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Cytotoxic activity and Antioxidant Potentials of hexane and Methanol extracts of IR64 Rice bran against Human Lung (A549) and Colon (HCT116) Carcinomas

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Abstract

Rice bran is a byproduct of rice milling process which contains significant amount of natural phytochemicals. The IR64 rice variety is a high-yielding, semi-dwarf and mostly grown variety in the tropics. This study aims to determine the cytotoxic and antioxidant potentials of IR64 rice bran. The sample was subjected to sequential extraction using hexane and ethanol. The concentrated extracts were diluted in dimethyl sulfoxide (DMSO). The cytotoxic activity of hexane and ethanol extracts was analyzed using the Methyl Thiazol Tetrazollium (MTT) Assay for human lung and colon carcinomas. Both extracts were also analyzed by Cytotoxicity Assay using lymphocytes from normal blood. The antioxidant property was evaluated by free radical diphenyl-picrylhydrazyl (DPPH) scavenging assay. The result of MTT Assay revealed that ethanol extract inhibit proliferation of human lung and colon carcinomas at 10181 µg/ml and 6650 µg/ml, respectively. The ethanol extract demonstrated 79.79% inhibition against DPPH. Based on the cytoxicity assay conducted, it shows that the sample is not toxic to normal cells (lymphocytes) having an average of only 14 cells died at a 193 cell population (7.25 per 100 cells) compared to the base cell medium as untreated control and 2.5% DMSO in phosphate buffered saline (PBS). No cytotoxicity and antioxidant potentials were exhibited by the hexane extract. It was concluded that IR64 rice bran ethanol extract is a potential source of bioactive compound/s against both human lung and colon carcinomas and a potential antioxidant against free radicals.

Keywords: IR64, antioxidants, anticancer, phytochemicals.

Introduction

Many studies have indicated that stabilized rice bran is a unique whole food that naturally contains protein, vitamins, minerals, complex carbohydrates, phytonutrients, phospholipids, essential fatty acids, and more than 120 antioxidants[1]. Dietary rice bran intake and rice bran components have demonstrated chronic disease fighting activity, particularly for protection against cardiovascular disease and certain cancers[2-8]. It is also popular as a dietary supplement or functional food ingredient[9-11]. Studies have shown that polar rice bran extracts inhibit tumor promotion of lymphoblastoid B cells, and rice bran agglutinin inhibited growth of monoblastic leukemia U937 cells[12]. Some studies have shown that rice bran contains a number of metabolites with reported anticancer effects, notably phenolics and phytosterols[13-15]. Emerging evidence supports additive and/or synergistic effects of rice bran components for protection against certain cancers[10,16,18,19]. In this study, we evaluated if methanolic and hexane-extracts of the rice bran of a popular rice variety IR64 have potential against human lung and colon carcinomas.

Material and Methods

Sample Preparation. The IR64 rice bran was obtained by milling rice grain in a local grinding mill. The sample was obtained from the third batch of the milling process. Only 500g bran was taken and stabilized by freezing to avoid chemical reactions or oxidation and later serially extracted. Solvent extraction made use of 1.5L 95%-Hexane soaking the rice bran for 24 hours and followed with another 1.5L 95%-Ethanol for another 24 hours. The extracts were filtered through filter paper and spin-dried using rotary evaporator for 3 hours. The spin-dried sample was further placed in the evaporating dish for 24 hours for further drying. Only 1g of the extract was dissolved with dimethyl sulfoxide (DMSO).

In vitro Cell Cytotoxicity Activity (MTT Assay): The MTT Assay was tested in the Institute of Biology, University of the Philippines, Diliman. The quantitative colorimetric methyl thiazol tetrazollium (MTT) assay used to measure mammalian cell survival and proliferation was used to determine how effective an extract or drug is in killing cancer cells[20] and is widely chosen as the optimal endpoint[21,22]. The assay involves three main steps: seeding of the cells in microtiter plates, treatment of cells using an extract or sample of choice, and termination and absorbance reading on a multiwell scanning spectrophotometer or ELISA reader.

The basic technique of culturing animal cells was adapted from Freshney[23]. A549 and HCT116 cell lines were separately seeded

in sterile 96-well flat-bottom microtiter plates at seeding densities of 4.0 x 10^4 cells per milliliter and incubated for 24 hours at 37°C and 5% carbon dioxide. The 4mg/ml Doxo and 1g/ml of IR64 rice bran extracts were serially diluted to concentrations of 1000µg/mL, 500µg/ml, 250µg/ml, and 125µg/ml in a master dilution plate (MDP). From the MDP, 10µl of each extract or fraction concentration was dispensed onto the seeded cells in the 96-well microtiter plate to obtain final concentrations of 25µg/ml, 12.5µg/ml, 6.5µg/ml, and 3.125µg/ml for Doxorubicin (Doxo) and 12500µg/ml, 6250µg/ml, 3125µg/ml, and 1562.5µg/ml for IR64 rice bran. Doxo served as the positive control, while DMSO was the negative control. Three replicate wells were used per extract concentration. The treated plates were then incubated for three days at 37°C and 5% carbon dioxide.

After the incubation period, the media in the wells were discarded, and 20µl of five milligrams MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) per milliliter 1x PBS was added to each well. After four hours of incubation at 37°C and 5% carbon dioxide, 150µl DMSO was added to each well. The absorbance was then read at 570nm.

Inhibition concentration 50 (IC_{50}) was computed from the absorbance reading obtained using the program A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICpin) Approach Version 2 from US Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN 55804.

DPPH – Free Radical Scavenging Assay: The DPPH – Free Radical Scavenging Assay was also tested in the Institute of Biology, University of the Philippines, Diliman. Numerous methods are available for determining the presence and quantification of the degree of anti-oxidant activity present in herbal extracts. Most of these methods make use of a color reaction and indicator to assess the degree of antioxidant activity. Phytomedicine program uses the diphenyl-picrylhydrazyl (DPPH) and trolox equivalent anti-oxidant capacity assay (TEAC)[24].

The DPPH assay is a qualitative indicator of free radical scavenging assay. DPPH is reduced from a stable free radical that is purple in color to diphenylpicryl hydrazine that is yellow, in the presence of antioxidant. The visual color change is observed on the chromatograms. This technique shows the number of anti-oxidant compounds separated by Thin Layer Chromatography (TLC) and also gives an indication of the polarity of the separated compounds.

The results were read through the ELISA or absorbance reader to determine the percent inhibition of the sample anti-oxidant activity. The positive control is the Vitamin C while the DMSO is the negative control. The Gallic Acid is used for the computation of the percent inhibition of the antioxidant activity.

Toxicity Test: To test the toxicity of the IR64 rice bran ethanol extract, the cytotoxicity test using lymphocytes from normal blood were administered by the extract. The obtained extracts were conducted to Biological Research and Services Laboratory of the Natural Science Research Institute (NSRI), University of the Philippines, Diliman. The lymphocytes from normal blood were cultured in this research institution. The cytotoxicity test used three treatments, the supplemented Roswell Park Memorial Institute (RPMI) as untreated control, 2.5% DMSO in PBS and the IR64 rice bran extract (treated control) in order to compare the number of cell death. This supplemented RPMI served as the base medium in which it was supplemented with fetal bovine serum (FBS) that supplements food and nutrients to the cells.

However, the PBS which is a buffer was used to dilute the DMSO since it is toxic if higher concentration. As the DMSO was diluted in PBS, it was used to dilute the IR64 rice bran extract to dissolve the oily nature of the extract. Since the buffer is osmoregulated saline solution, it is safe to cells preventing lysing of cells when treated by the sample. The results of the cytotoxicity test was obtained after 24 hours of application.

Results and Discussion

The results of this study show the potential of rice bran extracts against human and lung carcinomas. Positive toxicity was based on color change from brown to yellow (figures 2 and 3). Based on the computation of $IC_{50,}$ the IR64 rice bran extract is effective against human colon and lung carcinomas (table 1). The extent of effectivity of the extract is dependent on concentration (figure-3). The highest concentration at 12500µg/ml is the most effective.

Table -1
Inhibition Concentration of the different samples where 50% mortality of cancer cells (IC_{50})

Samples	Human Lung (A549) Carcinoma	Human Colon (HCT16) Carcinoma
IR64 Rice Bran	10181 µg/ml	6650 µg/ml
Doxorubicin (+ control)	1.8959 µg/ml	2.1006 µg/ml
DMSO (- control)	Negative	Negative

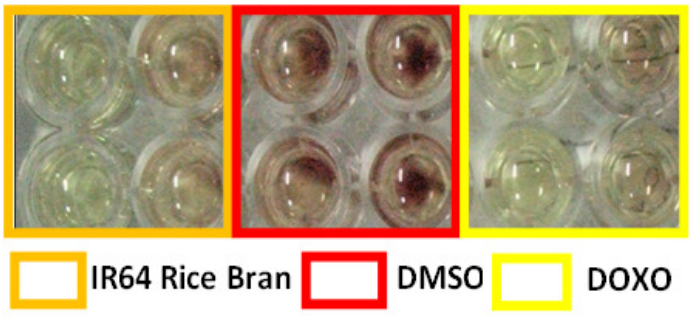

Figure -1
Color change of wells from purple to yellow containing cultured Human Lung Carcinoma exposed to IR64 rice bran extract for 72 hours

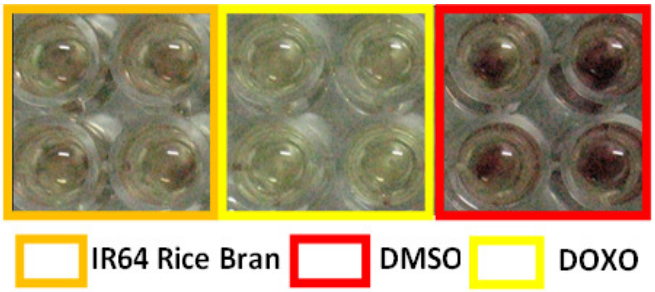

Figure -2
Color change of wells from purple to yellow containing cultured Human Colon Carcinoma exposed to IR64 rice bran extract for 72 hours

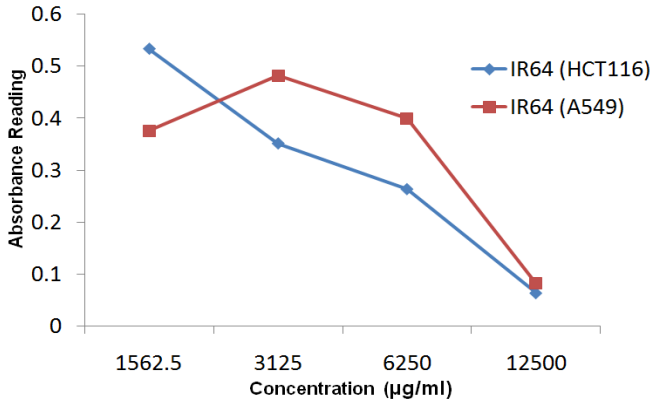

Figure - 3
Mean Absorbance Reading of IR64 rice bran extract with increasing concentration

Table 2 shows that IR64 rice bran has percent inhibition of 79.79% against free radical DPPH comparable to Vitamin C as the positive control which is 100%. This suggests that the IR64 rice bran is potential antioxidant against free radical DPPH.

Table - 2
Inhibition of free radical DPPH Scavenging Assay (Antioxidant Activity)

Inhibition against free-radical DPPH	
Treatments	Percent Inhibition
Vitamin C (+ control)	100%
IR64 rice bran	79.79%
DMSO (- control)	Negative

Table 3 shows that the IR64 rice bran ethanol extract has an average of only 14 cells at a 193 cell population (7.25 per 100 cells) died in an assay consisting of three replicates. In comparison, DMSO, which is a known mutagenic and toxic compound exhibited an average of 18 cell deaths per 207 cell population (8.70 per 100 cells) while supplemented RPMI exhibited a higher average cell death of 22 per 195 cell population (11.28 per 100 cells). Of all the treatments, the IR64 rice bran shows the least toxicity against lymphocytes from normal blood. Results show that rice bran oil extract exhibited very minimal toxic effects to normal healthy lymphocyte culture. This shows that rice bran oil can be a promising anticancer agent in future drug development programs.

Table - 3
Average Number Dead Cells from Total Number of Cells of Different Treatments

Treatments	No. of Dead Cells	No. of Total Cells	No of cells died per 100 cells
Supplemented RPMI	22	195	11.28
0.25% DMSO in PBS	18	207	8.70
Rice Bran Extract (Ethanolic)	14	193	7.25

*Cell density is expressed as n x 10^4 cells/ml, where n is the number of cells

The result of inhibition concentration 50 (IC_{50}) reveals that IR64 rice bran ethanol extracts expresses positive cytotoxicity against human lung and colon carcinomas that exhibits 10181µg/ml and 6650µg/ml, respectively. The percent inhibition of antioxidant activity is 79.79%. Based on the toxicity test conducted, it shows that the sample is not toxic to normal cells since it has the least number of cells death having an average of only 14 cells died at a 193 cell population (7.25 per 100 cells) compared to the base cell medium as untreated control and 2.5% DMSO in phosphate buffered saline (PBS). No cytotoxicity and antioxidant potentials were exhibited by the hexane extract. Moreover, the sample has no significant difference between the number of cell death of different treatments. Thus, the sample is not toxic to normal cells (lymphocytes). Moreover, the cytotoxic effect of the sample to both human lung and colon carcinomas exhibit dose-dependent effect and have the same concentration of effectiveness at 12500µg/ml.

The results of the study is an added information to the growing knowledge of the potentials of rice brans as source of compounds that have anticancer activities[2-8,25,26]. Evidence supports additive and/or synergistic effects of rice bran components for protection against certain cancers[10,12,16,18,19]. Rice bran contains metabolites with anticancer effects such as phenolics and phytosterols[13-15], phytic acid[17] and the most popularly studied tocotrienols[27-37]. These studies have revealed that tocotrienols can induce apoptosis in a wide variety of tumor cells mediated through activation of both extrinsic and intrinsic pathways by the vitamin. However few studies have examined differences in bioactive contents in commercially available rice varieties. Many studies have shown that rice varieties are not equal in content and composition of bioactive rice bran components[38,39]. The results of the current study show that the IR64 rice bran has potential as source of anticancer compounds.

Conclusion

Results of this study have shown that there was a cytotoxic activity of IR64 rice bran ethanol extract against human lung (A549) and colon (HCT 116) carcinomas. There was also a significant effect on the percent (%) inhibition of the IR64 rice bran ethanol extract against free radical DPPH indicating that IR64 rice bran contains antioxidants against free radical induced oxidative stress. Since there was no significant toxicity effect of

the extract to lymphocytes from normal blood, the rice bran has the potential as good source of compounds for anticancer treatments.

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