BAICALEIN-RICH FRACTION AS A POTENTIAL RADIOSENSITIZER OR RADIOPROTECTIVE FOR HDR BRACHYTHERAPY: A PRELIMINARY STUDY

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ABSTRACT

Usage of anti-cancer drugs as radiosensitizers is deemed necessary for the improvement of cancer radiation treatments. In avoiding the negative aftereffects of the drugs, natural compounds are being considered. However, plant compounds are usually highly antioxidative. Baicalein-rich fraction (BRF) which had been found to have an anti-cancer effect, could be a radiosensitizer or radioprotector. In this study, cytotoxicity of BRF was first investigated in MCF-7, MDA-MB-231, and NIH/3T3 cells. Brachytherapy was conducted with dose of 0 to 4 Gy before measuring reactive oxygen species (ROS) production and clonogenic assay. 25% of inhibition concentration by BRF treatment on MCF-7 cell line was found to be the lowest with value of 2.71 µg/ml. Following this concentration, the cells were treated before brachytherapy. The findings show higher post-irradiation ROS induction for BRF treated compared to the control. The highest ROS level was found in MDA-MB-231 cells. However, sensitization enhancement ratios calculated from survival curves show values of 0.96, 0.87. 0.60 for MCF-7, MDA-MB-231, and NIH/3T3 cells, respectively. The amount of ROS generated may be insufficient to damage the cells and was immediately counter-reacted by the BRF-induced antioxidants. Thus, the BRF is inferred to be a radioprotective natural chemical.

ABSTRAK

Penggunaan ubat-ubatan anti-barah sebagai radiosensitizer dianggap perlu untuk peningkatan rawatan radiasi barah. Untuk mengelakkan kesan negatif ubat-ubatan, sebatian semula jadi dipertimbangkan. Walau bagaimanapun, sebatian tumbuhan biasanya sangat antioksidan. Fraksi kaya Baicalein (BRF) yang didapati mempunyai kesan anti-barah, boleh menjadi radiosensitizer atau radioprotector. Dalam kajian ini, sitotoksisitas BRF pertama kali diselidiki di sel MCF-7, MDA-MB-231, dan NIH / 3T3. Brachytherapy dilakukan dengan dos 0 hingga 4 Gy sebelum mengukur pengeluaran spesies oksigen reaktif (ROS) dan pengujian klonogenik. 25% kepekatan perencatan oleh rawatan BRF pada garis sel MCF-7 didapati paling rendah dengan nilai 2.71 µg / ml. Berikutan kepekatan ini, sel-sel dirawat sebelum brachytherapy. Hasil kajian menunjukkan induksi ROS pasca penyinaran lebih tinggi untuk rawatan BRF berbanding kawalan. Tahap ROS tertinggi dijumpai dalam sel MDA-MB-231. Walau bagaimanapun, nisbah peningkatan pemekaan yang dikira dari keluk kelangsungan hidup menunjukkan nilai 0.96, 0.87. 0.60 untuk sel MCF-7, MDA-MB-231, dan NIH / 3T3, masing-masing. Jumlah ROS yang dihasilkan mungkin tidak mencukupi untuk merosakkan sel dan segera bertindak balas oleh antioksidan yang disebabkan oleh BRF. Oleh itu, BRF disimpulkan sebagai radiopelindungan semula jadi bahan kimia.

Keywords: Baicalein-rich fraction; brachytherapy; breast cancer cell; radioprotection; radiosensitizer.

INTRODUCTION

Cancers are still the most dreadful disease in this period. Breast cancer cells are the second most frequent cancer identified worldwide (IARC, 2014). In Malaysia, it is reported that breast cancer ranked number one for new cases in the year 2018 (WHO, 2019). With a high percentage of new cases each year, the need to improve the cancer radiation therapy had also increased. One of the problems with radiotherapy is the death of the healthy cells surrounding the cancer cells (Jiang & Iwahashi, 2019). The usage of radiosensitizer that could increase the dose of radiation at the cancer site while protecting the normal cells are still being researched.

There are chemotherapeutic drugs that served as radiosensitizers such as cisplatin, gemcitabine, and doxorubicin (Amouzegar-Hashemi, Akbari, & Esmati, 2013; Guo et al., 2019). Due to the toxicity of these commercial synthetic chemo drugs, researchers started to try in exploring the option for nontoxic radiosensitizers, possibly from natural chemicals and derivatives (Jiang & Iwahashi, 2019). Latest studies had discovered the radiosensitizing effects of curcumin, Dictyota dichotoma extract and Agelas extracts (Choi, Son, Lee, Lee, & Park, 2018; Liang et al., 2019; Malyarenko, Usoltseva, Zvyagintseva, & Ermakova, 2018).

However, plants are also well known for their antioxidant properties. Walnut and passionflower extracts were found to have anticancer activity on Colo-205 colon cancer and HeLa cervical cancer cell lines, as well as high antioxidant and antiradical levels (Anjum et al., 2016; Sisin, Suláin, & Abdullah, 2017). The high content of phenolics in pomegranate extract also might help in scavenging free radicals (Lantzouraki, Sinanoglou, Zoumpoulakis, & Proestos, 2016). Furthermore, extract of *Oroxylum indicum* (OI) plant leaves was also had been investigated for anti-cancer effect and radiotherapy (W. N. Rahman et al., 2019). Main compounds from IO leaves were baicalein, baicalin, chrysin, oroxylin-A, scutellarin and Aloe-emodin (Ahad et al., 2012; Dinda, Silsarma, Dinda, & Rudrapaul, 2015).

However, the application of baicalein-rich fraction (BRF) from the OI leaves for brachytherapy has not been studied. In this study, the BRF is investigated for its cytotoxicity, ROS induction and radiosensitization effect on MCF-7, MDA-MB-231 and NIH/3T3 cell lines for high dose rate (HDR) brachytherapy.

MATERIALS AND METHODS

Cell culture: MCF-7, MDA-MB-231, and NIH/3T3 cells

This study used three cells lines: MCF-7, MDA-MB-231, and NIH/3T3 cells. MCF-7 and MDA-MB-231 cells are both human breast cancer cell lines but different subtypes, while NIH/3T3 cells are normal mouse fibroblast cells. All cells were cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) added with 5% of fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). The three cell lines were grown in 25 cm^2 flasks and subcultured using trypsin-EDTA (Gibco, USA). The cells were incubated in a humidified condition of 5% CO_2 and temperature of 37°C.

In Vitro Cytotoxicity of BRF

Baicalein-rich fraction (BRF) was extracted and fractionated from OI plant leaves as reported by previous literature (Wahab & Mat, 2018). The cytotoxicity test was conducted according to Sisin et al. (2017). BRF was dissolved in dimethyl sulfoxide (DMSO). Each cell line was seeded in 96-well-plated overnight and treated with two-serial dilutions of the BRF ranging from 100 µg/ml to 0.39 µg/ml. The well-plate was incubated in temperature of 37°C with the presence of 5% CO_2. After 24 hours, each cell viability was measured using Prestoblue assay. Old media was removed before 10 µl of Prestoblue reagent (Invitrogen, USA) and 90 µl of

fresh media was incubated for 4 hours. The fluorescence was read using microplate reader at 535 nm excitation and 615 nm emission wavelength. The graph was plotted and fitted according to Dose-Response model.

Irradiation Set Up

The irradiation was conducted at Nuclear Medicine, Radiotherapy and Oncology Department (Hospital Universiti Sains Malaysia, Kelantan, Malaysia). The brachytherapy was performed using Microselectron HDR 178 Brachytherapy V14.23 system (Nucletron Corp, Columbia, Maryland) with an iridium-192 source. The chosen concentration of BRF used to treat all the cells before irradiation was chosen from the cytotoxicity data.

Post Irradiation ROS Measurement

The cells were seeded in 96-well plate and incubated overnight. Then, the old media was discarded and new media added with BRF. 15 µl from 100 µM of 2', 7'- dichlorodihydrofluorescein diacetate ($DCFH_2$-DA, Sigma- Aldrich) reagent was added to a final concentration of 9 µM. The plates were irradiated with dose of irradiation of 3 Gy. Both control and irradiated plates were read using a microplate reader at 485 nm excitation and 530 nm emission, before and after the irradiation. The percentage of ROS generation is calculated as shown in equation (1).

$$\text{ROS}\ (\%) = \frac{\text{value control or treated cells, for 0 or 3 Gy}_{\text{after radiation time}}}{\text{value of control cells for 0 Gy}_{\text{before radiation time}}} \text{ x } 100 \qquad (1)$$

Clonogenic Survival Assay

The cells were suspended in 200 µl tubes and treated with BRF. The dose of irradiation consisted of 0, 100, 200, 300, and 400 cGy. After the radiation, cell suspension was transferred into 6-well-plates and added 1.5 ml complete media per well. The plates were incubated for 5 to 10 days before the cells were fixed with cold methanol for a while and stained with 0.5% crystal violet for 1 hour. The image of colonies formed was scanned using flatbed scanner with minimum 300 dpi and calculated using ImageJ software. The cell survival fractions were plotted and fitted according to linear-quadratic (LQ) model.

Quantification of SER and Theoretical Dose Enhancement Factor (DEF)

SER values were extrapolated from the survival curves, and theoretical DEF value was estimated from mass-energy absorption coefficient of baicalein compound to water, acquired from the National Institute of Standard and Technology database, as stated by previous literature (Abdul Rashid et al., 2019; W. N. Rahman et al., 2014). As the composite of the BRF from IO is still unidentified, the composite of well-known baicalein compound (main compound in the BRF) was used. The calculated effective atomic number (Z) if baicalein compound is generally closer to carbon (Z=6). Thus, the theoretical DEF was predicted using Z of carbon.

Statistical Analysis

The data were stated as the mean ± standard error of the mean (SEM) and mean ± standard deviation (SD). All graphs and statistical tests were plotted and performed using OriginPro 2018 software (OriginLab Corporation, US).

RESULTS AND DISCUSSION

Cytotoxicity of Baicalein-rich Fraction (BRF)

Inhibition concentrations of 25% (IC_{25}) and 50% (IC_{50}) of BRF obtained from each cell line were shown in Fig. 1 and listed in Table 1. IC_{50} values were the gold standard to speculate about the anticancer effect of plant extracts. Treatment agent with IC_{50} values of less than 30 µg/ml could be considered as an anticancer agent (Sisin et al., 2017).

As presented in Table 1, BRF showed high toxicity on the breast cancer cells MCF-7 and MDA-MB-231 cells, while not toxic to normal cells NIH/3T3. Cytoselectivity of an agent towards the types of cells is excellent characteristics of a potent anticancer agent. This result is also supported by a previous study that demonstrated anticancer properties of BRF on SiHa and HeLa cells (Wahab & Mat, 2018). Furthermore, a commercial anticancer agent such as cisplatin is a good radiosensitizer (Cui et al., 2017). Thus, BRF is an excellent candidate to be a radiosensitizer.

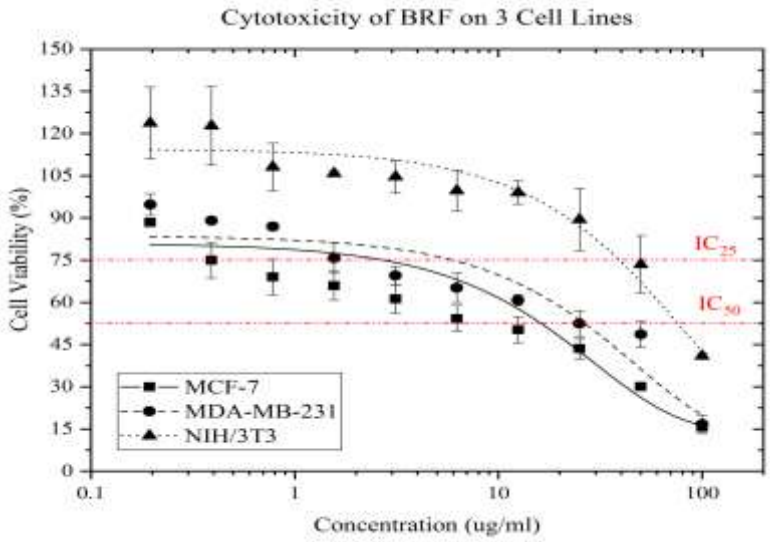

Fig. 1: Percentage of the three cell lines viability after treatment with a few concentrations of BRF. Both IC_{50} and IC_{25} levels were shown.

A lower inhibition concentration (IC_{25}) was selected for the radiation studies (Cui et al., 2017). This chosen concentration is to emphasize the effect of radiation in killing while BRF in assisting the radiation in subsequent experiments. Among the IC_{25} values recorded in Table 1, we chose the lowest value as it indicated the highest toxicity among the three cells.

Table 1: IC_{50} and IC_{25} values for BRF treatment on different cell lines.

	IC_{50} (µg/ml)	IC_{25} (µg/ml)
MCF-7	18.18	2.71
MDA-MB-231	29.22	5.95
NIH/3T3	80.87	39.19

Post-Irradiation ROS Measurement

ROS was one of the indirect cause of cell death after ionizing radiation (Tong, Chuang, Wu, & Zuo, 2015). Thus, the role of BRF in ROS induction after 3Gy dose of irradiation was tested. Fig. 2 showed that presence of BRF could induce more ROS than the control. Therefore, the presence of BRF may contribute to cell death after the irradiation.

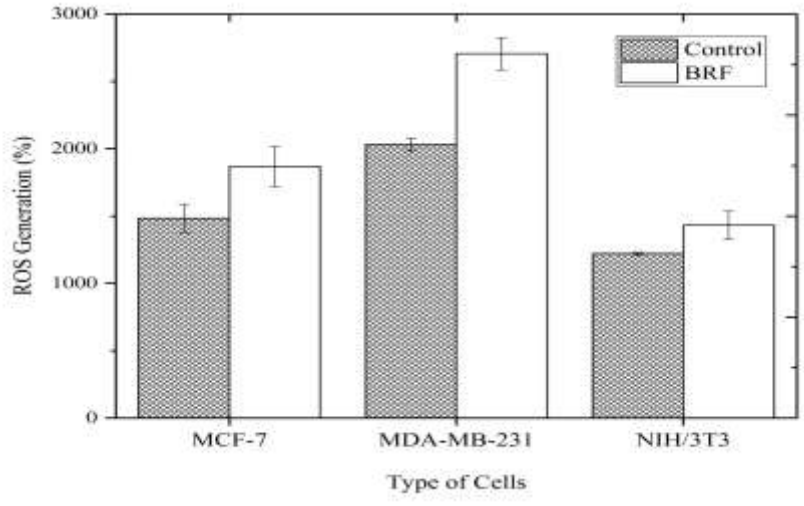

Fig. 2: Percentage of ROS generation of control and with BRF treatment after the brachytherapy.

In Figure 2, the highest intracellular ROS production was demonstrated in MDA-MB-231 cells, followed by MCF-7 cells and NIH/3T3 cells. It illustrates that MDA-MB-231 cells are more sensitive to radiation. Although MDA-MB-231 cells are known as radioresistant, there are several agents that could increase the radiosensitivity of MDA-MB-231 cells, such as curcumin-loaded lipid nanoparticles and mesenchymal stem cells conditioned media (Dinkelborg et al., 2019; Gray et al., 2019; He et al., 2018; Mina et al., 2019). Thus, BRF may be a radiosensitizer through the ROS mechanisms.

SER and Theoretical DEF Measurement

Fig. 3 shows the survival curve of each cell lines after irradiation with the presence of BRF. Sensitization enhancement ratio (SER) values were calculated from the 50% of survival fraction of control against the BRF-treated cells of the fitted lines. The SER and theoretical DEF values are presented in Table 2. α and β values exemplify the cell killings from single and double hits, respectively (W. N. W. A. Rahman et al., 2018).

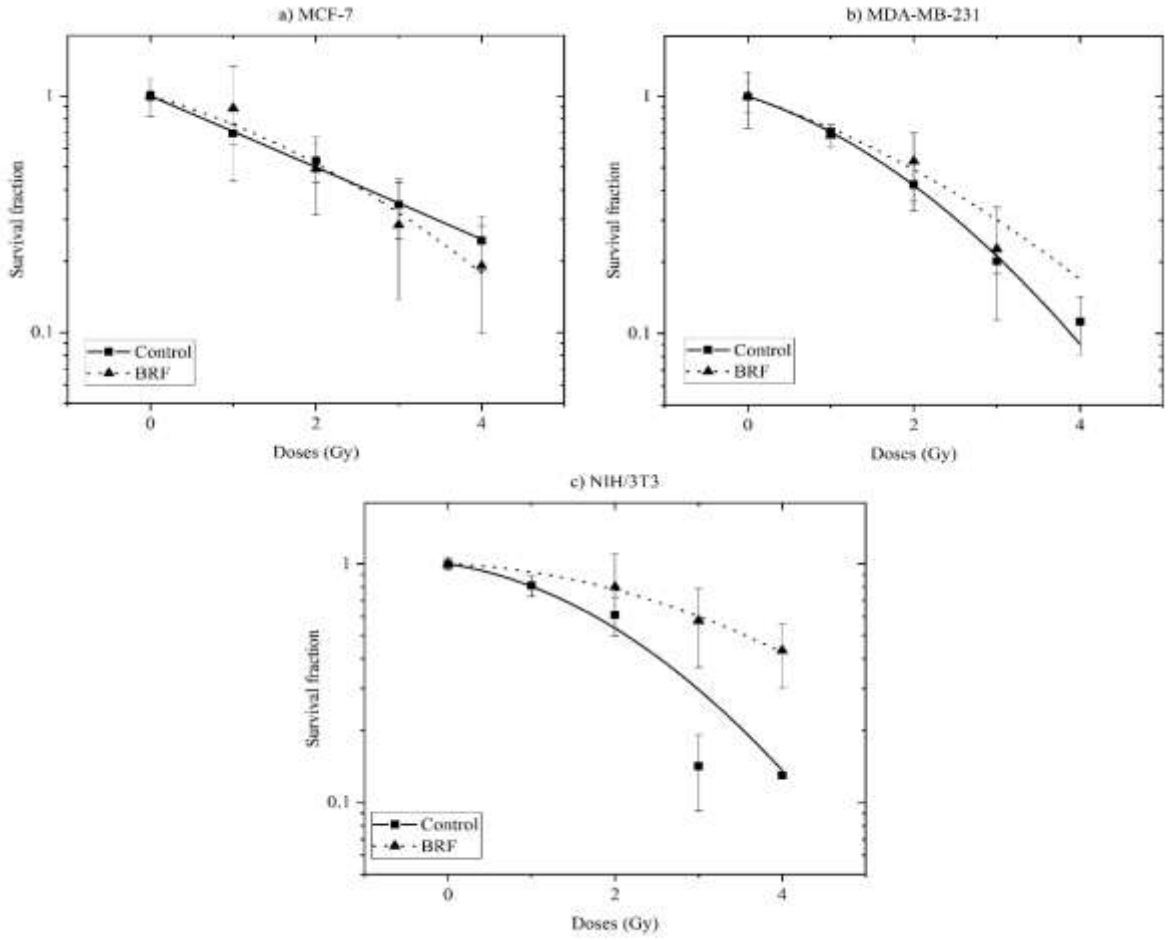

Fig. 3: Survival curves of three cell lines, treated with BRF and irradiated with brachytherapy.

Table 2: The calculated SER for BRF in different cell lines.

	SER	Alpha (α)	Beta (β)
Theoretical DEF	0.99	-	-
MCF-7 cells	0.96	0.22779 ± 0.07932	0.05074 ± 0.02427
MDA-MB-231 cells	0.87	0.26755 ± 0.17818	0.04404 ± 0.0679
NIH/3T3 cells	0.60	0.03511 ± 0.03241	0.0447 ± 0.00946

The SER values which are less than one indicate that there are more colonies of BRF-treated cells than that of control. The experimental results conformed to the theoretical DEF. However, these discoveries are in contrast to the aforementioned post-irradiation ROS measurement. Past works had deduced that ROS were secondary stimulus in exterminating the cancer cells (Park et al., 2011; Sisin et al., 2019). The DNA strands breakage in cells might also undergo a very rapid repair mechanism (Phillips, McBride, & Pajonk, 2006). A study on a radiosensitizer also found that gold nanoparticles could not inhibit the DNA repair mechanism in normal L132, prostate cancer DU145 cell lines (Jain et al., 2011).

Moreover, baicalein compound from *Scutellaria baicalensis* was protective against radiation-induced cell death (Oh et al., 2013). The parent plant of the BRF, OI was also found to have a high antioxidant level and successfully protected the cellular DNA from radiation-induced damages (Thokchom, Shantikumar, & Sharma, 2014). The difference in this study also suggested that the amount of the post-irradiation ROS was not high enough to immediately cause the damage to the cells due to the antioxidant potentials from the BRF. Thus, the BRF is inferred to be a radioprotective natural chemical.

CONCLUSION

BRF is found to be a safer anti-cancer agent and is predicted to be a good radiosensitizer. The ROS measurement after brachytherapy with the present of BRF were highest in MDA-MB-231 and followed by MCF-7 and NIH/3T3 cells. However, all the SER values were less than value 1. This discrepancy happened might be due to the antioxidant properties of BRF that originated from a plant extract. In conclusion, BRF is an excellent anti-cancer agent, and it also has good radioprotective activity.

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