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Biological Activities of a Thai Luminescent Mushroom

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Abstract

Wild fruit bodies of luminescent mushrooms were collected from wood stumps over a period covering August to October 2011 in the Kosumpisai forest, Mahasarakham province, in the Northeast of Thailand. A study of the morphological and genetic characteristics of the luminescent mushroom suggested that it was *Neonothopanus nimbi* KS. The fruiting bodies and mycelium of *Neonothopanus nimbi* KS were assayed for their antimicrobial activities, antifungal activity, inhibitory activity against avian influenza H5N1 neuraminidase (NA), and anticancer activity, using organic solvent extracts. The results showed that only the methanol extract of mycelia was effective against *Bacillus sphaericus*, with the widest inhibition zone of 11.66 ± 2.71 mm, but this was not effective against the other 3 bacteria (*Pseudomonas aeruginosa*, *Serratia marcescens*, and *Escherichia coli*). On the other hand, all of the fruit body extracts were inactive against all four bacteria. The ethylacetate extract of mycelia inhibited the NCI-H187 small lung cancer cell line, KB oral cavity cancer cell line, and the MCF7 breast cancer cell line, including *Magnaporthe grisea* and *Curvularia lunata*. The methanol extract of mycelia inhibited the KB oral cavity cell cancer cell line, *Magnaporthe grisea*, and *Curvularia lunata* at 96.66, 95.32 and 95.41 %, respectively. The results imply that polar extracts of mycelia are a resource of bioactive compounds, whereas extracts of fruit bodies have less inhibitory activity against cancer, phytopathogenic-fungi and H5N1 neuraminidase.

Keywords: *Neonothopanus nimbi*, luminescent mushroom, antimicrobial activity, antifungal activity, anticancer

Introduction

Luminescent mushrooms have increased in large numbers because more and more new species are found, specifically in the subtropical and tropical zones of the globe where natural conditions are most favorable for their habitation [1]. Luminescent mushrooms have been found in North and South America, Europe, Asia, Australia and Africa, most of them belonging to the basidiomycetes class [2]. The luminescence of mushrooms has been studied for more than a hundred years. However, the structure of the luminescence system of luminescent mushrooms and the mechanism of light emission remain an enigma to date. Currently, conventional knowledge assumes there are 2 alternative concepts. According to the first, mushroom luminescence is determined by the functioning of the luciferase-luciferin system. According to the second, mushroom luminescence is associated with the oxidation of organic substrates and takes place without the participation of a specialized enzyme [3-5]. Luminescent mushrooms have been used as an indicator test object for bioassays. Luminescent mushrooms such as *Armillaria mellea*, *Mycena citricolor*, and *G. viridilucens* were used to develop toxicity tests. Luminescent mushrooms specifically were used to detect toxicity of 3,5 dichlorophenol, pentachlorophenol and salts of heavy metals [6]. In addition, a bioactive compound from *Neonothopanus nambi* can control the plant parasitic

nematodes *Meloidogyne incognita* and *Steinernema carpocapsae* and the plant pathogenic fungi *Pythium* sp. and *Phytophthora palmivora* [7,8]. Thai luminescent mushroom is a wild mushroom which is vulnerable to extinction and has been the subject of little research, and, therefore, it is of interest when studying bioactive compounds of Thai luminescent mushrooms. This study investigated a luminescent mushroom which was found in the Kosumpisai forest of Northeast Thailand during the rainy season of 2011 (**Figure 1**). The Thai luminescent mushroom was initially described as *Neonothopanus nambi* KS. We studied their biological activities; antibacterial, antifungal, anticancer and inhibitory activity against avian influenza H5N1 neuraminidase (NA), which may lead to applications for medical treatments and agriculture in the future.

Materials and methods

Collection of samples and isolation of a luminescent mushroom

All mushroom samples were collected from wood stumps at night, and were observed in the field as sources of luminescence over the period of August to October 2011 in the Kosumpisai forest, Mahasarakham province, in the Northeast of Thailand (**Figure 1**). They have a rather white color when in light conditions (**Figure 1a**) and express luminescent color when in dark conditions (**Figure 1b**). The fruiting bodies were found on the dead wood of a broadleaf tree, covered by a thick layer of rotten leaves. All fresh samples were placed in papers and put in plastic bags. The samples of the mushroom fruiting bodies were washed and then wiped with alcohol 70 % under aseptic lab conditions. Small pieces of the core part of the caps and stipes were obtained and transferred into (PDA; Merck, Germany) medium and incubated at 25 °C for fungal growth covered with mycelium. The culture transfer obtained a pure isolate. The mycelium was maintained in PDA slants at 4 °C.

(a) (b)

Figure 1 Thai luminescent mushroom in light conditions (a) and dark conditions (b).

Mushroom identification

Morphological characteristics of the luminescent mushroom

The fruiting bodies of the luminescent mushroom was determined by morphological characteristics in daylight, colony characteristics, type of areal hypae, and aerial mass color. The mycelium of the luminescent mushroom was observed under a microscope for growth of hyphae and spore formation.

Genetic characterization of the luminescent mushroom

Mycelium grown in potato dextrose broth (PDB; Merck, Germany) for 14 days was subjected to DNA extraction by using a Nucleospin Plant II kit (Machery-NagelTM, Germany). Quality of DNA solution was assessed by a spectrophotometer at 260/280 nm. PCR amplification : ITS regions were amplified using primer ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3'), described by White *et al*. [9]. Amplification was carried out in a 50 µl reaction mix containing 1× PCR buffer, 3 mM MgCl2, 0.2 mM dNTPs, ITS primers 10 picomole each, Taq polymerase 0.5 unit (InvitrogenTM, Brazil) and 250 ng fungal genomic DNA templates. Amplification was performed in a thermal cycler PTC-200 (MJ researchTM, USA) with the following program: denaturing at 94 °C 5 min, 40 cycles of 1 min at 94 °C, annealing 1 min at 56 °C, elongation 1 min at 72 °C, and final elongation of 10 min at 72 °C. PCR product (8 µl) was electrophoresised in a 1.5 % agarose gel using the QIA quick PCR purification kit (QiagenTM, USA) according to the manufacturing protocol.

Purified PCR product was sequenced at 1st base. ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') primer was used to sequence ITS1, ITS2 and 5.8S regions.

The sequence was used as a query to check for similar sequences in databases of DNA (Genebank) using the Basic Local Alignment Search Tool (BLAST) [10].

Extract preparation from fruit bodies

The fruiting bodies were dried at 50 °C and pulverized. The powdered material (50 g) was mixed with 600 ml of methanol for 3 days at room temperature and repeated for 3 times. The crude methanol extracts were collected and dried under a rotary evaporator, leading to a brown sticky paste (12.76 g). The resulting extracts were partitioned with 300 ml of hexane, dichloromethane, ethyl acetate, and methanol, respectively, at room temperature, and repeated for three times. The hexane extract (1.69 g), dichloromethane extract (0.50 g), ethyl acetate extract (0.20 g), and methanol extract (6.30 g) were screened for their biological activities (**Figure 2**).

Extract preparation from mycelia cultures

The mycelia were grown in PDB. Erlenmeyer flasks were inoculated by 5 mm. mycelia agar plugs from the PDA Petri cultures. The inoculated flasks were put on a shelf at room temperature. The mycelium pellets were separated from the culture fluids by filtration after 4 weeks of fermentation. After that, they were dried at 50 °C. The dried mycelium pellets (50 g) were extracted with 600 ml methanol for 3 days at room temperature, and repeated 3 times. The mycelium extracts were then partitioned with hexane, ethyl acetate 3 times for each solvent. All extracts were evaporated, leading to the hexane extract (2.53 g), ethyl acetate extract (31.66 g), and methanol extract (8.34 g), which were tested for their biological activities (**Figure 2**).

Microorganism

The antimicrobial studies were performed using pathogenic microorganism strains such as *Bacillus sphaericus* TISTR 1048, *Pseudomonas aeruginosa* TISTR 781, *Serratia marcescens* TISTR 1354, and *Escherichia coli*, which were supplied by the Thailand Institute of Scientific and Technological Research (TISTR). Cultures of these bacteria were grown in nutrient broth (NB, Merck, Germany) at 37 °C for 24 h, and the prepared density of the cell was 10^6 CFU/ml before use.

The phytopathogenic fungi (*Magnaporthe grisea* and *Curvularia lunata*) were cultured and tested at the National Center for Genetic Engineering and Biotechnology (NSTDA), Thailand.

Cancer cell line and avian influenza H5N1 neuraminidase

The NCI-H187 small cell lung cancer, KB oral cavity cell cancer, MCF7 breast cell cancer, and avian influenza H5N1 neuraminidase were obtained and tested by the National Center for Genetic Engineering and Biotechnology (NSTDA), Thailand.

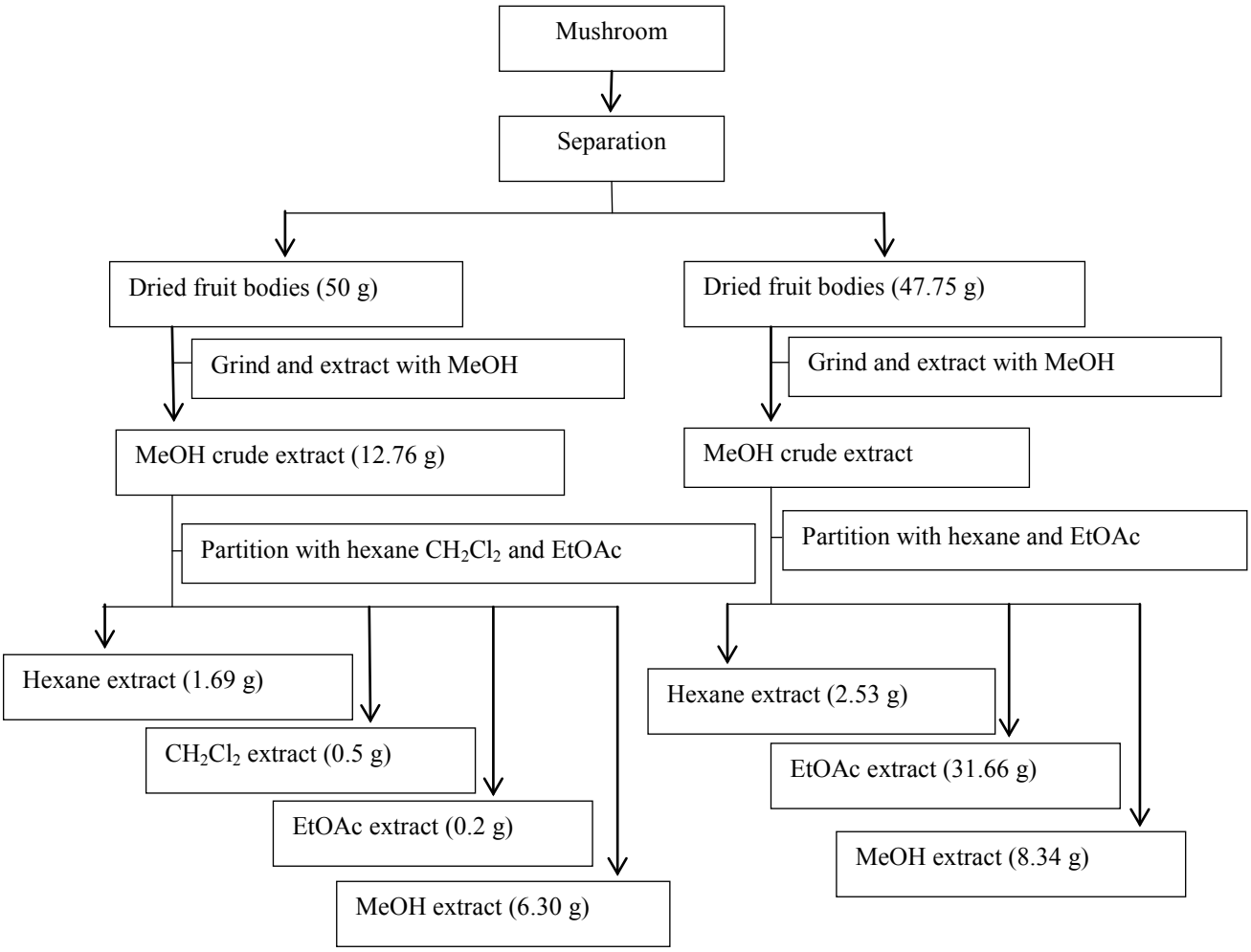


Figure 2 Extraction procedure of the Thai luminescent mushroom.

Antimicrobial activity

The antimicrobial activity tests were carried out by using the disc diffusion method [11]. The fruiting bodies and mycelium extracts were dissolved in 5 % DMSO, and 20 µl samples (100, 1,000 and 10,000 ppm) were dropped on paper disks with diameters of 6 mm. The disk with extract was placed on nutrient agar with compost microorganism 10^6 CFU /ml spread on top of agar, while the paper disk containing 5 %DMSO was used as a negative control. This assay was performed in triplicate and, after, incubated at 37 °C for 24 h; the antimicrobial was monitored based on the diameter of the clear zone or inhibition zone around the paper disk.

Antifungal activity

The fruiting bodies and the mycelium extracts of Thai luminescent mushroom were investigated for anti-phytopathogenic fungal activity. *Magnaporthe grisea* is a plant-pathogenic fungus that causes a serious disease affecting rice, and *Curvularia lunata* causes a black kernel of rice and severe grain discoloration. The fruiting bodies and the mycelium extracts of the luminescent mushroom were tested against *Magnaporthe grisea* and *Curvularia lunata* using the Fluorometric method; 5(6)-Carboxy

fluorescene diacetate assay (CFDA). The minimum inhibitory concentration (MIC) of positive control is Amphotericin B (1.56 - 3.13 µg/ml).

Inhibitory activity against avian influenza H5N1 Neuraminisase (NA)

Inhibitory activity against avian influenza H5N1 neuraminidase (NA) was determined by fluorometric determination (MUNANA-based enzyme inhibition assay). IC_{50} of positive control is Oseltamivir carboxylate (0.203 nM).

Anticancer activity assay

The fruiting bodies and the mycelium extracts of Thai luminescent mushroom were investigated for anticancer activity. The inhibitory activity of extracts on the NCI-H187-small lung cancer cell line, KB-Oral cavity cancer cell line, and MCF7-breast cancer cell line were tested by a resazurin microplate assay (REMA). IC_{50} of positive controls are Tamoxifen (7.78 µg/ml) and Doxorubicin (8.24 µg/ml).

Results and discussion

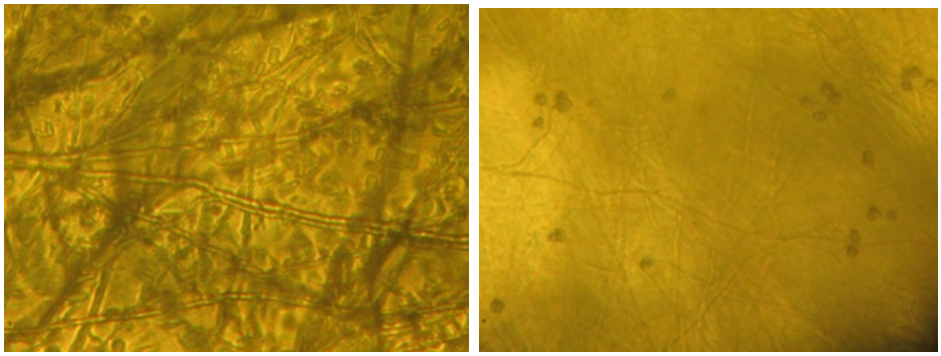
Mushroom identification

Morphological characteristics of the luminescent mushroom

The luminescent mushroom was observed and photographs taken in laboratory conditions. The fruiting bodies of the luminescent mushroom appeared in a variable white shape (**Figure 3**). The pileus was 3 - 10 cm long and 2 - 5 cm across, with a circular shape when young, becoming flabellate and convex, sometimes depressed toward the insertion of the stipe, when mature. The surface was smooth and white, with a margin acute to somewhat crenate, and whitish, thin flesh. The lamellae were white, long decurrently on the stipe. The stipe was cylindrical, 1 - 2 mm across, 2 - 4 mm length, eccentric to lateral, solid; solitary, grouped to somewhat clustered (**Figure 3**); basidiospore hyaline, cylindric - elliptic and smooth with white spore printing. In culture, the colonies on PDA were fast-growing, attaining a diameter of 30 mm in 6 days, and a full petri disk within 13 days at 25 °C. Colonies were white, smooth, and homogenous in growth. Neither conidiophores nor conidia were observed within 14 days of mycelium developing. The luminescent mushroom appeared in white mycelia (**Figure 4**). Their hyphae were white when observed with the naked eye, but transparent and colorless under an optical microscope. The appearance of mycelium and hyphae growing of the luminescent mushroom is illustrated in **Figure 5**. The luminescent mushroom had a white spore printing feature, which identified it with the Subdivision *Basidiomycota* [12,13], Class *Agaricomycetes*, Order *Agaricales*, Family *Marasmiraceae*, and Genus *Neonothopanus*, which was similar to the 3 isolate (isolatePW1, PW2 and KKU) of the luminescent mushrooms (*Neonothopanus nambi*) that Saksirirat *et al*. found in Kok Putaka (isolatePW1 and PW2), Khon Kaen and in Khon Kaen University (isolateKKU) [6,14,15].

Figure 3 The fruiting bodies of the luminescent mushroom.

Figure 4 Mycelium appearance of the luminescent mushroom on PDA for 13 days.

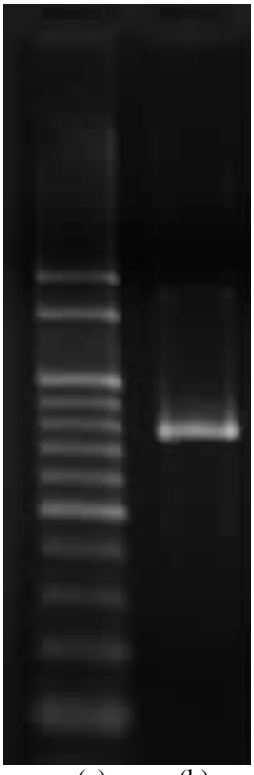


(a) (b)

Figure 5 Hyphae appearance of the luminescent mushroom.

Genetic characterization of the luminescent mushroom

PCR amplification had only one product (800 base pair) (**Figure 6**). The sequencing data is shown in **Figure 7**. The search for similar sequences in the GenBank using the BLAST program showed the *Basidiomycete* isolate belonged to the *Eukaryota*; *Fungi*; *Basidiomycota*; *Agaricomycetes*; *Agaricales*; *Marasmiraceae*; *Neonothopanus nimbi*, with a 99 % identical score. It showed similarity with three luminescent mushrooms, with accession numbers JN 571728.1, KC514805.1 and DQ 444307.1.



(a) (b)

Figure 6 Molecular characterization of the Thai luminescent mushroom; (a) lane showing 100 bp DNA ladder; (b) lane showing the band of the Thai luminescent mushroom DNA.

CTACTGTTTGAGGTCAACAAATCAATTAGTTGTCCGTCAAACAGACAGTTAGAGAGCAGACA
CCCCAAAGCCTCTTTGACAGTTAAAACAGACAGAGCAAACTCTCGTTTGCAACTATCCCAAC
CAAAGAGACCCTTTTGTCAGATAGAACACCAAGGCGTAGATAATTATCACACCAAAGGTAG
TCCAACAATTGGTTTCTACTAATGCTTTTAAGAGGAGCCAATGCATCTTAGATGCCAGCAAG
CCTCCAACAATCCAAGCCTCAGAAGCTACAAACTTGTGAGGTTGAGAAATTAATGACACTCA
AACAGGCATGCCCCTCGGAATACCAAGGGGCGCAAGGTGCGTTCAAAGATTCGATGATTCA
CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCAA

Figure 7 DNA sequence of the Thai luminescent mushroom.

Antimicrobials activity

Of all mycelia extracts used against *Bacillus sphaericus*, the methanol extract of mycelia (10,000 ppm) had the widest inhibition zone of 11.66±2.71 mm (**Figure 8**, **Table 1**). However, mycelia extracts was inactive against other 3 bacteria; *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Escherichia coli*. In addition, all fruiting bodies extracts were inactive against all 4 bacteria. For bactericidal effect, Bua-art *et al*. found that the bioactive compound from *N. nambi* was not effective against *Bacillus subtilis*, *Rhizobium* sp. and *Aspergillus* sp. [8].

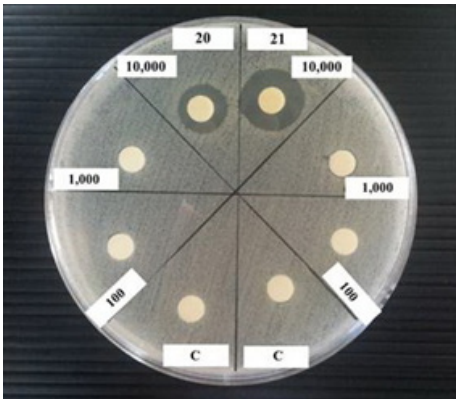


Figure 8 No. 20 (Left) shows the ethyl acetate extract of mycelia inhibit *B. sphaericus* and no. 21 (Right) shows the methanol extract of mycelia inhibit *B. sphaericus*.

Table 1 Inhibition zone diameter of extract from Thai luminescent mushroom.

	Extracts (10,000 ppm)	**Inhibition zone diameter ± SD (mm)**			
		B. sphaericus	***P. aeruginosa***	***S. marcescens***	***E. coli***
Fruit bodies	Hexane	NA	NA	NA	NA
	CH_2Cl_2	NA	NA	NA	NA
	EtOAc	NA	NA	NA	NA
	MeOH	NA	NA	NA	NA
Mycelia	Hexane	9.61±0.82	NA	NA	NA
	EtOAc	9.88±1.71	NA	NA	NA
	MeOH	11.66±2.71	NA	NA	NA

NA = indicate no antibacterial effect

Antifungal, anticancer activity and inhibitory activity against avian influenza H5N1 neuraminidase (NA) assay

All extracts were examined for anticancer, anti-phytopathogenic fungal activity, and inhibitory activity against avian influenza H5N1 neuraminidase. The ethylacetate extract of mycelia inhibited the NCI-H187 small lung cancer cell line, KB oral cavity cancer cell line, MCF7 breast cancer cell line including *M. grisea* and *C. lunata* while dominant inhibit *M. grisea* at 94.20 % (**Table 2**) but was inactive against avian influenza H5N1 neuraminidase. The methanol extract of mycelia inhibited the KB oral cavity cancer cell line, *M. grisea*, and *C. lunata* at 96.66, 95.32 and 95.41 %, respectively. The results

imply that extracts of mycelia are a resource of bioactive compounds, whereas extracts of fruit bodies have less inhibitory activity in cancer, phytopathogenic-fungi, and H5N1 neuraminisase. Bua-art *et al*. studied the effectiveness of the bioactive compound from *N. nambi* against fungi. They found that the bioactive compound was effective against *Pythium aphanidermatum* and *Phytophthora palmivora*, but not *T. harzianum*. In addition, the bioactive compounds showed nematicidal effect on the root-knot nematode *Meloidogyne incognita*, but not on the entomopathogenic nematode *Steinernema carpocapsae* [8]. The two isolates of luminescent mushroom, *Neonothopanus nambi* PW1 and PW2, were found in naminones A-C (1-3), 1-epi-anmbinone B (4), nambinone D (5), aurisin A, and aurisin K [16]. Aurisin A and aurisin K exhibited antimalarial activity against *Plasmodium falciparum*, and antimycobacterial activity against *Mycobacterium tuberculosis*. Nambinone C, aurisin A, and aurisin K showed cytotoxicity against NCI-H187 cancer cell lines. Aurisin A and aurisin K showed cytotoxicity against the cholanginocarcino ma cell line [16].

Table 2 Inhibitory activity of extracts from Thai luminescent mushroom.

Mushroom	Solvents	Percentage inhibition (%)					
		NCI-H187 small lung cancer cell line	**KB oral cavity cancer cell line**	**MCF7 breast cancer cell line**	***M. grisea***	***C. lunata***	**H5N1 neuraminidase (NA)**
Fruit bodies	Hexane	NI	NI	NI	NI	NI	NI
	CH_2Cl_2	74.90	NI	55.50	NI	NI	NI
	EtOAc	NI	NI	NI	NI	NI	NI
	MeOH	NI	NI	NI	NI	NI	NI
Mycelia	Hexane	NI	NI	NI	95.27	NI	NI
	EtOAc	88.30	85.50	60.09	94.20	91.11	NI
	MeOH	85.10	96.66	NI	95.32	95.41	NI

NI = no inhibition

Conclusions

A Thai luminescent mushroom was collected from wood stumps during the rainy season in the Kosumpisai forest of Northeast Thailand. The morphological and molecular identification of the Thai luminescent mushroom showed that it was *Neonothopanus nimbi* KS. The fruiting bodies and mycelium extracts of the Thai luminescent mushroom were examined for antimicrobial activity against four bacteria, *B. sphaericus*, *P. aeruginosa*, *S. marcescens* and *E. coli*, and 2 phyto-patogenicfungi, *M. grisea* and *C. lunata*, including anticancer activity and inhibitory activity of avian influenza H5N1 neuraminidase.

The sources of bioactive compounds were mostly found in ethylacetate and methanol extracts of mycelia, which showed inhibitory activities against *B. sphaericus*, *M. grisea*, and *C. lunata*, and anticancer actions against the NCI-H187 small lung cancer cell line and KB oral cavity cancer cell line. However, no fruiting bodies or mycelium extracts could inhibit avian influenza H5N1 neuraminidase. The results of this study give new insight into a useful way to apply bioactive compounds of luminescent mushroom for medical treatment and agriculture.

Acknowledgements

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