



Cytotoxic and cyto-protective activities of four Thai indigenous *Russula* mushroom extracts on RAW 264.7 cells

Taengphan W¹, Muangman T², Klungsupya P^{2*}, Pradermwong K³

¹ Ph.D Student, Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 21000, Thailand

² Pharmaceuticals and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Techno Polis, Pathum Thani 12120, Thailand

³ Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 21000, Thailand

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Objectives: This study was to evaluate the cytotoxicity and cyto-protective activities of ethanolic extracts from four selected *Russula* mushrooms; *R. medullata*, *R. virescens*, *R. helios* and *R. alboareolata* on RAW 264.7 cell line.

Methods: Four *Russula* mushrooms extracts including *R. medullata*, *R. virescens*, *R. helios* and *R. alboareolata* were prepared individually by maceration with 95% ethanol. Then, their cytotoxicity and cyto-protective activities on RAW 264.7 murine macrophage cells were determined using water-soluble tetrazolium salt (WST-1) assay. Results were expressed as 50% lethal concentration (LC₅₀) value percentage of cell viability following 24 hours exposure time.

Results: The four ethanolic extracts of *Russula* mushrooms indicated a slight cytotoxic activity on RAW 264.7 cell line as follows: *R. medullata* (IC₅₀ = 484.44 ± 07.43 µg/ml), *R. virescens* (IC₅₀ = 907.14 ± 52.37 µg/ml), *R. helios* (IC₅₀ = 541.78 ± 14.35 µg/ml) and *R. alboareolata* (IC₅₀ = 760.05 ± 28.95 µg/ml). Interestingly, the only extract from *R. alboareolata* at 125 µg/ml exhibited the cyto-protective activity by enhancing the survival of RAW 264.7 cells when treated with presence of known mutagen agent (mitomycin C, MMC, 10 µg/ml).

Conclusion: The results from this study revealed that extracts of *R. medullata*, *R. virescens*, *R. helios* and *R. alboareolata* possessing a slight cytotoxic. Among these, *R. alboareolata* expressed cyto-protective property against MMC toxicity. The findings on these *Russula* mushrooms activities can be used to support the utilisation of *Russula* mushrooms for a healthy dietary supplement.

* Corresponding author: Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Techno Polis, Pathum Thani 12120, Thailand; Tel. +66(0)2577-9122; Fax. +66(0)2577-9100

E-mail address: prapaipat@tistr.or.th

Introduction

Russula is a genus of Basidiomycota mushroom, belonging to the family of Russulaceae. They have thin clear cap with the gills underneath and the stem which make their shapes resemble an umbrella. The mushroom is fresh, soft, fragile and perishable^[1]. It was reported that 750 species of *Russula* distributed worldwide including the United States of America, Sweden, France, Norway, Madagascar, Italy, Belgium, Taiwan, China, Japan and Thailand^[2]. In Thailand, the presence of *Russula* mushroom have been reported in 17 provinces of the northeastern part and some of them have been consumed as food such as *R. monspeliensis*, *R. virescens*, *R. alboareolata*, *R. medullata* and *R. helios*. Some *Russula* mushrooms have an established history of the uses in traditional medicines for the treatments of various diseases as follows: *R. cyanoantha* and *R. nobilis* for treatment of fever, *R. luteotacta* for wound healing, *R. delica* and *R. parazurea* for the treatments of gastritis and hypertension, *R. acrifolia* for treatments of skin cancer and *R. luteotacta* as a sleep promoting agent.^[2,3] Biological activities of some *Russula* mushroom have been previously reported. *R. delica* showed antimicrobial activity against various bacteria and fungi including *Salmonella enteritidis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Micrococcus flavus*, *Bacillus cereus* and *Candida albicans*^[4]. *R. griseicarnosa*, *R. albonigra*, *R. laurocerasi* and *R. delica* exhibited antioxidant activities tested by *in vitro* assays such as reducing power, hydroxyl radical scavenging chelating ability of ferrous ion, 2,2-diphenyl-1-picrylhydrazyl (DDPH) radical scavenging, superoxide radical scavenging assay^[4,5]. However, there are still some *Russula* mushrooms of Thailand those have never been studied on biological properties. Therefore, this study aims to evaluate cytotoxic and cyto-protective activities of ethanolic extracts of four selected *Russula* mushrooms on RAW 264.7 cell line.

Methods

Collection of *Russula* Mushroom samples: Fresh samples of *R. medullata*, *R. virescens*, *R. helios* and *R. alboareolata* were collected in rainy season during August-October 2013-2014 from three provinces including Kalasin, Mukdahan and Yasothon in the Northeastern part of Thailand. The collected mushrooms were identified by the mushroom specialist,

Mr. Winai Klinhom of Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, Mahasarakham, Thailand.

Extraction of mushroom: Collected *R. medullata*, *R. virescens*, *R. helios* and *R. alboareolata* were dried in hot air oven at 50°C for 18-20 hours until dried then they were ground into powder using an electronic grinder. For extraction, all powder samples were macerated in 95% ethanol (plant: solvent ratio 1:10 w/v) for 5 times. Each ethanolic extract solution was evaporated using the rotary evaporator to yield dried *Russula* crude extract. The extract was stored in darkness at -20°C until utilization. The percentage yield extracts were calculated based on dry weight as following equation:

$$\text{Yield (\%)} = (W1 \times 100) / W2$$

Where W1 = weight of extract after solvent evaporation; W2 = Weight of the ground mushroom powder.

Culturing and maintaining of RAW 264.7 cell line: The mouse monocyte macrophage cell line (RAW 264.7) was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were grown as adherent in Dulbecco's Modified Eagle Medium (DMEM, GIBCO®) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO®) and 1% (v/v) penicillin-streptomycin (GIBCO®). The cells were propagated in tissue culture flask (Corning®) in humidified atmosphere incubator with 5% CO₂ at 37°C, sub-culturing every 2-3 days by scraping to allow detachment of cells and adding fresh culture medium, aspirating and dispensing into new culture flasks. For experiments, cells were harvested by scraping as explained before in phosphate buffered saline (PBS, GIBCO®), plated in 96-well plates at a density of 2x10⁴ cells/well and incubated for 24 hours before treatment.

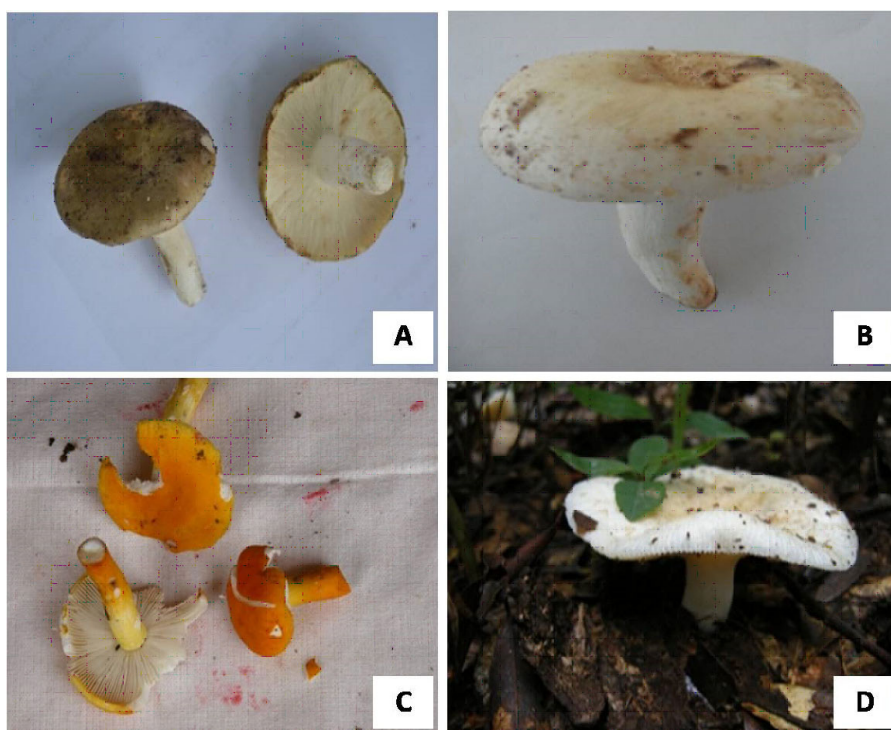


Figure 1: Morphology of selected *Russula* mushrooms used in the study: *R. medullata* (A), *R. virescens* (B), *R. helios* (C) and *R. alboareolata* (D).

Determination of cytotoxic activity of extract: The cytotoxic property of *R. medullata*, *R. virescens*, *R. helios* and *R. alboareolata* mushroom extracts on RAW 264.7 cells was determined by 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) assay (BioVision, Milpitas, CA, USA). Its principle is as follows: the tetrazolium salt will be converted to soluble formazan crystals by succinate dehydrogenase in the mitochondria of metabolically active cells and hence in the dead cells, this reaction will not be occurred. For experimentation, each cell line at a density of 2x10⁴ cells/ml was seeded onto 96-well plate and incubated at 37°C of 5% CO₂ for 24 hours prior to being treated with various concentrations of *Russula* mushroom extracts for 24 hours. By the end of *Russula* treatment, 100 µl of WST solution was added to each well. The plates were kept in darkness for 30 mins before measuring the absorbance at 450 nm by the microplate reader system. Values of the three independent experiments obtained from WST assay were used to calculate the percentage viability of the cells using the equation demonstrated below. A graph of absorbance (Y-axis) plotted against sample concentration (X- axis) was constructed. The cytotoxicity of *Russula* extracts was presented as 50% inhibitory concentration (IC₅₀), the concentration of test samples required to reduce the absorbance to half (50%) that of the negative control

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells (with extract)}}{\text{Absorbance of untreated cells (without extract)}} \times 100$$

Determination of cyto-protective activity of extract against MMC toxicity: Prior to determine the cyto-protective activity carried out on the mouse monocyte macrophage RAW 264.7 (ATCC TIB-71™), cytotoxicity of *Russula* mushroom extracts was evaluated at 125, 250, 500, 1,000 and 2,000 µg/ml concentrations using the WST-1 assay described above. The extract concentration that yielded % cell viability greater than 80% was selected for cyto-protective activity study. For experimentation, 100 µl of RAW 264.7 cells at a density of 2x10⁴ cells/ml were seeded onto 96-well plate and incubated at 37°C of 5% CO₂ for 24 hours. The chemotherapeutic drug which is also a known mutagen mitomycin C (MMC) was used as cytotoxic-inducer. Media was removed and replaced with 200 µl of each *Russula* extract at 125 µg/ml in the presence of MMC (10 µg/ml) for 24 hours incubation. By the end of treatment time, cell viability was assessed as previously described for WST assay. Data were expressed as percentage of cell viability of untreated control (untreated) cells *versus* treated cells (in presence of extract, extract + MMC and MMC alone).

Results and Discussion

The yields of ethanolic extracts from four selected *Russula* mushrooms were in the range of 18 – 33 % w/w as shown in Table 1.

Table 1. Percentage (yield) of four selected *Russula* mushroom extracts

<i>Russula</i> samples	Yield (% w/w)
<i>R. medullata</i>	32.95
<i>R. virescens</i>	27.90
<i>R. helios</i>	18.73
<i>R. alboareolata</i>	24.67

Cytotoxicity of four *Russula* extracts: The cytotoxicity of *Russula* mushroom extract was assessed by WST-1 assay in RAW 264.7 cells. Cells were pre-treated with different concentrations of the extracts for 24 hours and the viability of cells was determined according to its principle as described above. The anti-proliferative activity of extracts was determined as principle described in WST-1 assay where the IC₅₀ (inhibitory concentration inhibited cell growth by 50%) value was calculated and used as a parameter of cytotoxicity. Results obtained from three different experiments demonstrated the IC₅₀ values of *R. medullata* extract at 484.44 ± 07.43, *R. virescens* at 907.14 ± 52.37, *R. helios* at 541.78 ± 14.35 and *R. alboareolata* at 760.05 ± 28.95 µg/ml. Regarding the classification of the cytotoxicity for natural ingredients described by Farshad H. Shirazi (2004)^[6], the four selected *Russula* extracts were potentially harmful (100 µg/ml < IC₅₀ < 1,000 µg/ml).

Cyto-protectivity of four *Russula* extracts: Before cell protection against MMC was undertake on RAW 264.7 mouse monocyte macrophage cells, the initial cytotoxicity screening of four selected *Russula* extracts was examined by using WST-1 assay. To verify the cyto-protective activity of four *Russula*, the WST-1 assay was used to determine viability after exposure of cells to MMC as a cytotoxic agent. The RAW 264.7 cells were supplemented with non-toxic concentrations of extracts in the presence of 10 µg/ml MMC for 24 hours. Then, cell viability values were assessed and % cell viability calculated as shown in Table 2 and Figure 2.

Table 2. Cytotoxicity screening of four *Russula* extracts against RAW 264.7 cells by WST-1 assay.

<i>Russula</i> extract Concentration (µg/ml)	Cell viability (%)			
	<i>R. medullata</i>	<i>R. virescens</i>	<i>R. helios</i>	<i>R. alboareolata</i>
125	83.71 ± 0.60 ^a	85.66 ± 0.74 ^a	78.50 ± 0.80 ^a	85.18 ± 2.27 ^a
250	76.71 ± 2.83 ^a	75.25 ± 1.59 ^a	60.62 ± 0.28 ^b	65.39 ± 1.02 ^b
500	47.27 ± 1.81 ^a	64.37 ± 1.04 ^c	50.70 ± 0.77 ^a	59.67 ± 1.96 ^b
1000	41.07 ± 0.67 ^a	44.46 ± 2.87 ^a	36.21 ± 1.09 ^b	43.52 ± 0.44 ^a
2000	32.96 ± 1.90 ^a	38.92 ± 2.54 ^b	34.48 ± 0.83 ^a	36.33 ± 1.96 ^a

Each value is mean ± S.D (n = 3). ANOVA and Tukey test (P < 0.05). Different letters indicate statistically significant differences.

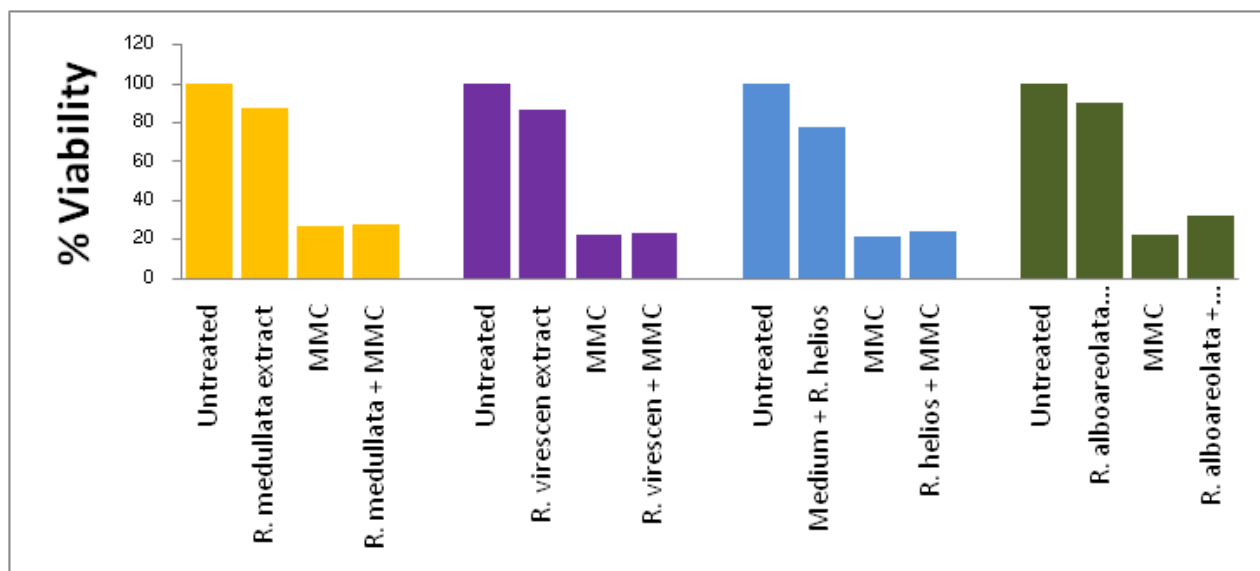


Figure 2. Histogram of RAW 264.7 illustrates % cell viability following the treatments of four selected *Russula* extracts (125 µg/ml), mitomycin C (MMC, 10 µg/ml) and a combination of each selected *Russula* extract and MMC assessed by WST-1 assay. Each value is mean ± S.D. Statistical analysis were tested by a multiple comparison Tukey test at 95% confidence, *p< 0.05

As reported in Table 2 and Figure 2, results were shown that treatments of four *Russula* extracts at various concentration ranging from 125 to 2,000 µg/ml produced cell toxicity in a dose-dependent manner (Table 2). Therefore, to avoid cytotoxicity effect caused by the extracts, the lowest dose of extracts at 125 µg/ml was chosen for cyto-protective activity study. MMC (10 µg/ml) was used as cytotoxic induction in all treatments. It found that MMC treatment decreased cell viability to 22.49 ± 0.18% and its cytotoxic effect was attenuated in the presence of *Russula* extract (125 µg/ml). The protection activity of *R. alboareolata* extract on RAW 264.7 cells was obviously seen by an increase in cell viability to 32.53 ± 3.00 %. The results suggest a cyto-protective ability of *R. alboareolata* extract against mitomycin C induced cell death.

Conclusion

The yields of ethanolic extracts from four selected *Russula* mushrooms were in the range of 18 – 33 % w/w. The ethanolic extracts of four selected *Russula* mushrooms were found to be potentially harmful (100 µg/ml < IC₅₀ < 1,000 µg/ml) to RAW 264.7 cells. Surprisingly, at 125 µg/ml, only *R. Alboareolata* extract exhibited the cyto-protective effect against MMC-induced cell death. The information obtained from this study can be used to support the uses of *Russula* mushrooms for a healthy dietary supplement in the future.

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