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Anti-inflammation and Active Compounds of Four Indigenous Thai *Russula* Mushrooms

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Abstract: *Russula* mushroom is mostly found in Northeastern part of Thailand. It has been used as foods and for the treatments of various diseases for a long time. However, less information of the anti-inflammatory activity of R. mushroom is revealed. The aim of this study was to evaluate the phytochemical constituents the anti-inflammatory mediator effect of Russula mushroom extracts including *R. alboareolata* (Nam-Pang in Thai), *R. medullata* (Ko-Kab-Yang in Thai), *R. virescens* (Khai-Domg in Thai) and *R. helios* (Ko-Reng-Som in Thai) on lipopolysaccharide-induced RAW 264.7 cells. The extract was prepared by maceration of dried mushrooms with 95% ethanol. Phytochemical components of extracts were analyzed by high performance liquid chromatography (HPLC) technique. It was found that all extracts possessed similar specific chromatographic fingerprints that exhibited peaks which corresponded to oleanolic acid. Results of water-soluble tetrazolium salt (WST-1) assay exhibited IC50 value of *R. alboareolata* (760.97 ± 28.95 µg/mL), *R. medullata* (484.44 ± 07.43 µg/mL), *R. virescens* (907.14 ± 52.37 µg/mL) and R. helios (514.78 ± 14.35 µg/mL) indicating their slight-cytotoxic effect. In inflammatory inhibition, *R. alboareolata* extract exhibited the highest in nitric oxide inhibition and prostaglandin E2 inhibition on lipopolysaccharide-induced RAW 264.7 cells at 78.80 ± 6.70 % and 66.62 ± 9.53 %, respectively. Including COX-2 inhibition, this extract showed the highest % COX-2 inhibition at 57.41 ± 2.27. These results suggest that the *R. alboareolata* extract is potential of anti-inflammatory activity on RAW 264.7 without affecting.

Key words: Anti-inflammation, *Russula* mushroom, active compound, RAW 264.7

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1. Introduction

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 activitiesas well as a less toxic effect. *Russula* mushrooms are of Basidiomycota genus and belonging to the family of Russulaceae. It was reported that 750 species of *Russula distributed* worldwide. In Thailand, the presence of *Russula* mushroom havebeen reported in 17 provinces of the Northeastern part and some of them have been consumed as food such as *R. monspeliensis* (Khai-Na-Lae-Paeng in Thai), *R. virescens* (Khai-Dong in Thai), *R. alboareolata* (Nam-Pang in Thai), *R. medullata* (Ko-Kab-Yang in Thai) and *R. helios* (Ko-Rang-Som in Thai). 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. nobilisfor* 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[1]. Biological activities of some *Russula* mushroom have been previously reported. *R. delica* showed antimicrobial activity against various bacteria and fungi including *Salmonella enteritidis, Stephylococus aureus, Micrococus luteus, Micrococus flavus, Bacilus cereus* and *Candida albicans*. *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-picryhydrazyl (DDPH) radical scavenging, superoxide radical scavenging assay[2]. However, less information of the anti-inflammation of *Russula* mushroom is revealed. Therefore, this study aims to evaluate the anti-inflammatory activities as well as cytotoxic of *Russula* extracts on RAW 264.7 cell line.

2. Materials and Methods

Preparation of *Russula* mushroom extracts: Fresh samples of *Russula* mushrooms (Fig 1) were collected in rainy season from the Northeastern part of Thailand. The powder mushrooms were extracted by macerating in 95% ethanol (plant: solvent ratio 1:10 w/v) for 24 hrs and 5 times. The aqueous suspension was evaporated under vacuum at 40 ℃ and stored for experimentation.

Fig 1. The four selected *Russula* mushrooms. A) *R. medullata* B) *R. virescens* C) *R. helios* D) *R. alboareolata*

Phytochemical analysis: All four *Russula* extracts were analysed on phytochemical constituents by HPLC techniques. HPLC analysis was performed using Water 600 with an Empower software equipped with ultraviolet (UV) detector using the analytical condition for terpenoid compounds. The retention times of peaks in HPLC chromatograms were monitored and recorded.

RAW 264.7 culture: The RAW 264.7 mouse monocyte macrophage cell line was purchased fromthe American Type Culture Collection (ATCC) (Manassas, VA, USA). The RAW 264.7 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO®) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO®) and 1% (v/v) penicillin-streptomycin (GIBCO®) in humidified atmosphere incubator with 5% CO_2 at 37 ℃. For experiments, cells were seed in 96-well plates at a density of $2x10^4$cells/well and incubated for 24 hrs before treatments.

Determination of cytotoxic activity: The cytotoxic property of extracts on RAW 264.7 cells were determined by 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Bio Vision, Milpitas, CA, USA). After overnight incubation, RAW 264.7 cells were treated with various concentrations of extract for 24 hrs. Then, 100 µL of WST-1 solution was added to each well and incubated for 30 mins before measuring the absorbance at 450 nm by the microplate reader and was taken to calculate the percentage (IC50) of viable cell.

Determination of anti-inflammatory activity: RAW 264.7 cells were treated with 100 µL of extracts at 125 µg/mL or β-glucan at 100 µg/mL and incubated for 24 hrs. Culture media was replaced with 100 µL of LPS at 10 µg/mL and incubated for 24 hrs. After incubation, the culture media was analyzed as indicator of nitric oxide and PGE2 and production. The nitrite accumulated in culture media was measured as indicator of NO production using Griess reagent assay. The culture media was mixed 100 µL of Griess reagent (1%

sulfanilamide, 5% phosphoric acid and 1% Naphthyletylenediamine dihydrochloride) and incubated at room temperature for 10 mins, and then the absorbance at 540 nm was measured using microplate reader. The amount of nitrite presented in the samples was measured with the sodium nitrite serial dilution standard curve. The ability of extracts to inhibit PGE2production was assessed by prostaglandin E2 ELISA kit-monoclonal (Cayman, USA) according to the manufacturer's instruction.

Determination of COX-2 inhibition: The extracts at concentration of 10 µg/ml were analyzed on COX-2 inhibition. The inhibition of COX-2 activity was measured by COX-2 fluorescence inhibitor screening assay kit ELISA (Cayman, USA) according to the manufacturer's instructions**.**

Statistical analysis: All data were expressed as mean ± standard error (Mean ± SE) of triplicated determination. The significance of difference was used to compare mean ($p<0.05$). The percentage of anti-inflammatory inhibition; NO, PGE_2 and COX-2 were analyzed by One-way ANOVA. The percentage of cell viability was analyzed by Two-way ANOVA followed by Tukey's Honestly Significant Difference test, where $p< 0.05$ was considered statistically significant.

3. Results and Discussion

HPLC analysis: 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 (Figure 2).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).The amount of oleanolic acid in the samples was measured with the oleanolic acid standard curve.

Cytotoxicity: After RAW 264.7 cells were treated with various concentrations 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). Results obtained from three different experiments demonstrated the IC50 values of *R. medullata, R. virescens, R. helios* and *R. alboareolata* were respectively at 484.44±07.43, 907.14± 52.37, 541.78±14.35 and 760.05±28.95 µg/mL. Regarding the classification of cytotoxicity for natural ingredients described by Shirazi, these *Russula* extracts were slight-cytotoxic effect (100 µg/mL<IC50< 1,000 µg/mL).

Table 1 The percentage of cell viability of *Russula* extracts on RAW 264.7 cells

Russula extracts	% Cell Viability				
	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)	1,000 (µg/ml)	2,000 (µg/ml)
R. medullata	83.71 ± 0.60^{Aa}	76.71 ± 2.83^{Ba}	47.27 ± 1.81^{Ca}	41.07 ± 0.67^{Da}	32.96 ± 1.90^{Ea}
R. virescens	85.16 ± 0.74^{Aa}	75.25 ±1.59^{Ba}	64.37 ± 1.04^{Cc}	44.46 ± 2.87^{Da}	38.92 ± 2.54^{Eb}
R. helios	79.50 ± 0.80^{Aa}	60.62 ± 0.28^{Bb}	50.70 ± 0.77^{Ca}	36.21 ± 1.09^{Db}	34.48 ± 0.83^{Ea}
R. alboareolata	85.18 ± 2.27^{Aa}	65.39 ± 1.02^{Bb}	59.67 ± 1.96^{Cb}	34.48 ± 0.83^{Ea}	36.33 ± 1.96^{Ea}

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.

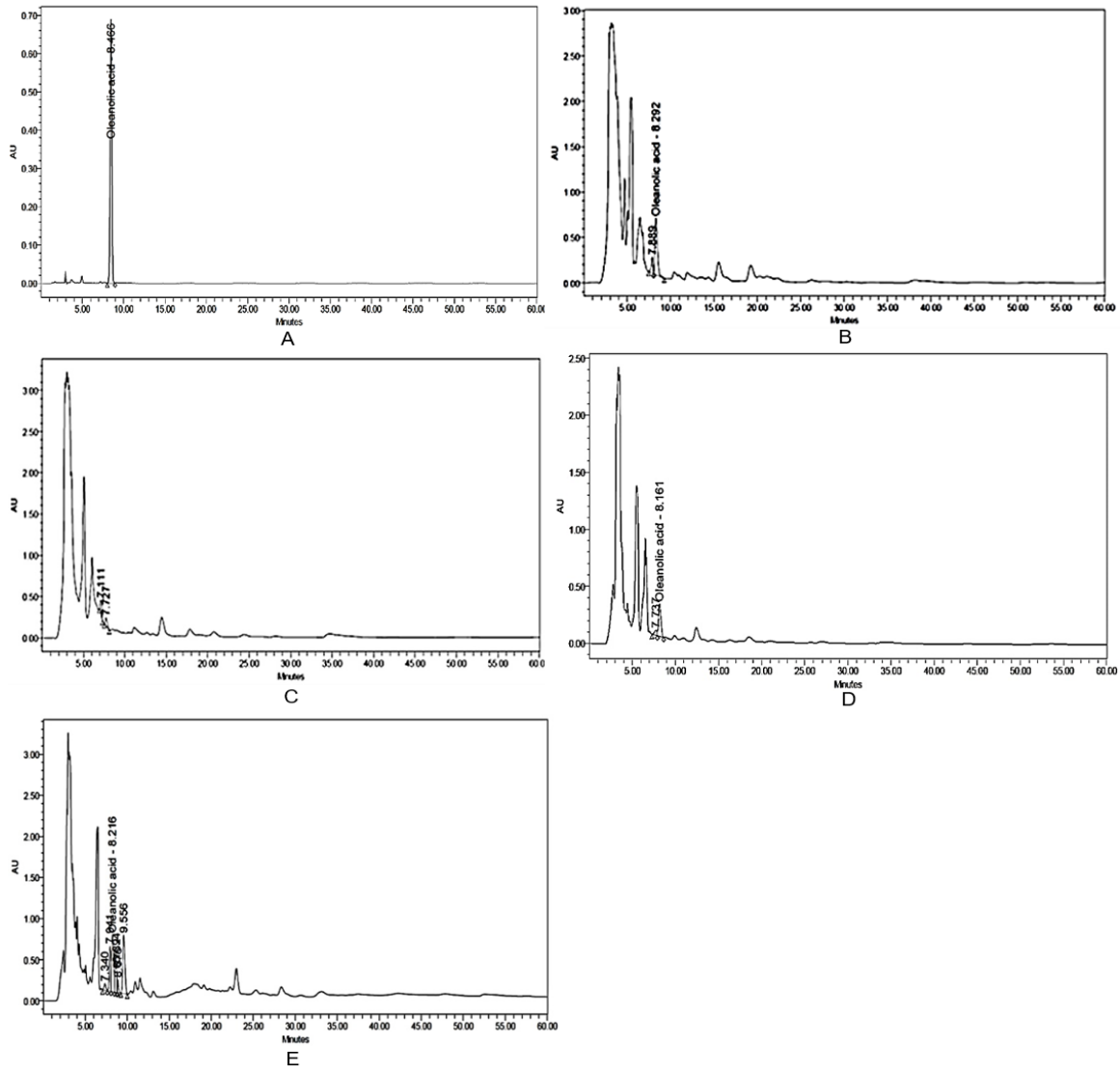

Fig 2. HPLC chromatograms of oleanolic acid contained in *Russula* extracts and standard compounds A) Oleanolic acid B) *R. medullata* C) *R. virescens* D) *R. helios* E) *R. alboareolata*

Anti-inflammatory activity: PGE2 is an important inflammatory mediator and, like NO, is involved in normal biological functions and inflammatory process. After RAW 264.7 cells were pre-treated with extracts at 125 µg/ml for 24 hrs prior to treatment with LPS at 10 µg/ml. The presence of *R. alboareolata* extract was statistically significant ($p<0.05$) exhibited the % NO and PGE2 inhibition at 76.80±6.71 and 66.62± 9.53 (Fig 3). While treatment of *R. medullata* extract exhibited the % NO and PGE2 inhibition at 68.68±2.60 and 38.90±0.39. The % PGE2 production of *R. virescens* and *R. helios* extracts were not different when compared to the treatment with LPS alone. Among the four *Russula* extracts, the data suggested that *R. alboareolata* extract shown in the most potential in an inhibition of PGE2 production in the LPS-stimulated RAW 264.7 cells.

COX-2 inhibition: COX-2 is enzyme which plays a major role in prostaglandins biosynthesis in inflammatory cells and in proliferative diseases. The inhibitory effect on COX-2 by *Russula* mushroom extracts at concentration of 10 µg/ml was measured by COX fluorescence inhibitor screening. Among the four *Russula* extracts, *R. alboareolata* extract significantly exhibited the highest % COX-2 inhibition at 57.41 ± 2.27%. The % COX-2 inhibition of the other three extracts, *R. virescens, R. helios* and *R. medullata* were not difference that compare to the control (Fig 4). This result indicates that only *R. alboareolata* extract shown in the most anti-inflammatory activity as inhibition of COX-2 activity.

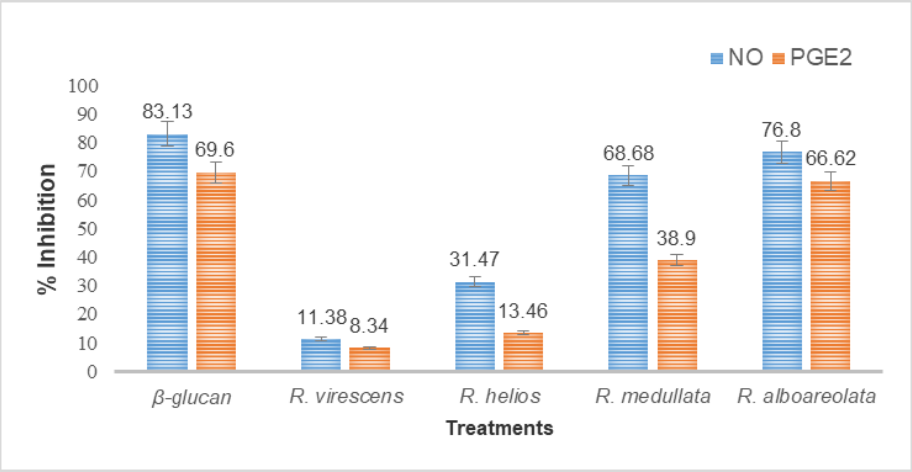

Fig 3. Histogram of RAW 264.7 cells illustrates % NO and PGE2 inhibition following the treatments of *Russula* extracts, β-glucan and followed by stimulated with LPS. Each value is mean ± SE Statistical analysis were tested by a multiple comparison Tukey test at 95% confidence, *$p< 0.05$

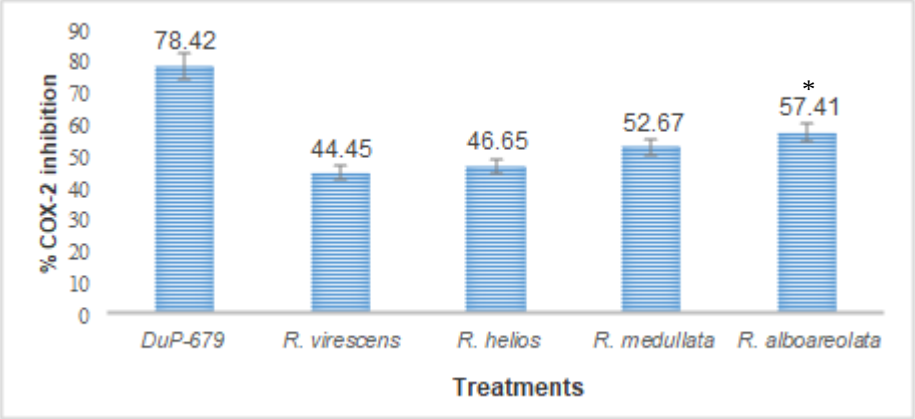

Fig 4. Histogram of the four selected *Russula* mushroom extracts and DuP-679 at concentration at 10 µg/ml illustrates % COX-2 inhibition. Each value is mean ± SE Statistical analysis were tested by a multiple comparison Tukey test at 95% confidence, *$p < 0.05$*

Regarding results demonstrated that the ethanolic extracts of four *Russula* mushrooms were found to be potentially harmful to RAW 264.7 cells. Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, (OA)) is a natural occurring pentacyclic triterpenoid compound which widely distributed in vegetables, fruits, mushrooms, medicinal herbs and many plants3. At a concentration of 100 mg/ml of *Russula* mushroom 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, respectively. In the present study, the *R. alboareolata* extract shown in the potential in an inhibition of NO and PGE2 production in the LPS-induced RAW 264.7 cells without affecting cell viability. The present finding had been supported by the reports that the extract of *R. vinosa* and *I. obliquss* could significantly inhibit NO and PGE2 production in LPS-induced RAW 264.7 macrophage cells4. Furthermore, only *R. alboareolata* extracts obviously showed the highest % COX-2 inhibition thought the mechanism presumably reflect bind tightly to the active site of COX-2 enzyme. *T. camphoratus* and *A. camphorate* inhibited the production of TNF-α, IL-8 and PGE2 as well as iNOS and COX-2 expression. The reports of triterpeniods from mushrooms were revealed from many researches. The triterpenoid extracts of *F. pinicola* and *G. lucidum* could decrease the secretion of PGE2 and could downregulation of iNOS and COX-25.

4. Conclusion

The information obtained from this study can be used to support the uses of *Russula* mushrooms. *R. alboareolata* extract can be considered as a natural source supplement that may be useful in the

anti-inflammation. It can be used in development of healthy food and dietary supplement products in the future.

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