Biological activities and phytochemical constituent assessments of Thai Russula mushroom extracts

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Introduction

Mushrooms have been used as food and traditional medicines since ancient time. In Asian countries, numerous mushrooms have been utilized as alternative medicine for the treatments of various diseases such as cancer, inflammatory disease and enhancement of immunity (1,2). Edible mushrooms accumulate a variety of secondary metabolites as bioactive compounds for medicine including phenolic compounds, polyketides, flavonoids, terpene, steroid, lectin, polysaccharide and proteoglycan. Some of these compounds have tremendous important to health promoting in antioxidant, antimicrobial, antiviral, anticancer, anti-inflammatory, immunostimulatory effect and pharmaceutical activities as well as a less toxic effect (3).

In Thailand, 5,000 species of indigenous mushrooms have been reported (4). The Northeastern part is found to be the most in diversity that 16 species of Thai mushrooms used for traditional medicine are included. Russula is a genus of the mushroom that has been reported in 17 provinces of the Northeastern part. Some of them have been consumed as food and some have been used in traditional medicines for the treatments of various diseases (5). However, less information of toxicity and immunomodulatory activities of Thai Russula mushrooms is revealed. Therefore, this study was carried out to evaluate the cytotoxic, cyto-protective, phagocytic, production of ROS activities of four selected Russula mushrooms including R. medullata, R. virescens, R. helios and R. alboareolata collected from various provinces in Northeastern part of Thailand.

Methods

Preparation of mushroom extracts: The dried powder of R. medullata, R. virescens, R. helios and R. alboareolata was separately extracted in 95% ethanol. The aqueous suspension was evaporated under vacuum at 40°C and stored for use.

Phytochemical analysis: All four Russula extracts were analysed on phytochemical constituents by TLC and HPLC techniques. The TLC plate was detected under ultraviolet light at 254 and 366 nm with TLC-densitometer (CAMAG, Germany) then sprayed with anisaldehyde-sulphuric acid reagent (BDH chemicals LTD, England) for terpenes detection. HPLC analysis was performed using Water 600 with an Empower software equipped with ultraviolet (UV) detector using the analytical condition for terpeniod compounds. The retention times of peaks in HPLC chromatograms were monitored and recorded.

Cytotoxicity Assay: WST-1 assay was conducted to evaluate the viability of RAW 264.7 cells following treatment with various concentrations (125 to 2,000 µg/mL) of four Russula extracts. The formazan dye produced by viable cells were quantified by measuring the absorbance at 450 nm (Tecan Infinite 200 PRO, Tecan Group Ltd., Mannedorf, Switzerland). The amount of formazan was taken to calculate the percentage of viable cells.

Cyto-protective Assay: RAW 264.7 cells (2x10^4 cells/ml, 200 µl) were exposed to each Russula extract at 125 µg/mL in the presence of 10 µg/mL MMC (Sigma, USA) which was cytotoxic-inducer. The cells were incubated at 37°C with 5% CO2 for 24 hr. By the end of treatment time, cell viability was assessed as previously described for WST-1 assay.
**Phagocytic activity assay:** The RAW 264.7 cells (2x10⁴ cells/ml, 200 µl) were incubated with each *Russula* mushroom extract at 125 µg/mL or 10 µg/mL lipopolysaccharide (LPS) (Sigma, USA) or 100 µg/mL beta-glucan (β-glucan) (Sigma, USA) for 24 hr. Cells were gently washed with PBS and then 100 µl of 0.075% neutral red solution (Sigma, USA) was added to each well and incubated for 60 min. The supernatant was removed and the cells were washed with PBS twice. Then, 100 µg/mL cell lysed solution (ethanol and 0.01% acetic acid at the ratio of 1:1 w/w) was added and incubated at 37°C for 120 min. Cell lysate was removed to measure the quality of dye incorporated into cells by spectrometry at the wavelength of 540 nm. The percentage of phagocytosis activity was calculated as following equation:

\[
\% \text{ Phagocytic activity} = \frac{A2-A1}{A1} \times 100.
\]

When: A1 = Absorbance of control, A2 = Absorbance of sample.

**Respiratory burst assay:** RAW 264.7 cells (2x10⁴ cells/ml, 200 µl) were incubated with each *Russula* mushroom extract at 125 µg/mL or 10 µg/mL LPS or 100 µg/mL β-glucan at 37°C for 24 hr. After incubation, plate was centrifuged at 1,500 rpm for 4 min to pellet cells. Then, 100 µl of DHE solution (ENZO, USA) (dihydroethidium and DMEM at the ratio of 2:100 w/w) was added to each well. The plate was covered to protect from light and further incubated for 60 min. Cells were collected by centrifugation (1,500 rpm for 4 min) and then 2 µg/mL of Hoechst 33342 solution (Sigma, USA) diluted in DMEM at the ratio of 1:1 (v/v) was added to each well and incubated for 20 min. The stained cells were analyzed by fluorescent imaging system using the InCell Analyzer 2200 (GE Healthcare, UK) with Cy3 (excitation 550/ emission 620 nm) filter set. For superoxide anion (O₂•⁻) analysis, the dye Hoechst 3342 was utilized as a co-nucleus (DNA) counterstain (excitation 361/ emission 486 nm). The percentage of superoxide activity was calculated as follows:

\[
\% \text{ Superoxide activity} = \frac{F2-F1}{F1} \times 100.
\]

When: F1 = Florescent intensity of control and F2 = Florescent intensity of sample.

**Results**

**TLC and HPLC analyses:** All *Russula* extracts exhibited similar specific chromatographic fingerprints with dark quenching chromatographic bands under the detection of UV 254 nm (Figure 1A). The blue fluorescence chromatographic bands under the detection of UV 366 nm represented to coumarin (Figure 1B). After spraying with anisaldehyde - sulphuric acid reagent for detection of the terpene compounds, there were major bands at Rf value of 0.60 which promoted dark red spot detected under 366 nm (Figure 2C) and appeared as dark violet spot when detected under white light (Figure 2D) which could correspond to oleanolic acid. HPLC chromatograms of terpenoid compounds analysis were found in the extracts of *R. medullata*, *R. helios*, *R. alboareolata* and oleanolic acid, but not in *R. virescens* extract. Among the peaks in HPLC chromatograms, *R. medullata*, *R. helios* and *R. alboareolata* were monitored at tR of 8.292, 8.161 and 8.218 min, respectively which could correspond to standard oleanolic acid (tR of 8.466 min).

![Figure 1. Chromatographic fingerprints of four *Russula* extracts by TLC.](image)

Effects of extracts on cytotoxicity: After RAW 264.7 cells were treated with various concentration of extracts ranging from 125 to 2,000 µg/mL and assessed by WST-1 assay, all extracts exhibited the decrease of % cell viability in dose-dependent manner (Table 1).
Table 1. The percentage of cell viability of four *Russula* extracts on RAW 264.7 cells

<table>
<thead>
<tr>
<th><em>Russula</em> extracts (µg/mL)</th>
<th><em>R. medullata</em></th>
<th><em>R. virescens</em></th>
<th><em>R. helios</em></th>
<th><em>R. alboareolata</em></th>
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<tbody>
<tr>
<td>125</td>
<td>83.71 ± 0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.16 ± 0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.50 ± 0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.18 ± 2.27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>250</td>
<td>76.71 ± 2.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.25 ± 1.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.62 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.39 ± 1.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>500</td>
<td>47.27 ± 1.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>64.37 ± 1.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50.70 ± 0.77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>59.67 ± 1.96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td>41.07 ± 0.67&lt;sup&gt;d&lt;/sup&gt;</td>
<td>44.46 ± 2.87&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36.21 ± 1.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43.52 ± 0.44&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2000</td>
<td>32.96 ± 1.90&lt;sup&gt;e&lt;/sup&gt;</td>
<td>38.92 ± 2.54&lt;sup&gt;e&lt;/sup&gt;</td>
<td>34.48 ± 0.83&lt;sup&gt;e&lt;/sup&gt;</td>
<td>36.33 ± 1.96&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each value is mean ± SE (n=3). Two way ANOVA by Tukey’s Honestly Significant Difference (p<0.05). Difference capital letters in the same column are significantly different. Difference lowercase letters in the same row are significantly different.

**Effects of extracts on cyto-protective activity:** It was found that MMC alone decreased the cell viability to 22.49 ± 0.18% and its cytotoxic effect was attenuated in the presence of *Russula* extracts at =125 µg/ml as data illustrated in Table 2.

Table 2. The percentage of cell viability and cyto-protective of RAW 264.7 cells treated with four *Russula* extracts, mitomycin C (MMC) and a combination of each *Russula* extract and MMC assessed by WST-1 assay

<table>
<thead>
<tr>
<th>Treatments</th>
<th><em>Russula</em> extracts</th>
<th>MMC</th>
<th><em>Russula</em> + MMC</th>
<th>% Cyto-protective activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. virescens</em></td>
<td>86.40 ± 2.31&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>22.92 ± 1.96&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>23.55 ± 1.73&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>2.84 ± 1.54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>R. helios</em></td>
<td>78.01 ± 0.35&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>21.76 ± 0.21&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>24.03 ± 0.50&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>10.43 ± 1.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>R. medullata</em></td>
<td>87.30 ± 1.58&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>23.87 ± 0.27&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>27.77 ± 0.83&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>18.14 ± 4.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>R. alboareolata</em></td>
<td>90.83 ± 3.00&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>22.49 ± 0.17&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>33.52 ± 3.00&lt;sup&gt;Cc&lt;/sup&gt;</td>
<td>46.10 ± 5.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each value is mean ± SE (n=3). Two way ANOVA by Tukey’s Honestly Significant Difference (p<0.05). Difference capital letters in the same column are significantly different. Difference lowercase letters in the same row are significantly different.

**Effects of extracts on phagocytic activity:** Phagocytic function of RAW 264.7 cells were determined by meaning the amount of neutral red internalized in phagosomes (Figure 2). It was found that phagocytic activity of RAW 264.7 cells was stronger dominated by the exposure to LPS and *R. alboareolata* extract (118.97 ± 4.96%) than by *R. medullata* (96.87 ± 1.79%) and *R. helios* (50.92 ± 6.89) extracts and β-glucan (74.21 ± 5.36%).
Figure 2. The neutral red uptake ability of RAW 264.7 cells after exposed to various chemicals and extracts. (A) Control, (B) LPS, (C) β-glucan, (D) R. medullata extract, (E) R. virescens extract, (F) R. helios extract (G) R. alboareolata extract.

Effects of extracts on respiratory burst activity: The respiratory burst was evaluated thought the measurement of $\text{O}_2^{-\bullet}$ production. After RAW 264.7 cells were treated with Russula extracts at concentration of 125 µg/ml, LPS and β-glucan as the activator to induced $\text{O}_2^{-\bullet}$ production assessed by dihydroethidium (DHE) assay as (Figure 3). Among the four Russula extracts, R. alboareolata extract was significantly ($p<0.05$) exhibited the highest % $\text{O}_2^{-\bullet}$ production at 143.71 ± 0.23% and showed the highest % $\text{O}_2^{-\bullet}$ activity at 43.71 ± 2.34. The second rank was R. medullata extract whereas the activity of the R. virescens and R. helios were not different when compared with control.

Figure 3. Superoxide production in RAW 264.7 cells after exposed to various chemicals and extracts: (A) Control, (B) LPS, (C) β-glucan, (D) R. medullata extract, (E) R. virescens extract, (F) R. helios extract and (G) R. alboareolata extract

Discussion
Regarding the classification of cytotoxicity for natural ingredients described by Farshad H. Shirazi (6) the four selected Russula extracts were classified as potentially harmful substance (100 µg/mL < IC50 < 1,000 µg/mL). Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, (OA)) is a natural occurring pentacyclic triterpenoid compound which widely distributed in medicinal herbs medicinal herbs vegetables, fruits, mushrooms, medicinal herbs and medicinal herbs many plants (7). We found out that at a concentration of 100 mg/ml extracts, oleanolic acid was respectively detected in R. helios, R. medullata and R. alboareolata at 548.598, 1,231.155 and 1,504.715 mg/l. MMC is a chemotherapeutic drug that has been used in cancers treatment. It works by intercalating the DNA together so that it cannot come apart during cell division resulting in cells cannot divide and apoptosis. Damage to nuclear DNA is thought to be its primary mechanism of cell death in MMC action (8). In our study, Russula extracts at 125 µg/mL could protect RAW 264.7 cells from MMC (10 µg/mL)-induced cell death. It indicated the DNA-protective activity (or anti-mutagenic activity) of these Russula extracts where the most potent activity was observed in R. alboareolata extract. It is clearly known that macrophages contribute to the activation of immune responses against infectious agents. They detect gram negative bacteria particularly on binding of the cell wall component LPS to their pattern-recognition receptors (PRRs), comprising TLR4, CD14 and MD2, which recognize pathogen-associated molecular patterns (PAMPs). Among the different PAMPs, the response to LPS is one of the best characterized (9). Interestingly, phagocytic activity of RAW 264.7 cells was stronger dominated by the exposure to LPS and R. alboareolata extract than those of R. medullata extract, R. helios extract and β-glucan. The % phagocytic activity seemed to be gradual decrease in dose-dependent manner of oleanolic acid contained in the extracts. Based on results obtained in this study, R. alboareolata extract was previously detected the oleanolic acid contained in the highest amount by HPLC. Its high % phagocytosis activity of R. alboareolata extract was likely to be from oleanolic acid that had been identified as one of peroxisome proliferator activated receptors (PPARγ) ligand which binded to activate PPARγ, a family of nuclear hormone receptors. Results of respiratory burst assay demonstrated that R. alboareolata extract was significantly ($p<0.05$) exhibited the highest % $\text{O}_2^{-\bullet}$ production over other three extracts as well as β-glucan which was a well-known immune-stimulant compound found in mushrooms.

Conclusion
Data obtained from this study can suggest and promote the utilization of Russula mushrooms. R. alboareolata extract and R. alboareolata extract can be considered as a natural source supplement that may be useful in the treatment of bacterial infection. It can be used in development of healthy food and dietary supplement products in the future.
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References


