The 6th International Conference on Biochemistry and Molecular Biology

Toxicity investigation of amanitas wild mushrooms from local market in Khon Kaen

Prakaymars Panumars[1,2], Sompong Klaynongsruang[2,3], Nisachon Jangpromma[1,2,*]

[1]Forensic Science Program, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[3]Departments of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand
*E-mail: nisaja@kku.ac.th

Abstract

Mushrooms have high protein content and are recognized as the human diet as well as versatile medicinal properties. However, some species of mushroom are dangerous because of their toxicants. In present, cases of people eating toxic mushrooms in Thailand increase annually, especially in the Northeast. This evidence may result from the inability of consumers to distinguish edible from inedible species. The most common lethal mushrooms are *Amanita* species. Hence, the wild Ra York mushrooms, which are considered as edible mushroom from a local market in Khon Kaen were preliminary identified by ITS gene amplification. Ribosomal nucleotide sequencing result indicated that these mushrooms are identical to *Amanita princeps* and *Amanita hemibapha*. Both of wild mushrooms were investigated for the toxicity effect against the murine macrophage cells (RAW 264.7). The result showed that *A. hemibapha* mushroom extract exhibited toxicity against RAW 264.7 cells higher than *A. princeps* mushroom extract. Thus, the apoptosis induction of *A. hemibapha* mushroom extract was further determined. The result demonstrated that mushroom extract treatment at 25 and 50 μg/ml resulted in the enhancement of late apoptotic cells with dose-dependent manner. This finding suggested that the *A. hemibapha* could involve possible risks to human. Therefore, careful identification is required to ensure safe consumption of wild mushrooms.

Introduction

Mushroom contains rich protein, non-fat, dietary fiber and has enough vitamins and minerals for healthy benefits. The contents in mushroom are contributing to high valued in therapeutic uses and an alternative source of health[1]. However, mushroom food poisoning has increased annually, especially in the Northeast of Thailand. This evidence continues to be a problem faced by health care professionals[2]. Several types of toxins are responsible for mushroom poisoning, including cyclopeptide, orellanine, monomethylhydrazine, disulfiram-like, hallucinogenic indoles, muscarinic, isoxazole and gastrointestinal specific irritants[3]. Consumption of toxic mushrooms can cause various signs depending on the mushroom toxin such as gastroenteritis, CNS disturbances, and liver failure. Moreover, ingestion of toxic mushrooms may result in serious toxicity, including death[4]. The most common lethal wild

mushrooms are *Amanita* species. Although, some *Amanita* species are edible and some of them are poisonous.

In Thailand, the rainy season starts in June and terminates in September. Within this period, various groups of wild mushrooms grow in the forests and some villagers collect and consume them. Outbreaks of poisonous mushroom in the Northeastern area of Thailand are usually occur[5]. Especially, *Amanita princeps* and *Amanita hemibapha* which commonly named as "Hed Ra York Kao" and "Hed Ra York Laung", respectively, were generally collected from the wild for consumption and sale. Some of *Amanita* species are edible mushrooms such as *A. princeps* and *A. hemibapha*. However, these mushrooms are sometimes misidentified as their young fruiting bodies resemble those of the edible species and may be contain some toxic substances. In addition, there are misconceptions about ethno mycological knowledge such as only toxic mushrooms can tarnish a silver spoon, mushrooms that are ingested by animals are safe for humans, and boiling of mushrooms can detoxify toxins. In most cases of poisonous mushroom, a rapid species identification of samples consumed by the patients is required for appropriate medical treatments. Generally, the available mushroom samples are not always well-preserved for clinical diagnosis, especially those from uncooked mushroom debris, stomach contents and meals[6]. Thus, the toxicology of wild mushrooms should be concerned. Herein, this study aimed to estimate the effective toxicity of wild mushroom extracts on the murine macrophage cells (RAW 264.7). In addition, the mechanism of apoptotic cell death pathway was investigated.

Methodology

Mushroom samples and identification

Two wild mushrooms were purchased from a local market in Khon Kaen, Thailand. The samples were cleaned with double distilled water (DDW) then separated and collected in a plastic bag. Mushrooms sample were cut into small pieces using blades sterile and the weight were recoded. All mushrooms were dried at 40 °C until a constant weight will be obtained. Then, the mushroom samples were DNA-extracted and identified by DNA sequencing.

DNA extraction and PCR amplification

The fresh mushroom samples containing 2-15 mg of wet weight were ground in liquid nitrogen. DNA was extracted using the GF-1 Nucleic acid extraction kit (Vivantis, USA) according to the manufacturer's instructions. The entire ITS region was amplified with the primers: ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS 4 (5´-TCCTCCGCTTATT GATATGC-3´)[7]. The 25 µl of PCR reaction contained 10x *Taq* buffer (10 mM Tris pH 8.8, 50 mM KCl, 0.1% Triton X-100), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, 0.4 µM of each primer and 5 µl of DNA template. PCR conditions were initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation 94 °C for 30s, annealing at 55 °C for 30s, extension at 72 °C for 30s, and final extension step at 72 °C for 10 min. Five microliters of reaction were determined by electrophoresis on 2% agarose gels in 1% TAE buffer. The gels were photographed with Gel Doc (Bio Rad, USA). The DNA sequencing of ITS regions were identified and confirmed by searching in Genbank.

Extraction of mushroom

All samples were dried to obtain a constant weight and ground with a mortar and pestle. The mushrooms were then coarsely powdered and extracted follow by the method of Kawaji[8] with a minor modification. Briefly, the mushroom powder was mixed with double distilled water (DDW) at a ratio of 1 g of mushroom powder: 5 ml of water. Then the mixture was allowed to boil at 100 °C for 25 min and immediately kept on ice. After centrifugation the supernatant was collected. The amount of protein was determined using Bradford assay[9]. The protein solutions of wild mushroom extracts were stored at -80 °C until use.

Cell culture

RAW 264.7 cells, a murine macrophage cell line were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic:antimycotic were purchased from Gibco, USA. Cell culture were incubated in CO_2 incubator (5% CO_2) at 37 °C. The cells were passaged prior to confluence 90-95% by removing cells with trypsin, following by centrifugation and count cells using Haemacytometer (Bright-Line™, USA) for seeding.

Cell viability assay

The MTT assay was used to assess the *in vitro* cytotoxicity of the wild mushroom extracts on RAW 264.7 cells. The cells ($2x10^4$ cells/well) were seeded in 96 well-plates with RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic:antimycotic (Gibco, USA). Cell cultures were incubated in CO_2 incubator (5% CO_2) at 37 °C for 24 h. The medium was then removed from the wells and replaced with 100 µl of sterilized medium containing the wild mushroom extracts at different concentrations (12.5 to 50 µg/ml). After incubated for 24 h, MTT assay was performed by adding 100 µl of 0.5 mg/ml MTT solution (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromine) to each well and further incubated for 30 min. The MTT solution was then removed and 100 µl of DMSO was added. The absorbance at 570 nm was recorded using microplate reader (Varioskan LUX, USA). The cell viability was expressed as percentage of control[10].

Apoptotic morphology assay by cell staining

The fluorescence microscopy was used to assess the apoptotic morphology of the wild mushroom extracts on RAW 264.7 cells. The cells ($1.5x10^5$ cells/well) were seeded in 48 well-plates with RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic:antimycotic (Gibco, USA). Cell cultures were incubated in CO_2 incubator (5% CO_2) at 37 °C for 24 h. Then, the medium was removed and replaced with 300 µl of sterilized medium containing the wild mushroom extracts with different concentrations (12.5 to 50 µg/ml). The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. Subsequently, the cells were harvested and washed with PBS. Then, the pellet cells were collected by centrifugation at 3,000 rpm for 3 min and resuspended in 30 µl of Acridine orange/Ethidium bromide (AO/EtBr) (stock solution 1 mg/ml in water, diluted 1:90 in PBS) (Biotium, USA). The cells were incubated for 15 min in the dark at room temperature and the cell pellets were resuspened in 10 µl of PBS. The morphology of apoptotic cells was determined using a ZEISS Axio Vert.A1 Inverted Microscope (Carl Zeiss Microscopy, USA.). The experiment was repeated at least 3 times.

Statistical analysis

The data were presented as the mean of triplicated independent experiments. Statistical analysis was evaluated by One way ANOVA and Duncan's new multiple range test using SPSS ver. 19.0. The different letters indicate to be not significantly different ($p < 0.05$).

Results and Discussion

Mushroom identification

The wild mushroom samples were purchased from the local market in Khon Kaen, Thailand. Wild mushrooms were identified up to the species level using the standard taxonomic keys (Figure 1). Mushrooms have been represented in different in morphological characterization. Figure 1A and Figure 1B represented Hed Ra York Kao and Hed Ra York Laung, respectively, which both of them were considered as edible mushroom. The identification of wild mushrooms was confirmed using ITS gene region. The BLAST results revealed the highest pairwise of nucleotide identities with 99% of *Amanita princeps* (Genbank accession No. KT213713.1) (Figure 1A) and 96% of *Amanita hemibapha* (Genbank accession No. LC068790.1) (Figure 1B). Even if, they are edible mushrooms[11,12] however, in China the *A. hemibapha* var. *ochracea* has been reported to cause dizziness and nausea after eaten in large amount[13]. Hence, the further experiments were carried out with the aim of finding a scientific answer regarding cytotoxic effect of both mushrooms.

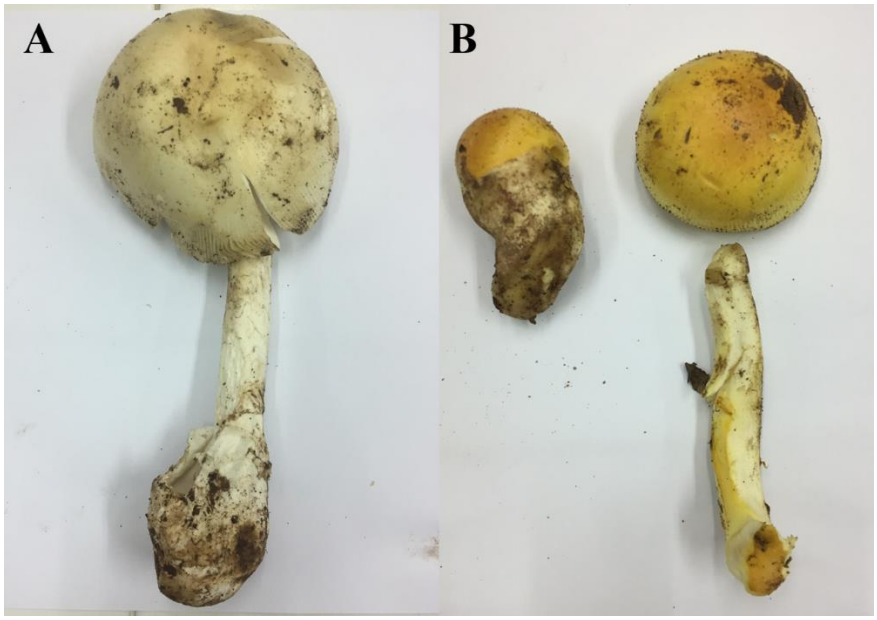

Figure 1. Morphology of *Amanita princeps* (A) and *Amanita hemibapha* (B).

Effect of wild mushroom extracts on RAW 264.7 cells

Macrophages are known to play the important roles in the cellular and humoral immunities[14]. They have been widely used in various scientific researches such as toxicological, immunology, hematological and screening for drug candidates[15]. Therefore, RAW 264.7 cell was selected for evaluated the effect of wild mushroom extracts by MTT assay. Each of the wild mushroom extracts at the concentrations of 12.5, 25 and 50 μg/ml indicated that the *A. princeps* was less cytotoxic towards RAW 264.7. At 12.5 μg/ml, *A.*

princeps extracts showed cell viability more than 80%. In addition, the concentration at 25 and 50 μg/ml was showed 77.8% and 75.5% of cell viability, respectively (Figure 2A). In contrast, *A. princeps* and *A. hemibapha* showed the cytotoxic effect towards RAW 264.7 cells. The cell viability less than 20% was observed from the concentration of 12.5 μg/ml up to the concentration of 50 μg/ml (Figure 2B). Taken together, our data demonstrated that *A. hemibapha* had toxicity to immune cell. Recently, it is well known that *Amanita* includes various both of edible and inedible species which contain dangerous poisoning[16]. Due to the possibility of poisoning combined with the difficulty of distinguishing edible from poisonous species, consumers should be careful about eating any *Amanita* specimen. Additionally, some edible mushroom can become poisonous mushrooms by taking up and accumulate high levels of chemicals or toxic substances, especially several heavy metals[16]. Hence, besides sufficient knowledge or suitable methods to classify poisonous mushrooms, toxicity studies are highly mandatory to ensure the safe utilization of mushrooms.

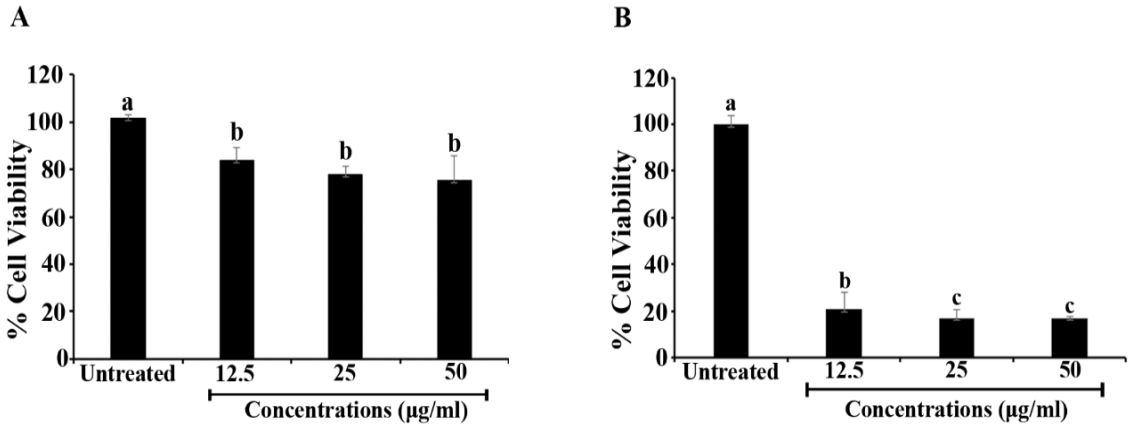

Figure 2 The cytotoxicity of *Amanita princeps* extracts (A) and *Amanita hemibapha* extracts (B) on murine macrophage RAW 264.7 cell line. RAW 264.7 cell was treated with 12.5, 25 and 50 μg/ml of wild mushroom extracts for 24 h. The cell viability was determined by MTT assay towards RAW 264.7 cells. The experiment was performed in triplicate. The different letters indicate that they are not significantly different at the 0.05 level ($p < 0.05$).

Effect of wild mushroom extracts on apoptosis of RAW 264.7 cells

RAW 264.7 cells were treated with wild mushroom extracts of *A. hemibapha* at concentrations of 12.5, 25 and 50 μg/ml for 24 h. After 24 h of co-incubation, treated cells were harvested and photographed. Each concentrations of *A. hemibapha* extracts demonstrated death of RAW 264.7 cells as a dose-dependent manner with the increasing of Ethidium bromide (EtBr) intensity and decreasing of live cells as the reduction of Acridine orange (AO) intensity. On the other hand, early apoptotic cells are presented in yellow-green. In addition, the late apoptotic cells are presented in orange-red (Figure 3). These results indicated that *A. hemibapha* showed cytotoxicity towards immune cells through apoptotic promotion. Apoptosis is a process of programmed cell death pathway that occurs in all multicellular organisms. It is triggered by various stimuli, including pathogenic bacteria and exogenous toxins[15]. Thus, the apoptosis induced by *A. hemibapha* extract might due to its containing toxins.

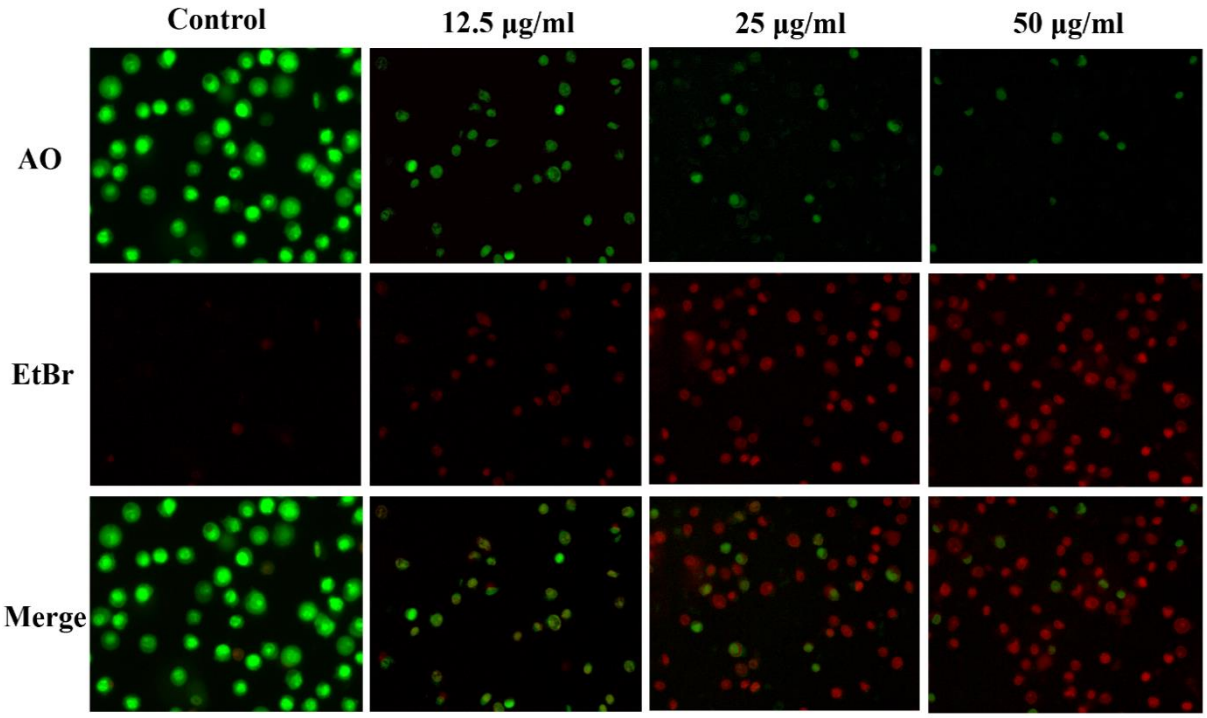

Figure 3 RAW 264.7 cells were stained with Acridine orange/Ethidium bromide (AO/EtBr) and the stained cells were observed under a fluorescence microscope equipped with UV excitation filters at 40x magnification. RAW 264.7 cells were co-incubated with 12.5, 25 and 50 µg/ml *Amanita hemibapha* extract for 24 h. After 24 h incubation, cells were harvested, washed with PBS and stained with 1 mg/ml AO/EtBr. The live cell or normal cell morphologies are presented in green. The cells are early apoptotic cells are presented in yellow-green, and the late apoptotic cells are presented in orange-red.

Conclusion

The correct identification of the wild mushroom species is difficult for laypersons, especially in *Amanita* spp. This study indicated that edible mushroom *A. hemibapha* showed a poisonous effect towards RAW 264.7 cells via apoptosis process. From this evidence, *A. hemibapha* may be hazardous to human. Thus, rapid detection experiments to aid in the identification of poisonous mushrooms are essential. Additionally, consumers should be careful about *Amanita* mushrooms consumption.

References

1. Lindequist U, Niedermeyer T.H. J. and Jülich W.D. eCAM 2(3): 285–299.
2. Parnmen S, Sikaphan S, Leudang S, Boonpratuang T, Rangsiruji A and Naksuwankul K. The Journal of Toxicological Sciences. 41(1): 65-76.
3. KJ Berger and DA Guss. The Journal of Emergency Medicine, 28(1): 53– 62.
4. SH Eren, Demirel Y, Ugurlu S, Korkmaz I, Aktas C, Güven FM. Clinics. 65(5): 491-6.
5. Sanmee R, Tulloss R, Lumyong P, Dell B and Lumyong S. Studies on *Amanita* (*Basidiomycetes : Amanitaceae*) in Northern Thailand. Fungal Diversity. 32: 97-123.
6. Cai Q, Tulloss R.E, Tang L.P, Tolgor B, Zhang P, Chen Z.H. and Yang Z.L. BMC Evolutionary Biology. 14: 143.
7. Dörnte B. and Kües U. Biotechnology and pharmacy, 7(4): 825-836.
8. Kawaji A, Sone T, Natsuki R, et al. The Journal of Toxicological Sciences, 5(3): 145-56.
9. Bradford M. Analytical biochemistry, 72, 248-254.

10. Mosmann T. Journal of Immunological Methods. 65: 55-63.
11. Zhang P, Tang L.P, Cai Q and Xu T.P. Mycology, 6(2): 86-93.
12. Semwal KC, Stepheson SL, Bhatt VK and Bhatt RP. Mycosphere, 5(3): 440-461.
13. Jing SH, Liping T, Miliang Y. Yunnan Wild Mushroom Poisoning Prevention. 1-18.
14. Martin S.B. Hematopoietic system, 153: 724-727.
15. Lalita R and Ian R.T. Molecular pharmacology, 53: 415-421.
16. Biagi M, Martelli L, Perini C, Lella LD, and Miraldi E. Natural Resources, 5: 225-232.

Acknowledgements

This study was supported by Protein and Proteomics Research Center for Commercial Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen, Thailand, The research capability enhancement program through graduate student scholarship, Faculty of Science, Khon Kaen University and Plant Genetic Conservation Project under The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindrorn : Khon Kaen University.