Neonothopanus nambi Speg., a new source of antibiotic and anti-inflammatory agents

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ABSTRACT

Neonothopanus nambi Speg. is a poisonous basidiomycete luminescent mushroom. The information on antibacterial and anti-inflammatory activities of the fungus has not been established. Study on antibacterial and anti-inflammatory potencies of *N. nambi* extracts using *in vitro* model were assessed. The results indicated that the mycelia of the luminescent mushroom extracted with $CHCl_3$, EtOAc, and culture filtrate extracted with EtOAc demonstrated excellent antibacterial activity against *Staphylococcus aureus* ATCC 25923 with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranged from 2-4 µg/ml, which is very closed to that of vancomycin (0.5-1 µg/ml). Moreover, hexane fraction of the collected mycelia exhibited obvious antibacterial potency against the pathogen with MIC and MBC values of 16 µg/ml. In contrast, all of the fractions were inactive against *Escherichia coli* ATCC 25922, a Gram-negative bacterium. For anti-inflammatory assay, $CHCl_3$ fraction of *N. nambi* mycelia showed the most potent inhibitory effect on nitric oxide-released macrophage cells with an IC_{50} of 10.9 µg/ml followed by EtOAc and hexane fractions with IC_{50} values of 19.7 and 25.6 µg/ml, respectively. Both of the culture filtrate and the collected mycelia extracted with $CHCl_3$ exhibited comparable effect to the positive controls, L-NA (NO synthase inhibitor, IC_{50} = 10.2 µg/ml), indomethacin (IC_{50} = 16.6 µg/ml), the standard used non-steroidal anti-inflammatory drugs. Purification, antibacterial mechanism of action, and anti-inflammatory activity of the extracts are still under investigations.

Keywords: *Neonothopanus nambi*, Luminescent mushroom, Antibacterial activity, Anti-inflammation

1. INTRODUCTION

Neonothopanus nambi Speg. is a poisonous basidiomycete luminescent mushroom which is found in Australia, South America, Central America and Malaysia. Pharmacological effects of sesquiterpenes isolated from the fungus exhibited pronounced cytotoxicity against cancer cell lines as well as antimalarial and antimycobacterial activities [1]. Moreover, the effect of bioactive compounds from *N. nambi* was investigated on infectious larvae of root-knot nematode (*Meloidogyne incognita*) *in vitro*. The results demonstrated that the compounds caused mortality of the nematode without adverse effects on beneficial organisms [2, 3]. However, antibacterial and anti-inflammatory activities of this luminescent mushroom have not been evaluated. Therefore, the objectives of this research were to study on antibacterial and anti-inflammatory activities of *N. nambi* extracts using *in vitro* model.

2. MATERIALS AND METHODS

Preparation of the luminescent mushroom extracts

Cultivation of *N. nambi* was carried out following a method from Kanokmedhakul et al. [1]. Briefly, the mushroom isolate No. 3 was collected from Ratchaburi province and the mushroom hyphae were grown in potato dextrose agar. The mycelia were transferred to potato dextrose broth and incubated in a dark room without shaking with 2 h of light per day at 25°C for 18 days.

Crude extracts from broth culture and mycelia were prepared as follows: liquid culture of *N. nambi* was filtered using Whatman filter paper No. 1 to collect culture filtrate. The filtrate was thrice partitioned and then evaporated to yield *n*-hexane-, $CHCl_3$-, EtOAc-, and H_2O-extracts (295, 25, 385 and 6,035 mg/l, respectively). The collected mycelia were macerated with MeOH (3 x 500 ml) for 3 days, under constant stirring at 250 rpm at room temperature. The mycelium mixture was filtered and the combined filtrate, once dried, was then added with 10% MeOH/H_2O mixture. The mixture solution was extracted three times with equal volume of *n*-hexane, $CHCl_3$, and EtOAc solvents. The water-crude fraction was freeze-dried and the organic crude extracts were dried over anhydrous Na_2SO_4 and then evaporated to obtain *n*-hexane-, $CHCl_3$-, EtOAc-, and H_2O-extracts with yield 56, 196, 114, and 366 mg/l, respectively. All of the mushroom extracts were kept at -20°C for future antimicrobial and anti-inflammatory assays.

Antibacterial assay

A modified broth microdilution method outlined by Clinical and Laboratory Standards Institute (CLSI) [4] was performed. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used in this study. The mushroom extracts were dissolved in 10% dimethyl sulphoxide and diluted two-fold to give final concentrations ranged from 1,024-0.5 µg/ml. One hundred microliters of the bacterial suspensions, containing approximately 10^6 colony forming unit/ml of the microorganism, was inoculated in 80 µl of Mueller-Hinton broth supplemented with 20 µl of the compound. The microtiter plates were incubated at 37°C for 16-18 h. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were recorded.

Nitric oxide measurement

Nitric oxide (NO) production by RAW264.7 cells was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent as previously described [5]. The % inhibition was calculated based on the following equation and IC_{50} values were determined graphically ($n = 4$):

$$\text{Inhibition (\%)} = [(A-B)/(A-C)] \times 100$$

A-C: NO_2^- concentration (µM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)].

Assay of cell viability

Viability of RAW264.7 cells was assayed using a MTT colorimetric method [5].

3. RESULTS

The luminescent mushroom extracts were tested in broth microdilution assays to determine their MICs. The activities of the extracts against human pathogens are indicated in Table 1. The results demonstrated that the collected mycelia extracted with hexane (M1), $CHCl_3$ (M2), EtOAc (M3) and its culture filtrate extracted with EtOAc (F3) elucidated pronounced antibacterial activity against *S. aureus* ATCC 25923. The MIC and MBC values of the extracts against the microorganisms ranged from 2-16 µg/ml. The results suggested that the main bioactive compounds may obtain from the mycelia and EtOAc fraction of the culture filtrate. Purification and antibacterial mechanisms of the extracts are still under investigation. In contrast, the culture filtrate extracted with hexane (F1),

$CHCl_3$ (F2), and H_2O (F4) showed no antibacterial activity against the tested Gram-positive bacterium. All of the mushroom extracts were inactive against *E. coli* ATCC 25922, a Gram-negative bacterium.

Lipopolysaccharide (LPS) induced RAW264.7 macrophage cells are widely used as screening platform for anti-NO production by induction the transcription and protein synthesis of iNOS, and increased NO production [6]. The result demonstrated that the inhibitory effects of the mushroom extracts on NO production in a concentration-dependent manner were observed. $CHCl_3$ fraction of *N. nambi* mycelia exhibited the most potent inhibitory effect with an IC_{50} of 10.9 µg/ml followed by EtOAc and hexane fractions with IC_{50} values of 19.7 and 25.6 µg/ml, respectively. In addition, the hexane, $CHCl_3$, EtOAc, and H_2O extracts of the culture filtrates elucidated anti-inflammatory activity against NO-released macrophage with IC_{50} values of >100, 64.6, 27.4, and >100 µg/ml, respectively. However, the cytotoxic effect of the culture filtrate and the mycelium extracts on RAW264.7 cells was observed at a dosage of 10-100 µg/ml (Table 1). This aspect has to be considered in the application of the extracts for therapeutic purposes. Both of the culture filtrate and the collected mycelium extracted with $CHCl_3$ exhibited comparable effect with positive controls, L-NA (NO synthase inhibitor, IC_{50} = 10.2 µg/ml), indomethacin (IC_{50} = 16.6 µg/ml), the standard used non-steroidal anti-inflammatory drugs. Therefore, it also suggested that the major active compounds of *N. nambi* may obtain from $CHCl_3$ fraction. $CHCl_3$ fractions might show potent inhibitory effect on NO production in LPS stimulated RAW264.7 cells through the suppression of iNOS and COX-2 genes.

Table 1. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *Neonothopanus nambi* extracts against pathogenic bacteria and its anti-inflammatory activity on inhibition of nitric oxide production in RAW264.7 cells

Sample	Solvent extraction	Yield (mg/l)	MIC/MBC (µg/ml)		Inhibition of NO production IC_{50}[1] (µg/ml)
			Staphylococcus aureus ATCC 25923	*Escherichia coli* ATCC 25922	
F1	Hexane	295	1,024/>1,024	1,024/>1,024	>100
F2	$CHCl_3$	25	512/>1,024	1,024/>1,024	64.6
F3	EtOAc	385	4/4	256/512	27.4
F4	H_2O	6,035	>1,024/NA[2]	>1,024/NA	>100
M1	Hexane	56	16/16	>1,024/NA	25.6
M2	$CHCl_3$	196	2/2	512/512	10.9
M3	EtOAc	114	4/4	512/>1,024	19.7
M4	H_2O	366	1,024/>1,024	>1,024/NA	-
Vancomycin	-	-	0.5/1	-	-
Indomethacin	-	-	-	-	16.6
L-NA	-	-	-	-	10.2

[1]Each value represents mean ± S.E.M. of four determinations.

[2]Not applicable.

4. CONCLUSIONS

The mycelia of *N. nambi* extracted with hexane, $CHCl_3$, EtOAc, and culture filtrate extracted with EtOAc elucidated pronounced antibacterial activity against *S. aureus* ATCC 25923 with MIC and MBC values ranged from 2-16 µg/ml. Moreover, the fractions exhibited anti-inflammatory activity against NO-released macrophage with IC_{50} values ranged from 10.9-64.6 µg/ml. Purification, antibacterial mechanism, and *in vivo* anti-inflammatory activity of the extracts are still under investigations.

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