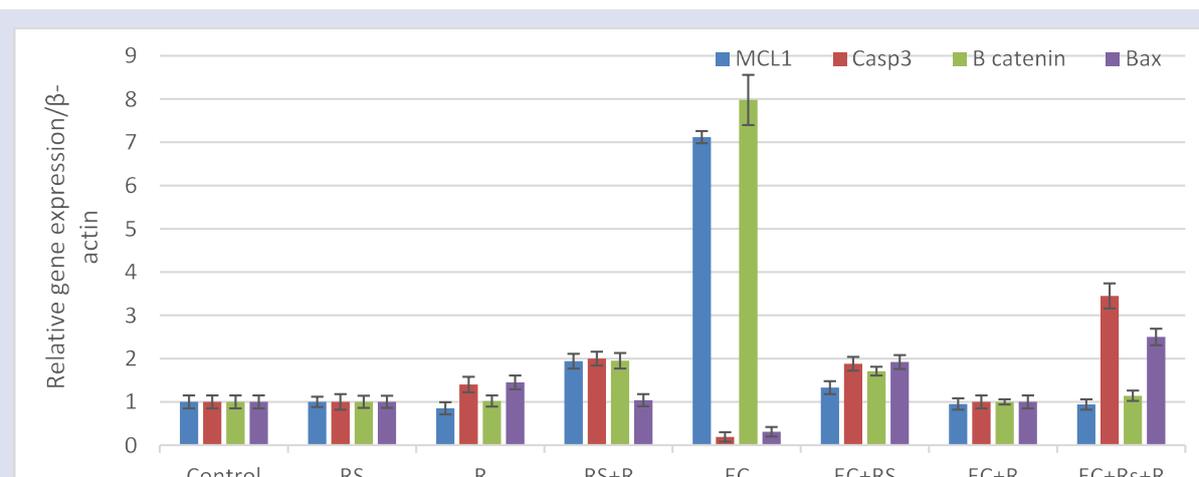
Compound name	Rt (min)	Area %
Gallic acid	4.87	1%
Vanillic acid	5.3	2%
P-coumaric acid	7.05	15%
Ferulic acid	7.30	19%
Sinapic acid	7.59	2.4%
Catechin	10.57	4%
Quercetin	23.7	7%
Apigenin	24.8	3%
Kaempferol	25.4	5%



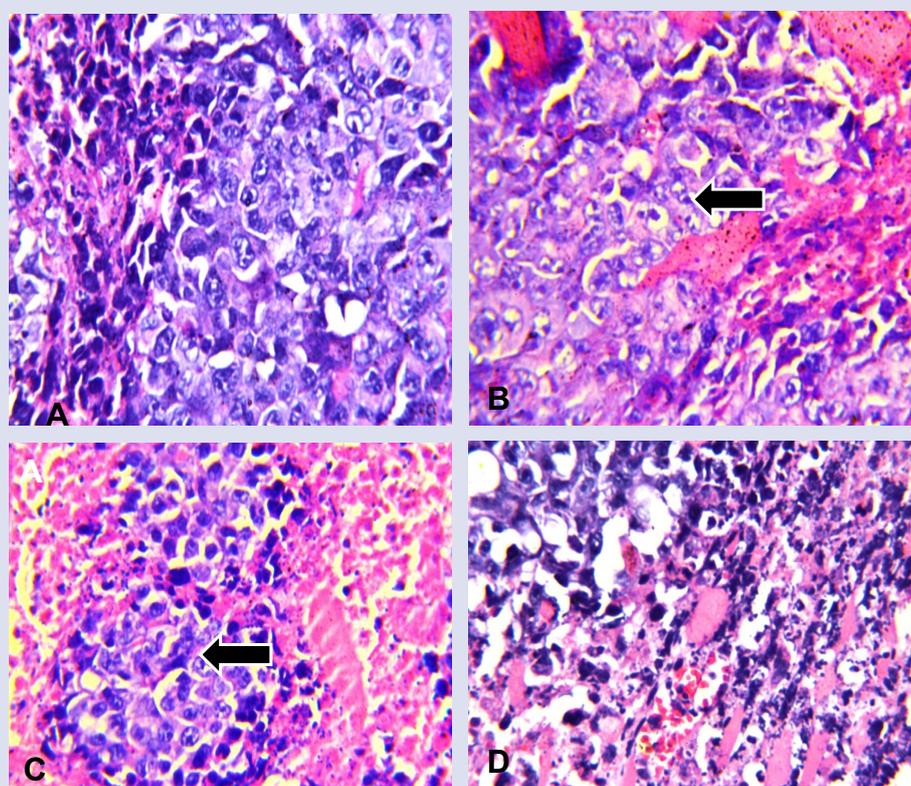
**Figure 1:** Rice straw extract (RS) and/or  $\gamma$ -radiation exposure 0.25 Gy (R) markedly delay tumor growth and volume (mm<sup>3</sup> A) and increase (% of growth inhibition B) of mice bearing solid tumor (EC).



**Figure 2:** Effect of rice straw extract (RS) and/or low dose of  $\gamma$ -radiation 0.25 Gy (R) on (A) IL-6, IL-10 in serum and (B) VEGF in tumor tissue of EC-bearing mice.



**Figure 3:** Effect of rice straw extract (RS) and/or low dose of  $\gamma$ -radiation (0.25 Gy) on MCL-1, Casp-3, B catenin and Bax gene transcription levels in tumor tissue of EC-bearing mice.



**Figure 4:** Photomicrographs of sections in solid Ehrlich carcinoma (EC) stained by H and E. (A): Section in muscular tissue of EC bearing mice; (B): Section in muscular tissue of EC treated with RS extract; (C): Section in muscular tissue of EC treated with gamma irradiation; (D): Section in muscular tissue of EC treated with RS extract and gamma irradiation, H and E,  $\times 80$ .

involved in tumor suppression and proliferation using qRT-PCR. The results revealed that treatment with RS caused a significant down regulation ( $P < 0.05$ ) in the gene transcription level of MCL1 and b-catenin, and a significant ( $P < 0.05$ ) up-regulation of Caspase-3 and Bax gene expression at the mRNA level in the tumor tissue compared to the untreated tumor group (EC).

**Histopathological** photos of solid Ehrlich carcinoma (EC) tissue sections under the light microscope are shown in Figure 4. According to histopathological findings, EC section exhibited massive aggregation of tumor cells spread within the muscular tissue, with muscle fibers

intermingled with tumor areas. Tumor cells (EC group) are intact in between the skeletal bundles (Figure 4A). Exposing female mice bearing EC to a low dose of  $\gamma$  radiation (EC + R group) showed a moderate form of necrosis and apoptosis found in all over tumor cells (Figure 4B). Treatment of animals bearing EC with RS extract (EC + RS) resulted in the appearance of apoptosis found in moderate form all over the tumor cells (Figure 4C). Treatment of animals bearing EC with RS extract and gamma radiation (EC + RS + R group) revealed that there was a mild form of necrosis with severe apoptosis in the tumor cells (Figure 4D).

## DISCUSSION

A previous published results clearly showed that rice straw extract is potential of anti-proliferation activity *in vitro* with a promising cytotoxic effect in low dose against cancer lines of liver (HepG2), prostate (PC3), and breast carcinoma (MCF-7) while it is of moderate effect on viable lung cell line.<sup>11</sup> The pronounced potential cytotoxic effects of RS is in agreement with Suffness<sup>21</sup> who stated that a compound with IC50 less than 100 µg/mL is a potential antitumor. That encouraged us to obtain further insights into the growth inhibitory effects of RS on solid Ehrlich tumor in female mice.

In this study, a significant delay in the tumor growth was recorded in mice bearing tumor groups treated with EC + RS and EC + R. Significant and more pronounced delay in the Ehrlich solid tumor size recorded when mice were treated with combined treatment, compared to each single treatment, suggests a synergistic effect. This could be explained by the fact that RS extract is capable to inhibit tumor growth, mainly because of its antioxidant activity and the antitumor compounds present in the extract. In this study LC/MS/MS (Table 2) and previous HPLC analysis<sup>11</sup> revealed the presence of high content of ferulic acid, p-coumaric acid, vanillic acid, quercetin, kaempferol, apigenin and catechin, known of antitumor activity<sup>22,23</sup> and capable to inhibit tumor growth. Previously published studies reported that ferulic acid is of antiproliferative effect by inducing G0/G1 phase arrest and down-regulated the expression of cell cycle-related proteins, CDK 2, CDK 4, CDK 6. Additionally, ferulic acid upregulated Bax, downregulated Bcl-2, enhanced caspase-3 activity, and inhibited PI3K/Akt activation.<sup>24</sup> P-coumaric acid was found to protect rat's heart against doxorubicin (DOX)-induced oxidative stress<sup>25</sup> induce an inhibitory effect on HCT 15 and HT 29 proliferation and increased apoptotic cells at the sub-G1 phase of the cell cycle.<sup>26</sup> While, Vanillic acid was reported to possess antioxidant, hepatoprotective, cardioprotective, and antiapoptotic activities and showed no cytotoxic, antiproliferative or mutagenic effects while protecting cells from DNA damage.<sup>27</sup> Apigenin and other identified flavonols were reported to have antioxidant and anti-inflammatory functions and suppress various human cancers by multiple biological effects, such as activating cell apoptosis and autophagy, inducing cell cycle capture, suppressing cell migration and invasion, and stimulating an immune response. Anti-cancer effects of apigenin mechanisms are accomplished through the modulation of signaling pathways, including the PI3K/AKT, MAPK/ERK, JAK/STAT, NF-κB, and Wnt/β-catenin pathways.<sup>28</sup> Catechins are another components that were identified in the RS extract, possessing a variety of biological activities, showing powerful antioxidant activity by radical trapping,<sup>29</sup> affects the molecular mechanisms involved in angiogenesis, extracellular matrix degradation, apoptosis, and multidrug resistance in cancers and related disorders.<sup>30</sup>

On the contrary to the deleterious effect of high doses of ionizing radiation caused by the overproduction of reactive oxygen species<sup>31,32</sup> low-level ionizing radiation (< 1 Gy) was shown to enhance cell apoptosis.<sup>6,33</sup>

A low dose of ionizing radiation (LDR) is of antitumor activity and enhance the effectiveness of cancer therapeutics. Enhancing normal cell proliferation is a well-documented effect of low dose of gamma radiation, but this effect is absent in cancer cells, including leukemia and solid tumor cells, *in vitro* and *in vivo*<sup>34</sup> which favors recovery of damaged tissues during anti-cancer therapy. Previous studies pronounced antitumor effect of LDR on pulmonary tumor metastases.<sup>9</sup> Low dose of ionizing radiation induce several mechanisms to kill cancer cells; this could be mediated through the mild oxidative stress which raises cellular anti-cancer immunity via NK cells by activating the P38- mitogen-activated protein kinase (MAPK) pathway,<sup>35</sup> induces

hormesis in the immune system stimulating T cells proliferation, surge antibody secretion and boost the antibody-dependent cellular cytotoxicity response in tumor-bearing mice, which is well correlated with tumor regression.<sup>8</sup> Another mechanism is through increasing the level of various kinds of anti-oxidants function of SOD, CAT, GPX, HO-1, and NQO-1 through nuclear factor κB (NFκB).<sup>9</sup> Survival and death of living cell was found to be controlled by the LDR through MAPK superfamily (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK.<sup>36</sup>

The Results of the EC group showed a significant increase in the IL-6 and VEGF levels which interpreted to tumor growth, however RS and a LDR treatment effectively modulated it. Cytokines are the most important mediators by which cells of the immune system communicate. Antiangiogenic effect of RS could be via its direct inhibitory effect on IL-6 and IL-10 induction. A critical role of IL-6 in the pathogenesis of cancer was detected. IL-6- utilize their action via the signal transducers glycoprotein 130, LIF receptor and OSM receptor leading to the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) cascades. Apoptosis is preventing by IL-6 binding to IL-6 receptors which stimulate STAT3 phosphorylation and induce antiapoptotic gene transcription of MCL1, BCLx1, cmyc and VEGF in the nucleus.<sup>37</sup> In the same line, VEGF expression is induced by IL-6 which increases angiogenesis via STAT3 signaling pathway in gastric carcinogenesis<sup>38</sup> and cervical cancer.<sup>39</sup>

Results also pronounced low level of antiinflammatory IL-10 production in tumor cells which intercepts the inflammatory responses. RS treatment and a LDR elevated IL-10 production level compared to the EC group. Anti-inflammatory effect of IL-10 works effectively against tumor growth, as it activates STAT3 and induces the suppressor of cytokine signaling 3 (SOCS3), which binds and hinders the signaling of the proinflammatory IL-6 and IL-12/IL-23 receptors. Mice and humans deficient in IL-10 signaling show high rate of tumor induction, while its overexpression in models of human cancer or treatment with a pegylated IL-10 (PEG-IL-10) directed to tumor rejection and long-lasting tumor immunity. IL-10-induced tumor rejections are dependent on the expression of IFN-g and granzymes in tumor-resident CD8<sup>+</sup> T cells and the upregulation of MHC molecules.<sup>40</sup> While in support of anti-inflammatory and tumor-inhibitory function, IL-10-deficient mice and humans develop inflammatory bowel disease (IBD) and cancer.<sup>13,41,42</sup>

To gain a better understanding of the mechanism underlying tumor growth proliferation an anti-apoptotic (MCL1 and β-catenin) and apoptotic markers (caspase 3 and Bax) were investigated.

Results pronounced a significant inhibition in the gene expression of apoptotic markers: caspase 3 and Bax and significant elevation of antiapoptotic markers of MCL-1 and β-catenin transcription level in Ehrlich solid tumor tissue. Inhibiting of apoptotic markers is a character of tumor cells<sup>43</sup> and downregulation of caspase 3 and Bax in EAC is in agreement with this.<sup>44</sup> Another evidence for proliferation induction in EC, β-catenin accumulation in tumor cells is a marker of proliferation enhancement. β-catenin is involved in proliferation stimulation through binding to (TCF/LEF) transcription factors to induce unabated transcription of several oncogenes, including cyclin D1, enhancing cellular proliferation.<sup>45</sup> β-catenin expression inhibits apoptosis, in part by inhibiting Bax in a phosphatidylinositol-3 kinase/Akt-dependent manner, which gives neoplastic cells a growth benefit and favors the occurrence of additional genetic lesions with potential oncogenic properties.<sup>46</sup> MCL-1 is frequently amplified in cancer cells and considered as a major oncogene,<sup>47</sup> through exerting its anti-apoptotic activity where it antagonizes BAX and BAK activation,<sup>48</sup> downstreams of p53 and upregulation of p21 via a mechanism involving reactive oxygen species (ROS) production.<sup>49</sup>

It was observed that RS and/or LDR is effectively promoting apoptosis causing tumor cell death by the up-regulation of the Bax, and activation of caspase-3, with significant suppression of anti-apoptotic gene expression of  $\beta$ -catenin and MCL1 which enhances apoptosis pathway and decreases tumor volume. Beta-catenin gene expression is of an important marker in cancer cells, its blockade in metastatic melanoma cell lines efficiently induces apoptosis and inhibits proliferation.<sup>50</sup> WNT/ $\beta$ -catenin pathway modulate mitochondrial physiology by controlling MCL1 gene expression which is an anti-apoptotic and key regulator of mitochondrial function toward survival.<sup>35</sup> Combined treatment of RS extract with a LDR showed a synergistic effect in inducing gene expression of caspase 3 and Bax. That contributes to chemoradiotherapy approach in improving cancer treatment through modulating the mechanism by activation of caspases as it is a vital molecular aim in the progressions of apoptosis to eradicate cancerous cells without the activation of inflammatory responses.

## CONCLUSION

From the aforementioned results, it can be concluded that rice straw/low dose gamma radiation effectively and synergistically work towards inhibition of cancer cell proliferation, as it was shown in murine Ehrlich solid carcinoma model and stimulate apoptotic pathway gene expression and down-regulate gene expression for proliferation. Rice straw and/or exposure to a low dose of  $\gamma$ -radiation caused a marked suppression of tumor growth and induced significant reduction in IL-6 level with significant elevation in IL-10 serum level. VEGF level was significantly reduced in the treated tumor-bearing groups (EC + RS, EC + R, and EC + RS + R groups). Rice straw caused a significant down regulation in the gene transcription level of MCL1 and b-catenin, and a significant up-regulation of Caspase-3 and Bax gene expression at the mRNA level in the tumor tissue compared to the untreated tumor group (EC) and treatment of animals bearing EC with RS extract and gamma radiation (EC + RS + R group) revealed that there was a mild form of necrosis with severe apoptosis in the tumor cells. These findings were well appreciated Rice straw/low dose gamma radiation can serve as a good therapeutic agent against cancer which needs further clinical studies.

## DECLARATION OF CONFLICTING INTERESTS

### Ethical approval

All the experimental procedures were carried out according to the principles and guidelines of the Ethics Committee of The National Centre for Radiation Research and Technology approval number 10A (2018) following the 3Rs principle for animal experimentation (Replace, Reduce & Refine) and is organized and operated according CIOMS & ICLAS International Guiding Principles for Biomedical Research Involving animals 2012 and approval from Ethics Committee Faculty of Pharmacy Cairo University under approval number (MP 2272).

### Availability of data and materials

The database used and /or analyzed during the current study are available from the corresponding author on reasonable request

### Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The authors declare no competing interests.

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## Author contributions

KM Meselhy select the point of research and the aim of the research, collect and authenticate plant material, prepared extracts & fractions, analyze & conclude results of cytotoxic activity & and interpret the LC/ms/ms profile with Manal M.

Manal M carry out the laboratory work, analysis & interpretation of LC/ms/ms profile and shared in analysis & conclusion of the cytotoxic activity.

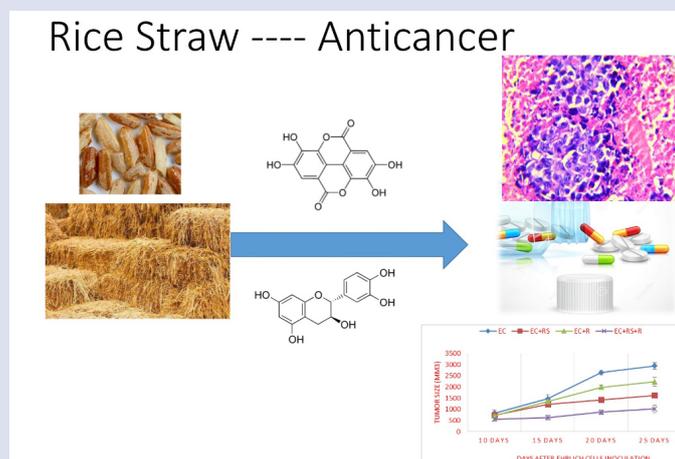
NH and Sawsan M carry out all laboratory work and analysis of cytotoxic activity. All authors reviewed the manuscript and concluded the general discussion.

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## GRAPHICAL ABSTRACT



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