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Chemical Components of *Hirsutella thompsonii* Crude Filtrate and their Biological Activities

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ABSTRACT

Hirsutella thompsonii # 966 was cultured and extracted with ethyl acetate. The ethyl acetate layer was then eluted on Sephadex LH-20 column with methanol and the eluted chemical components were analyzed on a Bruker DRX 400 NMR spectrophotometer. The analysis showed several components such as glycerol, ergosterol and diketopiperazine. This was the first time that diketopiperazine had been isolated from *H. thompsonii* and it was known that diketopiperazine compounds had certain biological activities. Hirsutellide A was not present in the crude extract. The freeze-dried broth and the residue from evaporation of the broth (crude solvent extract) were dissolved in dimethyl sulfoxide (DMSO) in order to test for their cytotoxicity against insect and mammalian cell lines. A bioassay revealed that the crude broth sample was weakly toxic and the crude solvent extract was non-toxic to all cell lines tested in the range of 0.78-100 µg/ml. The crude solvent extract had tendency to stimulate cell activity, especially in mammalian cell line (BHK(21)C13.

Key word: *Hirsutella*, chemical components, cytotoxicity, bioassay

INTRODUCTION

Hirsutella thompsonii (Fisher) is an important naturally occurring fungal pathogen of eriophyoid mites, especially citrus rust mite (*Phyllocoptruta oleivora* Ashmead) which inhabits numerous host plants in subtropical and tropical regions (Jeppson *et al.*, 1975; Keifer *et al*., 1982; McCoy *et al.*, 1988; Boczek *et al.*, 1989). *H. thompsonii* is a parasitic fungus belonging to the Phylum Deuteromycota (Fungi Imperfecti), Order Moniliales (McCoy and Kanavel, 1969) and was first found in citrus orchards in Florida, USA (Speare and Yothers, 1924). McCoy and Kanavel (1969) isolated this fungus on an artificial medium and confirmed its pathogenicity to mites. In Thailand, *H. thompsonii* var. *synnematosa* was first recorded on the citrus rust mite (*P. oleivora*) infesting citrus plants at Klong 7, Amphor Lum Luk Ka, Pathum Thani province (Chandrapatya, 1987). Preliminary investigation revealed that the fungus could be mass-produced successfully in both solid and liquid cultures.

H. thompsonii produces biological active metabolites. In 1992, Mazet identified two distinct protein toxins, hirsutellin A (HtA) and hirsutellin B (HtB) from *H. thompsonii* var. *thompsonii* (strain HTF-87) in a shake flask. Later, HtA was found to be highly toxic to *Galleria mellonella* (Linnaeus) larvae, *Drosophila melanogaster* (Meigen) adults, a cell line of *Bombyx mori* (Linnaeus) and *Spodoptera frugiperda* (Smith) larvae (Vey *et al.*,1993; Liu *et al*., 1996). Additionally, Omoto and McCoy (1998) reported that HtA increased

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mortality of *P. oleivora* and also decreased its fecundity.

Maimala *et al*. (2002) studied 125 isolates of *H. thompsonii* collected from Thailand compared with several isolates of *H. thompsonii* kept in the collection of the insect pathology laboratory, University of Florida, USA. The HtA gene in each isolate was detected using the HtA gene of *H. thompsonii* var. *thompsonii*, strain JAB04, GenBank Accession Number U86836 (Boucias *et al*., 1998). Subsequently, crude broth of each isolate was assayed against *G. mellonella* larvae. The results showed that 100 out of 162 isolates had the HtA gene and more than half of the broth filtrates exhibited more than 50% mortality when assayed against the larvae. Unfortunately, the presence of the HtA gene in some isolates was not associated with enhanced insecticidal activity. In addition, the results from both isolate groups (with or without an amplifiable HtA gene) induced a relatively similar mortality rate (65%) in the larvae. The conclusion was reached that there was no direct correlation between the presence of the PCR-amplified HtA product and the insecticidal activity against *G. mellonella* larvae. Hence, this fungus must secrete other chemicals that have not yet been characterized.

The objective of this study was to analyze chemical components of *Hirsutella* crude filtrate and to investigate their activities against insect and mammalian cell lines.

MATERIALS AND METHODS

Chemical extraction and isolation

H. thompsonii # 966, isolated from eriophyoid mite (*Aculops caricae* K.) collected from Chon Buri province Thailand, was cultured in three 2-liter Erlenmeyer flasks containing malt extract broth (MEB) pH 6.5. *Hirsutella* fungus was cultured without air flow and incubated at room temperature for 1 month. Fresh biomass was then separated from the broth by filtration though filter paper. Broth was extracted 3 times in a separating funnel using equal ratios of ethyl acetate (EtOAc) and crude broth. The top layer (ethyl acetate layer) was collected and evaporated yielding concentrated crude solvent extract. This crude solvent extract was divided into 2 parts: the first part was loaded in Sephadex LH-20 (eluted with MeOH) to separate chemical components, and then the Bruker DRX 400 NMR spectrometer operating at 400 MHz for proton and 100 MHz for carbon was used to analyse the chemicals present in the crude solvent extract (Vongvanich *et al*., 2002). The second part was used to determine the toxicity of the crude solvent extract against animal cell lines.

Fresh biomass was extracted by soaking in methanol (MeOH) for 2 days before filtering and subsequently soaking in dichloromethane (CH_2Cl_2) for a further 2 days. Both methanol and dichloromethane were evaporated separately in an evaporator to concentrate the organic compound soluble materials. Concentrated crude cell extracts collected from MeOH and CH_2Cl_2 were then extracted together 3 times with EtOAc. This combination was divided into two layers, the top layer was EtOAc (non-polar part) and the bottom layer was H_2O (polar part). Both layers were evaporated before loading in Sephadex LH-20 and the chemical components were analyzed in Bruker DRX 400 NMR spectrometer. The selected fractions were reloaded in the column and NMR was used to obtain the chemical compounds.

Bioassay of crude extract against cell lines

H. thompsonii #966 was cultured in 250 ml Erlenmeyer flasks containing 100 ml of MEB and incubated on a rotary shaker at 27°C, 180 rpm for 4 days. The crude broth, separated from the mycelia, was freeze-dried and used in the bioassay test.

Both crude broth (freeze-dried) and the crude solvent extract from the chemical extraction process were dissolved in dimethyl sulfoxide

(DMSO) to give a stock concentration of 100 mg/ml. These solutions were further diluted (1:2) in the respective growth medium of each cell line over the range of 0.78-100 µg/ml for crude solvent extract and 7.8-1000 µg/ml for crude broth sample.

The cytotoxicity of *Hirsutella* fungus was assessed against two insect cell lines, Sf9 (pupal ovarian tissue of *Spodoptera frugiperda* Smith = ECACC No. 89070101) and clone C6/36 (larval tissue of *Aedes albopictus* Skuse = ECACC No. 89051705) plus one mammalian cell line, clone BHK(21)C13 (Hamster Syrian kidney = ECACC No. 85011433). The Sf9 was grown in TC100 insect medium supplemented with 2mM L-glucosamine, 100U/ml Penicillin/Streptomycin and 10% fetal bovine serum. The C6/36 was grown in Minimum Essential Medium (MEM) supplemented with 2mM L-glutamine, 1% MEM-NEAA, 100 U/ml Penicillin/Streptomycin and 10% fetal bovine serum. These insect cells were incubated at 28°C. The mammalian cell line [(BHK(21)C13)] was grown in Glasgow MEM (GMEM) supplemented with 2mM L-glutamine, 100U/ml Penicillin/Streptomycin and 5% fetal bovine serum and incubated at 37°C in near 100% humidity, 5% CO_2: air atmosphere.

The Sf9, C6/36 and BHK(21)C13 were seeded separately in Corning 96-well tissue culture plates with 10,000, 20,000 and 1,000 cells/well respectively, incubated at 37°C for 48 h in near 100% humidity, 5% CO_2. The 1: 2 serial diluted crude solvent extract and crude broth were then added to the cells and incubated for another 24 h. The tested crude solvent extract, crude broth and inactive cell lines were then removed from the cell cultures using micropipette and the cells were re-incubated for another 24 h in fresh medium before being tested with the MTT assay. Two negative controls (medium + cell lines and medium + DMSO + cell lines) were used to compare the cytotoxicity of the fungal crude solvent extract and crude broth. The MTT assay was an indirect measurement of cell viability. This assay was a tetrazolium-dye based colorimetric microtitration assay where metabolism-competent cells were able to metabolize the tetrazolium (yellow) to formazan (blue). This color change was measured spectrophotometrically with a plate reader using Molecular device E max operated at wavelength 570 nm and the result was analyzed by SoftMax program. It was assumed that cells metabolically deficient would not survive (Skehan *et al*., 1990). Toxicity was expressed as the concentration of the sample required to kill 50% (LD_{50}) of the cells in comparison to the controls.

RESULTS AND DISCUSSION

Chemical extraction and isolation

The crude broth of *H. thompsonii* #966 had a yield of 260 mg concentrated crude solvent extract. Only fractions 1-6 were examined and the fourth fraction was subsequently rechromatographed through Sephadex LH-20 and examined with the Bruker DRX 400 NMR spectrometer. Fraction 4/6 yielded a pure peptide of 17 mg (Figures 1, 2).

Sequential extracts from mycelia with MeOH and CH_2Cl_2 produced a combined total crude cell extract of 260 mg. This combined cell extract was then partitioned with H_2O/EtOAc. The crude cell extracts from non-polar fraction (EtOAc) and polar fraction (H_2O) were 1.83 g and 185 mg respectively (Figure 1). The weight of crude extract increased from humidity during experiment. When the crude cell extract from the non-polar part was repeatedly purified with gel filtration on Sephadex LH-20, a glycerol derivative (9 mg) and ergosterol (17 mg) were obtained (Figure 3). The H_2O soluble (polar part) was also purified by Sephadex LH-20 column chromatography to yield diketopiperazines A and B, of 105 mg and 71 mg respectively (Figure 4).

Vongvanich *et al*. (2002) cultured *Hirsutella kobayashi* (BCC 1660) isolated from a cricket, in potato dextrose broth (PDB) (5 L) at

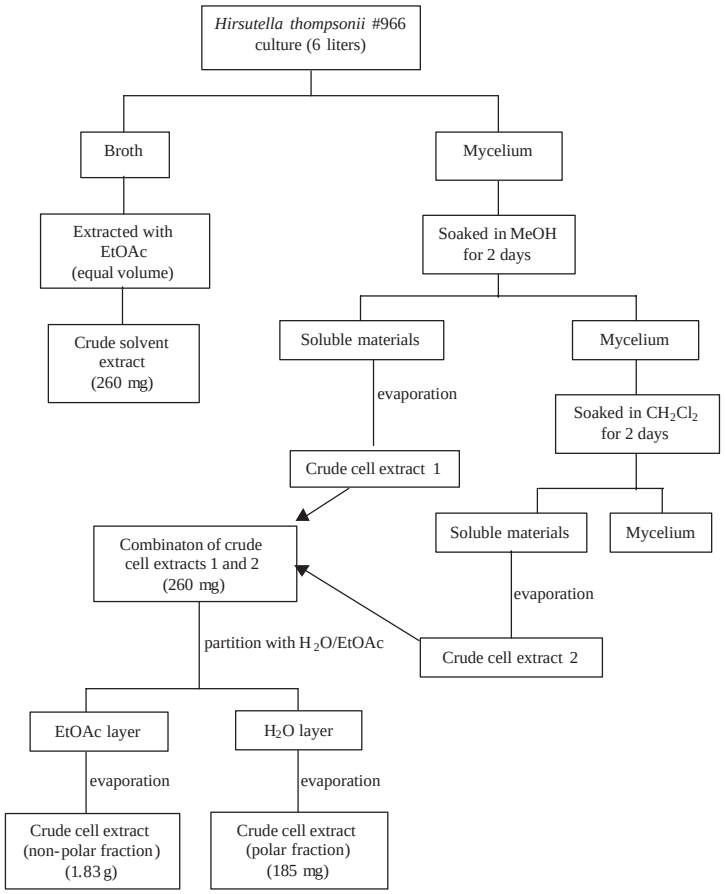

Figure 1 Chemical extraction and isolation.

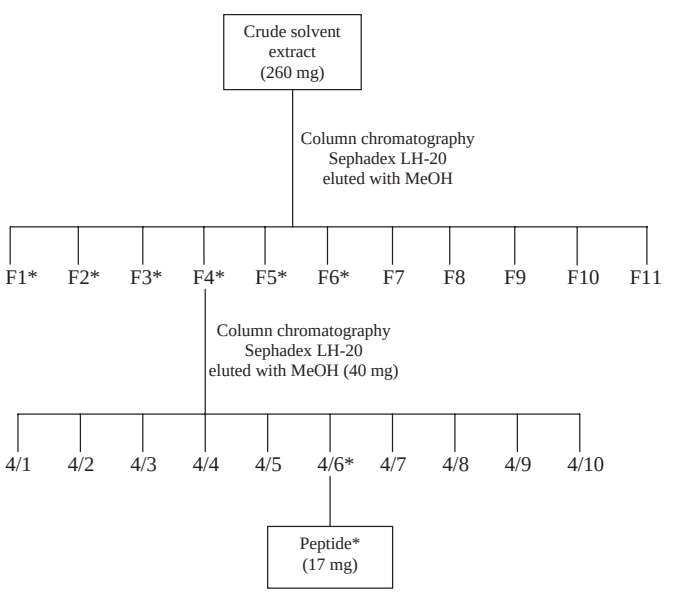

Figure 2 Isolation of the crude solvent extract from broth: F1, F2, F3, F4, F5, F6 and fraction 4/6 analyzed by NMR spectroscopy.

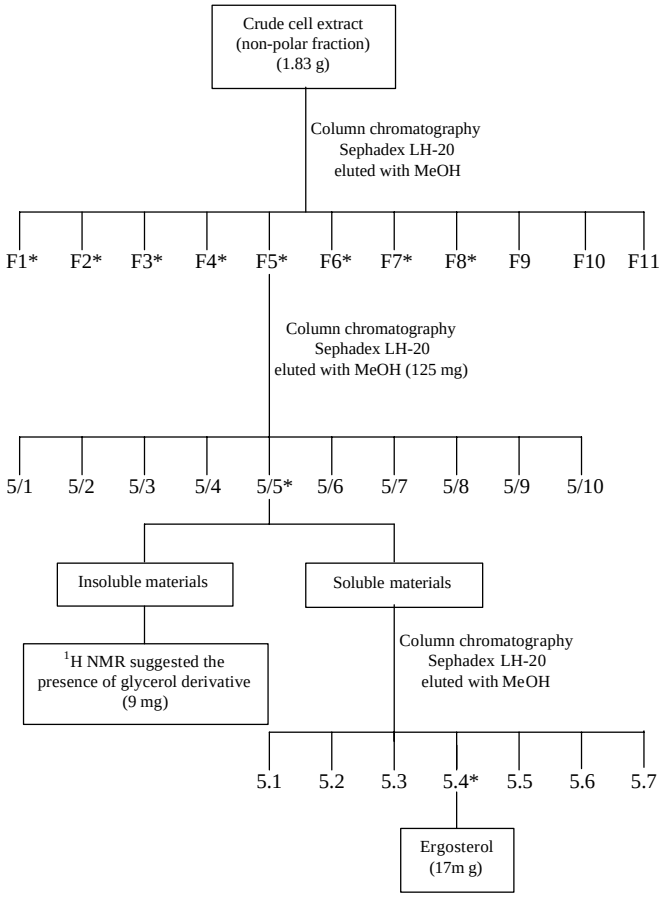

Figure 3 Isolation of the crude cell extract (non-polar fraction): F1, F2, F3, F4, F5, F6, F7, F8, fractions 5/5 and 5.4 analyzed by NMR spectroscopy.

25°C for 21 days. Crude broth was extracted with EtOAc and mycelia with MeOH and CH_2Cl_2. The crude extract was purified by Sephadex LH-20 (eluted with MeOH) and silica gel column chromatography to yield a new cyclohexadepsipeptide, named hirsutellide A. Unfortunately, hirsutellide A was not present in either crude solvent or crude cell extracts of *H. thompsonii* #966.

In this study, structure elucidation of the isolated compounds was not completed, yet, limited analysis did identify ergosterol, glycerol and diketopiperazines. The first two compounds are not known for having detrimental (toxic) activities but the third compound, diketopiperazines, are known for toxic activity. Diketopiperazines are secondary metabolites of fungi, and their structures contain two amino acids which condense to form a six membered-ring via amino and carboxylic moieties. Diketopiperazines have been reported to possess various biological activities including antimalarial activity (Nilanonta *et al*., 2003), antifungal activity (Byun *et al*., 2003) and antibacterial activity (Sugie *et al*., 2001).

Cytotoxicity test

The bioassay of crude broth derived from *H. thompsonii* #966 with insect (Sf9 and C6/36) and mammalian cell lines [BHK(21)C13] revealed that the crude broth was weakly toxic to all cell

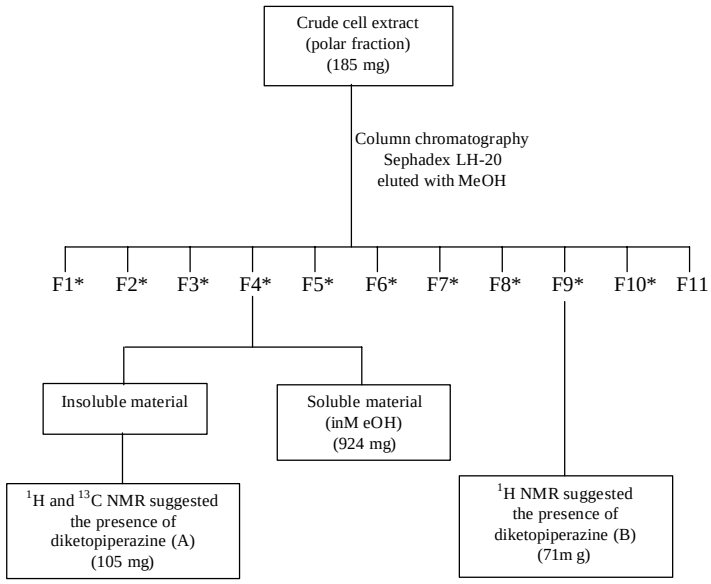

Figure 4 Isolation of the crude cell extract (polar fraction): F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10 analyzed by NMR spectroscopy.

lines in the tested range of 7.8-1,000 µg/ml (Table 1). Almost of all cell lines survived more than 50%, only cell lines of C6/36 and BHK(21)C13 treated with 1,000 µg/ml of crude broth survived 46.42% and 48.08%, respectively. The viable cells increased when the concentration of crude broth decreased. Some treatments gave the survival cells more than 100% (Sf9 treated with 31.25, 15.63 and 7.8 µg/ml; C6/36 treated with 15.63 and 7.8 µg/ml). This result indicated that after being treated with fungal crude broth, the viable cells could develop and duplicate themselves and also revealed that the fungal crude broth had no cytotoxicity against the tested cell lines (Table 1).

Fungal crude solvent extract derived from *H. thompsonii* #966 (after evaporation) was also non-toxic to all tested cell lines in the range of 0.78-100 µg/ml (Table 2). Cell lines in all treatments survived more than 50%. Cell lines of C6/36 and BHK(21)C13 showed viable cells more than 100% in every treatments, only cell lines of Sf9 gave viable cells more than 100% after treated with 0.78 µg/ml. This result indicated that the chemical components in crude solvent extract were non-toxic to all cell lines at low dosage (0.78-100 µg/ml). In contrast, these chemical components stimulated cell activity. Interestingly, the numbers of viable BHK(21)C13 cells were considerably higher than those of the controls, and more than Sf9 and C6/36 (Table 2). The results indicated that the crude solvent extract was non-toxic and probably stimulated cell activity and is non-toxic to mammalian cells.

The effectiveness of secondary metabolites of *Hirsutella* depends on the fungal variety and culturing conditions. For example hirsutellin A (HtA) extracted from *H. thompsonii* var. *thompsonii* strain HTF-87 and CBS 556.77D cultured in Czapek-Dox broth plus yeast extract for 4 days and the JAB 04 strain grew in PDY broth for 4 days are toxic to *G. mellonella* larvae, *Aedes aegypti* (Linnaeus), *Drosophila melanogaster* (Meigen and *Bombyx mori* (Linnaeus) (Vey *et al.*, 1993; Liu *et al*., 1995; Mazet and Vey, 1995).

In contrast, hirsutellide A extracted from *H. kobayashi* cultured in MEB for 21 days exhibited antimycobacterial and antimalarial activities with a MIC (minimum inhibitory concentration) of 6-

Table 1 Optical densities (OD) and survival percentages of different cell line after treatment with crude broth (freeze-dried) of *H. thompsonii* #966 for 24 h.

Standard	SF9		C6/36		BHK(21)C13	
(µl)	Mean ± SD	% survival	Mean ± SD	% survival	Mean ± SD	% survival
1000	0.65 ± 0.04	74.71 f	0.52 ± 0.03	46.42 h	0.25 ± 0.03	48.08 g
500	0.75 ± 0.05	86.21 e	0.63 ± 0.03	56.25 g	0.31 ± 0.02	59.62 f
250	0.83 ± 0.02	95.40 cd	0.69 ± 0.02	61.61 f	0.36 ± 0.02	69.23 e
125	0.81 ± 0.03	93.10 d	0.76 ± 0.05	67.86 e	0.40 ± 0.02	76.92 d
62.50	0.85 ± 0.02	97.70 bc	0.83 ± 0.02	74.11 d	0.42 ± 0.02	80.77 d
31.25	0.89 ± 0.05	102.30 b	0.97 ± 0.12	86.61 c	0.46 ± 0.02	88.46 c
15.63	0.88 ± 0.03	101.15 b	1.15 ± 0.03	102.68 ab	0.49 ± 0.02	94.23 bc
7.80	0.93 ± 0.02	106.90 a	1.19 ± 0.05	106.25 a	0.50 ± 0.03	96.15 ab
Control	0.87 ± 0.04	100.00 b	1.12 ± 0.04	100.00 b	0.52 ± 0.03	100.00 a

Table 2 Optical densities (OD) and survival percentages of different cell line after treatment with crude solvent extract (after evaporation) of *H. thompsonii* #966 for 24 h.

Standard	SF9		C6/36		BHK(21)C13	
(µl)	Mean ± SD	% survival	Mean ± SD	% survival	Mean ± SD	% survival
100	0.38 ± 0.02	79.17 d	0.52 ± 0.03	100.00 b	0.39 ± 0.02	121.88 e
50	0.40 ± 0.02	83.33 d	0.54 ± 0.03	103.85 b	0.44 ± 0.02	137.50 bc
25	0.44 ± 0.02	91.67 c	0.58 ± 0.04	111.54 a	0.47 ± 0.01	146.88 a
12.50	0.44 ± 0.02	91.67 c	0.61 ± 0.02	117.31 a	0.45 ± 0.02	140.63 ab
6.25	0.45 ± 0.04	93.75 c	0.54 ± 0.03	103.85 b	0.44 ± 0.02	137.50 bc
3.125	0.46 ± 0.02	95.83 bc	0.52 ± 0.04	100.00 b	0.42 ± 0.02	131.25 d
1.563	0.47 ± 0.04	97.92 bc	0.53 ± 0.03	101.92 b	0.43 ± 0.03	134.38 cd
0.780	0.53 ± 0.03	110.00 a	0.60 ± 0.03	115.38 a	0.45 ± 0.03	140.63 ab
Control	0.48 ± 0.03	100.00 b	0.52 ± 0.02	100.00 b	0.32 ± 0.02	100.00 f

12 µg/ml., but showed no cytotoxic effect toward the Vero cell line at 50 mg/ml (Vongvanich *et al*., 2002). Liu *et al*. (1995) extracted hirsutellin A from *H. thompsonii* var. *thompsonii* (strain JAB 04) and observed that the crude filtrates from 21-25 h old cultures contained detectable toxin, causing 60-100% mortality of *G. mellonella*. Maimala (2004) cultured *H. thompsonii* #966 in MEB for 4 days and reported that the crude filtrate containing HtA gene showed strong insecticidal activity by inducing 100% mortality of *G. mellonella* larvae. From this study, crude broth (4 d old) was weakly toxic to all cell lines while crude solvent extract (21 d old) was non-toxic to the tested cells. This phenomena was partly due to the prolonged fermentation period that probably caused degradation of some insecticidal metabolites. Hence, only secondary metabolites with antibiotic activity were most likely to be found.

CONCLUSION

The analysis of crude solvent and crude cell extracts was not completed showing only a group of ordinary components. The analysis detected glycerol a storage molecule, ergosterol a component of plasma membranes and diketopiperazines secondary metabolites of fungi. Unfortunately, the quantity of crude solvent extract used in this experiment was not great enough to induce cytotoxicity. Toxicity assays showed that the crude broth (freeze dried) and crude solvent extract were only weakly or non-toxic to all tested cell lines (Sf9, C6/36 and BHK(21)C13). In contrast, these extracts stimulated cell activity. Moreover, crude cell extract contained diketopiperazines, which might be useful for antibiotic treatments.

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