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Apoptotic activity of ethanolic extract of Thai indigenous mushroom *Russula alboareolata* against L929, HeLa and HepG2 cells by MMP assay

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Objectives: This study was carried out to investigate apoptotic activity of *Russula alboareolata* ethanolic extract against L929, HepG2 and HeLa cells

Methods: Fresh samples of *R alboareolata* from Kalasin, Mukdahan and Yasothon in the Northeastern part of Thailand**.** Dried samples were individually macerated with 95% ethanol with sonication for 1 hour to obtain crude extracts. Three cell lines including L929, HepG2 and HeLa purchased from ATCC were grown as adherent in DMEM at 37ºC in 5% CO_2 incubator. Cells ($1x10^5$ cells/ml, 200 µl) were seeded onto 24 well plate and further grown for 24 hr. Apoptotic activity of *R alboareolata* extract was determined by mitochondrial membrane potential (MMP) assay using JC-1 fluorescent method. The L929, HeLa and HepG2 cells were treated for 24 hr with the extract at 600, 500 and 1,000 µg/ml,respectively. The chemotherapeutic drug, valinomycin was used as an apoptotic inducer and kept as a positive control. Apoptotic cells of L929, HeLa and HepG2 were analysed by fluorescent imaging system using the InCell 2200 Analyser (GE Healthcare, UK) following being stained with JC-1 and Hoechst 3342 reagents.

Results: Valinomycin (1µg/ml) clearly induced apoptosis in L929, HeLa and HepG2 at 43.86, 36.85 and 40.00%, respectively. The percentages of apoptosis induced by *R alboareolata* extract was found as follows: L929 (77.20%, tested at 600 ug/ml), HeLa (73.69%, tested at 500 ug/ml), and HepG2 (30.00%, tested at 1,000 ug/ml),

Conclusion: The ethanolic extract *R. alboareolata* possesses apoptotic activity against both normal (L929) and cancerous cells (HeLa and HepG2). However, we were unable to compare the degree of its apoptotic induction among these three cell lines due to the different concentrations used. Further studies including active constituents and *in vivo* experiments are required if this mushroom will be used as a novel dietary supplement and/or botanical-drug for chemoprevention.

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Introduction

Mushrooms have been used for a food and traditional medicines since ancient time. Previous studies reported the presence of 1,147 mushroom species in the Northeastern part of Thailand. They composed of 647 edible mushroom species, 222 commercial mushroom species and 400 poisonous mushroom species[1]. Thirty-seven species of these mushrooms were used in traditional medicine. *Russula* mushrooms are in the family of Russulaceae[2] Their distributions are in several countries including the United States of America, Sweden, France, Norway, Madagascar, Italy, Belgium, Taiwan, China, Japan as well as Thailand[3,4]. Though *Russula* mushrooms have been consumed as food and used for the treatments of various diseases in the Northeastern part of Thailand for a long time, their biological properties are rarely studied. We recently determined cytotoxic and antioxidant activites of 10 selected *Russula* mushrooms including *R crustosa, R delica*, *R monspeliensis*, *R velenovskyi*, *R virescens*, *R lepida*, *R alboareolata, R paludosa* and *R medullata* collected in Chaiyaphum, Roi Et and Mukdahan provinces. Among these 10 *Russula* mushrooms, *R alboareolata* revealed a non-cytotoxic to L929, HeLa, MCF-7 and HepG2. Moreover, it possessed a high antioxidant capacity against superoxide radicals ($O_2^{-\bullet}$) when determined using the photochemiluminescence (PCL) assay. Therefore, we carried out this study with the aim to further investigate apoptotic activity of *R alboareolata* ethanolic extract against L929, HepG2 and HeLa cells.

Methods

Collection of mushroom samples: Fresh samples of *R alboareolata* were collected in rainy season during August-October 2014 from three provinces including Kalasin, Mukdahan and Yasothon in the Northeastern part of Thailand**.** It was identified by the mushroom specialist, Mr.Winai Klinhom of Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, Mahasarakham, Thailand.

Extraction of mushroom: Collected *R alboareolata* mushroom was dried in hot air oven at 50˚C for 18-20 hours and ground using an electronic grinder. For extraction, all dried sample was macerated in 95% ethanol (plant: solvent ratio 1:10 w/v) and sonicated for 1 hour. The ethanolic extract solution was evaporated using the rotary evaporator to yield *R alboareolata* crude extract. The extract was stored in darkness at -20˚C until utilization.

***Culturing and maintaining of cell cultures*:** Four cell lines including mouse fibroblasts (L929, ATCC® CCL-1) and two transformed or cancer cell lines including human hepatocellular carcinoma (HepG2, ATCC®HB-8065) and human cervical carcinoma (HeLa, HeLa,ATCC®CCL-2) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They 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®) at 37ºC in humidified atmosphere incubator with 5% CO_2, sub-culturing every 2-3 days by trypsinization with 1 to 2 ml of 0.25 % trypsin-EDTA to allow detachment of cells and add fresh culture medium, aspirate and dispense into new culture flasks. For experiments, cells were harvested by trypsinization as explained before in Hank's balanced salt solution (HBSS, GIBCO®) or phosphate buffered saline (PBS, GIBCO®), plated in 96 well plates at a density of 1 x 10^5 cells/well and incubated for 24 hours before treatment.

Determination of apoptotic activity: Apoptotic activity of *R alboareolata* extract was determined by mitochondrial membrane potential (MMP) assay using JC-1 fluorescent method. The L929, HeLa and HepG2 cells at density of 1x10^5 cells/ml were seeded onto 24 well plate and incubated at 37˚C of 5% CO_2 for 24 hours. Media were removed and replaced with 1,000 µl of fresh media containing extract at 600, 500 and 1,000 µg/ml for L929, HeLa and HepG2, respectively. Cells were further incubated for 24 hours. Valinomycin (1µg/ml) was used as an apoptotic inducer and kept as a positive control. Treatment of valinomycin was performed for 2 hours due to its high toxicity. At the end of treatment period, cells were harvested by trypsinization and stained with JC-1 reagent as procedure described by supplier (Sigma Chemicals, St.Louis, MO, USA). Apoptotic cells of L929, HeLa and HepG2 were analyzed by fluorescent imaging system using the InCell 2200 Analyser (GE Healthcare, UK) with Cy3 (excitation 542/ emission 697) and FITC (excitation 475/ emission 525) filters. For a precision of apoptotic cell analysis, the dye Hoechst 3342 (Sigma Chemicals, St. Louis, MO, USA) was utilized as a co-nuclear (DNA) counterstain.

Results

Figure 1 demonstrated the images of L929, HeLa and HepG2 cells stained with JC-1 and followed by Hoechst 3342 dye which is a part of a family of blue fluorescent dyes used commonly to stain DNA in cells[5]. Our aim of staining cells with JC-1 and Hoechst 3342 for apoptotic cells analysis since JC-1 dye will stain on cell membrane whereas Hoechst 3342 stain for nuclear DNA. Therefore, the location and number of individual cells were clearly identified during cell analysis/ scoring. In this study, at least 1,000 cells were scored for each cell line and treatment.

Summary of ratio of red to green fluorescence, % mitochondria health and % apoptosis obtained from each cell line and treatment were reported in Table 1. The ratio depends only on the membrane potential and not on other factors such as mitochondrial size, shape, and density, which may influence single-component fluorescence signals. Use of fluorescence ratio detection, therefore allows us to make comparative measurements of membrane potential and determine the percentage of mitochondria (% mitochondria health) within L929, HeLa and HepG2 cells that respond to *R. alboareolata* extract (Table 1).

Table 1. Summary of apoptotic effects on L929, HeLa and HepG2 cells after treatments with valinomycin and *R. alboareolata* extract mitochondrial membrane potential alteration and JC-1 stain.

Sample	Fluorescent Intensity (Red/Green)			% Mitochondria Health			% Apoptosis		
	L929	HeLa	HepG2	L929	HeLa	HepG2	L929	HeLa	HepG2
Untreated	0.57	0.19	0.10	100.00	100.00	100.00	-	-	-
Valinomycin	0.32	0.12	0.06	56.14	63.15	60.00	43.86	36.85	40.00
Russula alboareolata	0.13	0.05	0.07	22.80	36.31	70.00	77.20	73.69	30.00

Note: Valinomycin treatment performed at concentration of 1 µg/ml for 2 hours.
Russula alboareolata extract treatments performed at 500, 600 and 1,000 µg/ml for HeLa, L929 and HepG2 cells, respectively and for 24 hours.

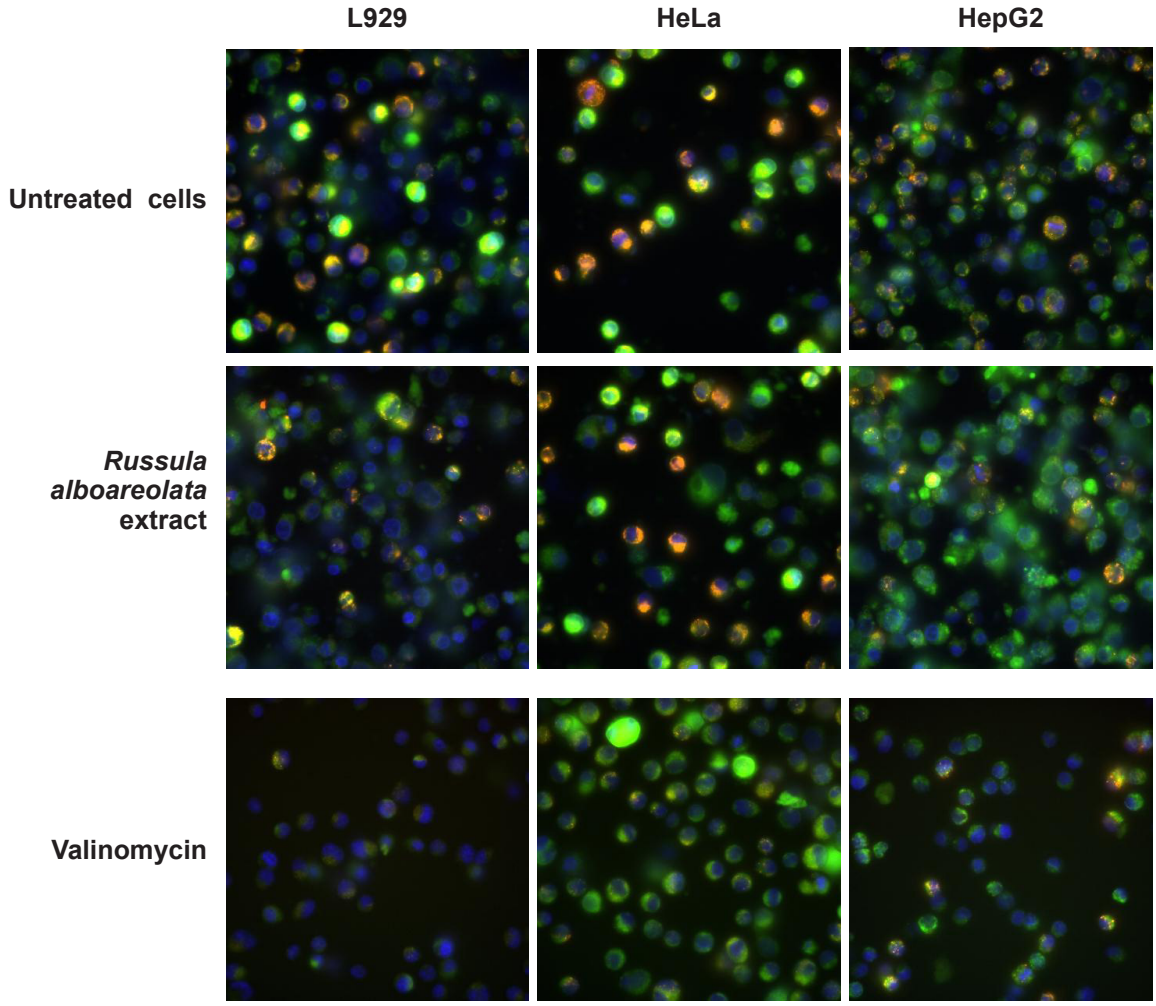

Figure 1. Effect of *Russula. alboareolata* extract on mitochondrial membrane potential (MMP) alterations occurred in L929, HeLa and HepG2 following treatment for 24 hours at concentrations of 600, 500 and 1,000 µg/ml extract, respectively. Valinomycin (positive control) was tested at 1 µg/ml for 2 hours. Images of cells were analyzed by JC-1 staining.

Discussion

Apoptosis is the pharmacodynamics endpoint of anticancer drug therapy as this phenomenon ensures that no cancer resistance to chemotherapy will occur[6]. Moreover, apoptosis is an autonomous dismantled process to remove individual components of cells and avoids inflammatory effect normally associated with necrosis; thus no toxicity to the normal surrounding cells will occur when cells undergo apoptosis[7]. In this study, we used the mitochondrial membrane potential (MMP) assay to evaluate the apoptotic activity of the *R. alboareolata* extract. The principle of MMP is based upon changes in the membrane potential and alterations to the oxidation–reduction potential of the mitochondria. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pore (MPTP), allowing passage of ions and small molecules. Probes that detect mitochondrial membrane potential are positively charged, causing them to accumulate in the electronegative interior of the mitochondrion. Regarding our study, the membrane-permeant JC-1 dye was employed and apoptotic cells were analyzed by Cell Imaging Analyser (InCell® 2200, GE Healthcare, UK) using the green (FITC, excitation 475/emission 525) and red (Cy3, excitation 542/emission 697) filters.

JC-1 dye is widely used in apoptosis studies to monitor mitochondrial health[8]. The dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. Regarding this principle, we demonstrated images of apoptotic activity induction by *R. alboareolata* extract on L929, HeLa) and HepG2 cells in Figure 1.

Besides JC-1, cells of all treatments were co-stained with Hoechst 33342 nucleic acid dye. Hoechst 33342 is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. Therefore, in our study this dye was employed to distinguish condensed pycnotic nuclei in apoptotic cells of L929, HeLa and HepG2. Regarding data of % apoptosis (Table 1), we found that valinomycin (1µg/ml) clearly induced apoptosis in L929, HeLa and HepG2

cells as indicated by a marked decrease in the red/green fluorescence ratio to 0.32, 0.12 and 0.06, respectively. The percentages of mitochondria (% mitochondria health) of the three tested cell lines were also illustrated and they were used to calculate % apoptosis of each cell lines and treatment as expressed in Table 1.

Conclusion

The ethanolic extract of *R. alboareolata* possesses apoptotic activity against both normal (L929) and cancerous cells (HeLa and HepG2). However, we were unable to compare the degree of apoptotic induction by *R. alboareolata* extract among these three cell lines due to the different concentrations used. Further studies including active constituents and *in vivo* experiments are required if this mushroom will be used as a novel dietary supplement and/or botanical-drug for chemoprevention.

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