International Journal of Agricultural Technology XXXX Vol. X(X):XXXX-XXXX
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Cultivation of *Phellinus* spp. for Bioactive Compound Production

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Puckpernchan, P., Unartngam A., and Unartngam, J. (2019). Cultivation of *Phellinus* sp. for Bioactive Compound Production. International Journal of Agricultural Technology X(X): XX-XX

Absract In Thailand, mushroom is reported in the traditional Thai medicine. Using of the name "Kratinphiman" covers the genus *Phellinus* sp. There are many species and different names, It is not clear to define which kind of them can be used to be a medicine because there is not research report yet about the culture and defining mechanism of biological synthesis pathway. Therefore, there is studying of the species and how to culture them for reducing the amount of natural storage that is decreasing. The methods are sampling a specimen and isolating various areas in the country then matches with morphological characteristics and the data on therapeutic in Thai traditional medicine list. In addition, mushroom was isolated in the culture with liquid medium, grain medium and woody sawdust at the different ingredient condition. Antibacterial activity was almost found in all recipes and all liquid culture conditions, but we are still in compound separation by chromatography technique. From the separation of antibiotics by HTLC and bioautrograpihc method, it was found that the proportion of chloroform: methanol: acetic acid: H2O has a polarity value of 0.436. All Phellinus species have low polarity antibiotics at a R*f* rate of 0.79. *P. noxius* and PKD644 found antibiotics two groups are clearly different. Most of the antibiotics are found 7-36 percent from isolated biological compounds. PKD644 contains the most antibiotics per unit, 2D TLC isolation results are also found to be single and co-activities. There found antitumor activities from Hep-G2, HT-29 and Chago-k1 at %survival 7.5, 23 and 13 PKD644 has an activities with Hep-G2 and Chago-k1 at %survival 38 and 50 respectively, With an average IC_{50} of 3.48 and 20 mg/ml. Also cultivating in the grain with potato powder and peptone in each treatment can help them grow faster and the net weight is the highest. Some of them have a layer of fruiting bodies when cultured them more than 4 months in grain and saw dust. In each proportion give a rapid growth and more fruiting bodies formation.

Keywords: *Phellinus* culture, *Phellinus linteus*, Bioactivecompoud activity, Anti cancer, Anti microbial, Bioautography

Introduction

Mushroom is important source of bioactive natural products with enormous potential for finding new molecules, drug discovery, industrial used and agricultural application. Varius new compounds which are isolated from mushroom that were reported. Many of these metabolites exhibit numerous biological activities and can be employed as supplement additives such as vitamins, sweetener and anti-microbial, autoxidation, antitumor also anticancer in pharmaceutical. (Blagodatski *et al*., 2018).

Phellinus (or Inonotus) is a genus of fungi which is found and growing on a living tree and wood carcass. The flesh is tough and woody or cork-like, brown in color and the skeletal hyphae are yellowish-brown. (Rizzo *et al*., 2003). Vast numbers of polypore fruit bodies invade old beech trunks in mountain, virgin forests and also in the other deciduous tree, live and dead trunks. They were found mostly in dipterocarp forest, mixed deciduous forest and the forest where located higher than 400 meters above sea level. Found that in some types of plant disease and almost all are endophytic fungi. (Huang *et al*., 2001; Panphut 1997).

In Thailand, mushroom is reported in the traditional Thai medicine. Using of the name "Kratinphiman" covers the genus *Phellinus* sp. There are many kinds of specie and also have different name such as "Keng", "Jik" and "Rung". (Masaladol and Makariya,, 1995). It is found that some of the same names have different species. *Phellinus* sp. is a medicinal mushroom used as different name in Japan, Korea, Russia and Indo-China for centuries (Mikhail *et al*., 2015; Glamoclija *et al*., 2014). , especially in Thailand,

Figure 1. The mushrooms specimen number PKD014, That grow in nature on the deciduous trees at the Sakaerat Environmental forest. Approximately 12 years old which is 2.8 meters high from the ground. The tree is about 35 cm in diameter

Phellinus sp. has been used as a traditional medicinal mushroom in North-east Asia before Historic Khmer era for the treatment of various diseases, including gastrointestinal cance, lymphatic diseases and heart disease. It was previously reported and prevent ailments as diverse as, haemorrhage. The extracts from them have an effect of stimulating immunity, and inhibiting tumor metastasis (Han *et al*., 1999).

Like other medicinal mushrooms, the bioactive compound in *Phellinus* sp. are manifoldly health-beneficiary. Other than being immunoregulatory and anticancerous, *Phellinus* sp. is an excellent herbal source of antioxidants. The mechanistic studies at molecular level provided evidence that *Phellinus* sp. differentially affected normal versus malignant cells, which might render different sensitivity to cyto toxic. By identifying and specific intracellular of PL compound, this herbal medicine can be developed for clinical usage (Chen *et al*., 2016; Kim *et al*., 2013; Sliva, 2010).

Materials and methods

Phellinus species and isolates

The fruiting body of PL (about 7 meters above from living tree, more 10 years old) was sampled and isolated with potato dextrose agar (PDA) media at founded site. Isolation in PDA contened rose Bangor media for a week at 37^{o}C chamber. In this experiment has got 4 isolated from wild (Table 2), *Phellinus linteus* from commercial culture and *Phellinus noxius* from The National Center for Genetic Engineering and Biotechnology (BIOTEC) Thailand.

Figure 2. Morphological characteristics and polyporus surface of *Phellinus* observed by stereo microscope. A, closed pores of specimen KRSK002; B, specimen KRSK002 from Lopburi dristric. C, opened pores of specimen SYI002. D, specimen SYI002 from Kanchanaburi. Bars = 1.5 mm

Fungal preparation

Phellinus linteus (PL), *P.noxius* (PN) and all *Phellinus* were cultured in liquid medium contented potato dextrose broth (PDB) and PDBx 1l, element ; MgSO4;0.2 g/l, Nitrogen source; Peptone;1 g/l, Yeast extract 0.5 g/l and monosodium glutamate 0.05 g/l (For comparing recipes with and without nitrogen sources) , (Siriwattanametanon *et al*., 2014). incubated with 120 rpm shaking at room temperature for 10 days. Rice grain medium (contented PDBx 100 ml : Rice grain 150 g) in bottle unit contains 150 g of rice soaking in a medium solution 100 ml for two hours and autoclave. (Jeon *et al*., 2012) And saw dust medium with a mixture of Corn cobs powder and bran. Seedling culture was prepared by agar medium from to the collections, and inoculate in each bottle unit, and then incubate at room temperature for 30 days. The raw material was harvested by hot air at 45 ^{o}C overnight before powdered. Based on the above results, and all PL were cultured in PDBx for completed synthetic compound.

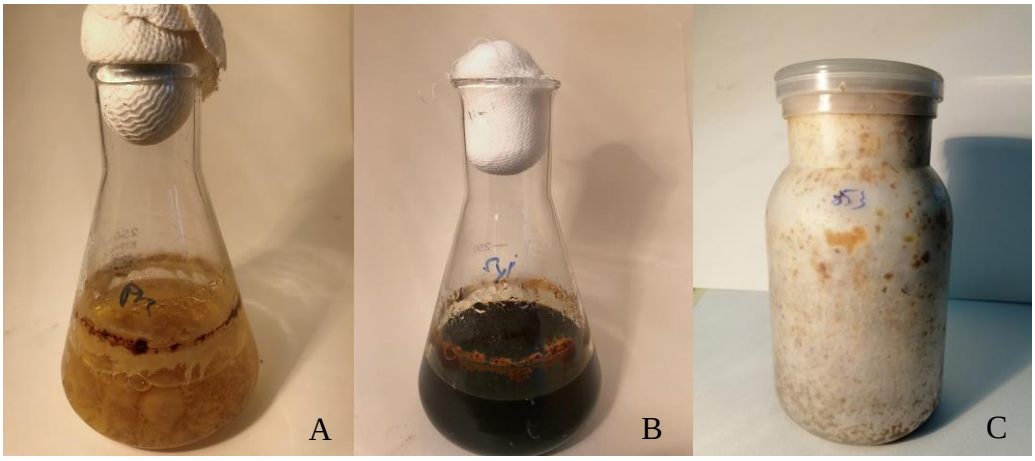


Figure 3. The culture of *Phellinus* grown on different medium. A, The culture broth of PL grown on PDB for 7 days. B, The culture broth of SYI002 grown on PDBx for 14 days. C, and PKD853 grown on saw dust medum for 30 days

Bioactive compound extractions

Crude material of PL in grain medium was powdered and extracted by 3 different solvents under sonication. Sort the ordered from the less to heavy solvent strength.

The primary extract was mixed with ethyl acetate by stirring the powder 20 g with 40 ml. Then filter with a separate funnel, Bringing sediment to extract with ethanol.

The ethanolic extract was obtained from above at same ratio with 95% ethanol and subsequently filtered through cotton paper. The water extract was used the same method. (Sonawane *et al*., 2012)

However, liquid medium mixed with solvent directly by ratio 1:1 v/v. Finally, the residue was extracted with 200 ml, and each extract was evaporated at 60°C (rotary evaporator Büchi R-210) to dryness. (Jeon *et al*., 2012) The extract was dissolved by chloroform and evaporated with nitrogen gas.

Separation

One-dimensional TLC

Thin layer chromatography (TLC) was performed on 10×10 cm glass Diol F254 and CN F254 high-performance TLC precoated plates (Merck, Darmstadt, Germany), and divide the substance along the length of 5 mm each, sample length 4 mm and distance from the edge 1 cm, by placing 50 µl of specimens in different, shining with UV light at a wavelength of 254 nm. Samples of 5 µL from the different proportions were loaded onto TLC plates and developed in 4 mobile systems S2-S5 (Table 1) The solvent system has been developed from research (Sharon et al., 2013; Shetty et al., 2014).

Two-dimensional TLC

2D-TLC was also performed on 10×10 cm. Chambers were conditioned with S1 for 15 minutes in eluent vapors. Samples 2 µL of 100 µg/ml solutions of the solutes in chloroform were spotted to the adsorbent layer. Secondary mixtures of modifier were applied S4 (Table 1). The second run used different mobile phase as S4 and S1 respectively (Basyuni *et al*., 2017).

Simultaneously, groups of antibiotic derivatives may have different polarity, and they are relatively easy for a group separation, certainly in the extraction step. However, in a particular group, they have similar chemical structures and antibiotic compounds properties. Then ensure, they have a single compound activity, the separation method will be used, but it was difficult to

separate in one run. Very often, the separation should be performed in reversed-phase (RP) systems when molecules differ in the polar group present or position; they frequently require a normal-phase system for separation. A multidimensional separation of closely related bioactive compounds should be performed.

Column chromatography

The concentrated ethyl acetated extract of 3 grams was fractionated by column chromatography on silica gel (60-120 mesh) sigma. The solvent was filled into the column, according to each proportion, the order of low to heavy polarity in the column until the end. (Raghava-Rao *et al*., 2017). Different solvent mixtures were used in elution systems (See table 1; S1, S4 and S6 respectively). The fractions were collected by 250 drops of fraction collector and subjected for further analysis. Each fraction was evaporated with a vacuum centrifuge at 55 °C for 30 minutes.

All dimensions TLC will be analyzed by HPTLC, As following after development, the plates were visualised under UV light at a 254 nm. And measured on the basis of the band intensity by using the Just TLC software (Sweday, Lund, Sweden). The separation experiment was selected the best for bioautography.

Chemicals and reagents

All chemicals used were of analytical grade. Bulk solvents and routine chemicals were obtained from molecular laboratories (Kasetsart Kampangsan District, Thailand) and Merck & Co. Inc (Whitehouse Station, NJ, USA). Ready-to use silica gel coated column and hispidin compound standard were obtained from Sigma-Aldrich. Commercial medium for PL culture were obtained from food grade trading.

According to cancer cell line is belonging to The American Type Culture Collection (ATCC: The Global bioresource center). While bacterial cell were obtaineded from Thailand Institute of Scientific and Technological Research (TISTR) and ATCC.

Table 1. The compound separated solvent system

Solvent	Conditions ratio				Strength	NO.
Toluene : acetone : hexane	70	20	10		0.316	S1
chloroform : acetone : hexane : methanol	20	20	40	20	0.405	S2
chloroform : methanol : acetic acid	81	11	8		0.424	S3
chloroform : methanol : acetic acid : H2O	79	11	8	2	0.436	S4
chloroform : methanol : acetic acid : H2O	80	11	8	1	0.387	S5
methanol	100				0.950	S6
Toluene : chloroform	60	40			0.398	S7

Qualitative phytochemical analysis

Phytochemical screening is available for using in the determination of extracts for the presence of certain chemical groups. The results of this test must be taken in conjunction with those of other analytical methods before conclusion can be drawn, There are simple but standard chemical tests to detect the presence of alkaloids, phenolic, tannins, saponins, carotenoids, anthraquinones, etc. in an extract.

Test for tannins: 0.2 g of the dried samples was boiled in 5 ml of distilled water and then filtered. Three drops of 0.1% $FeCl_3$ was added to the filtrate and observed for brownish green or blue-black coloration.

Test for flavonoids (Shinoda test): 0.2 g of the dried samples was extracted with 3 ml of 50% methanol and then filtered. Few fragments of magnesium metal were added in a filtrate and boiled. After the solution was cool, drop wise addition of concentrated HCl and observed for a magenta coloration.

Test for alkaloids (Dragendroff's test): 0.2 g of dried samples was warned 2-3 min in 15 ml of 2% H_2SO_4 and then filtered. Added a few drops of Dragendorff's reagent and observed for reddish brown precipitation.

Test for saponins: 0.2 g of the dried samples was boiled in 5 ml of distilled water and then filtered. A few drops of olive oil were added to the filtrate and shaken vigorously, then observed for formation of froth.

Test for terpenoids (Salkowski test): 0.2 g of the dried samples was extracted with 5 ml of chloroform for 3 min 3 times and then filtered. The extract was mixed with 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish-brown coloration of the inter face was formed to show positive results.

Test for cardiac glycoside: 0.2 g of the dried samples was extracted with 5 ml of petroleum ether for 3 times and dissolved in 80% methanol. Add 3 drops of glacial acetic acid and 3 drops of concentrated H_2SO_4 and observed blue or blue green coloration.

Test for phlobatannins: Add 10 ml of methanol and 1 ml of concentrated H_2SO_4 to 0.2 g of the dried samples. Deposition of red precipitate show positive results. (Reis *et al*., 2014)

Hispidin detection

The methanolic extract was obtained by *P. linteus* and PKD644 raw materials in each period (as 2, 7, 14 and 20 days for a liquid culture, and 10, 20 and 30 days for a grain medium contained nitrogen source) were soaked overnight. Then placed it in a sonicator bath for 30 minutes, filtered out the sediment and evaporated with a vacuum evaporator. After that the chromatograms were analyzed by HPTLC (used mobile phase S7, Table 1), scanned with 254 nm wavelengths and compared to standard substances.

Evaluation of the antioxidant activity of interested extracts of P. linteus

Antioxidative activities with DPPH assay (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method; 3 ml of a 3.6 × 10-5M ethanolic solution of DPPH were used. The decrease in the absorbance, at 515 nm, was continuously recorded with a Shimazu spectrometer for 16 minutes at room temperature. The scavenging effect was plotted against the time, and the percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 minutes duration (Kedare *et al*., 2011).

Evaluation of the bioactivity and extracts of P. linteus

Antibacterial activity

Bioassay

Dilute the concentrated was extracted by DMSO at a ratio of 1 µl: 100 µl. The Nutrient agar (NA) medium culture used a 7 cm plate. Firstly, inoculums, *Bacillus subtilist* (ATCC 6051) and streptococcus aureus (NCTC 10988) were prepared in the LB culture medium. Then they were used around volume 100 µl of them to spread plate, brought a 4 mm cellulose fiber disc onto 6 positions and 1 central position in the plate to be a solvent test control set. Finally, the extract diluted with DMSO on the cellulose fiber disc of 5 µl was dropped to the position, and incubated at 37 C for 3 days. (Sonawane *et al*., 2014)

Bioautography

A TLC bioautographic method was used to detect active components. Firstly, 25 ml of Water Agar (WA) was prepared on a 7 cm petri dish. The chromatogram was placed on WA and sterilized with ultraviolet-light for 20 minutes. Then 200 ml of NA medium at 45 °C was prepared in water bath and add 1 ml of certificated B. subtilist. (Silva *et al*., 2005) After that, it was shook at 110 rpm for 1 minute, and poured. Inoculated agar was distributed over the chromatogram inside with 50 ml of an appropriate culture medium, and incubated at 37 °C for 48 hrs.

Secondly, the dye was prepared by diluting the saffron mixture with 80% ethyl alcohol in the ratio of 7: 3 v/v, then stained it on an agar culture medium for 5 to 10 minutes. After that it was de-strained with 50% ethyl alcohol. At this stage, the inhibitions zone would appear. Finally, agar-gel was evaporated with Cellophane for storage. (Shahverdi *et al*., 2005)

Antitumor activity

Cell culture; Cancer cells line were HT-29 (Colon Adenocarcinoma), Hep-G2 (Liver, Hepatocellular Carcinoma) and Chago-K1 (Lung Bronchogenic Carcinoma) was used. All the cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat inactivated fetal bovine serum and 100 µg/ml streptomycin. Cultures were maintained in humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air until they reached confluence.

Anti-cancer assay; Cultivate individual cell in six-well plates with a starting cell volume of 2×10^5 cells/ml. The above extracts of Phellinus were dissolved in DMSO and diluted with medium. Then we put in a culture plate for test the growth inhibition screening, and cultivated in 96-well culture plates. After that, we counted survival cells along culture with the MTT test (3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide). After 3 hours Incubation, the culture medium was replaced with isopropanol at 1:1 v/v for the extraction of dye. All crystals were dissolved by repeated pipetting of the medium, and measured by ELISA reader (570 nm). Anti-cancer activity was calculated by the following survival ratio (%) =100. Finally, we selected some interesting activities for IC_{50} by diluted with different concentrations ranging from 0-200 µg/ml. (Jeon *et al*., 2012; Konno *et al*., 2015)

Results

Table 2. The fungal spaciment of *Phellinus* sp. used in the present study.

Code	Area	Locations	Host
PKD644	phu kradueng district, loei Thailand	16.8721, 101.8470	Shorea roxburghii
PKD853	phu kradueng district, loei Thailand	16.8724, 101.8231	Shorea roxburghii
SYI002	Saiyok distric, Kanchanaburi Thailand	14.4353, 98.8544	Wood carcass
PKD025	Sakaerat Environmental Research Station, Nakornrachasima Thialand	14.3059, 101.5555	Shorea siamensis

Culture method and isolation result

According to table 2, it has 4 isolated that almost found in 2 types of PL, symbiosis with living tree (*Lagerstroemia* sp., *Shored* sp. some found in *Lagerstroemia* sp.) and degrading mushroom with carcass, and also found in many areas of Thailand such as Fagaceae (Inthanon Chiangmai), Lecythidaceae (Phu Kradueng Loei), etc. For a mushroom in living tree, mostly it was found in a more 10-year-old tree in the ancient forest. Normally, the mushroom (Phellinus) grows in a living tree that is an entophytic fungus. It does not harm and become a plant's disease. Moreover, it is not found under 1 metre compare with the height of tree because the deciduous forest was fired; as a result, the mushrooms are damaged, and will grow at the cork cambium at the higher level. However, the other group (Degrading mushroom) cannot identify what a specific host is or it is similar to a symbiosis (it is different from the type that grow on a living tree). After decomposing the carcasses, the fruiting body continues to grow into the root as well.

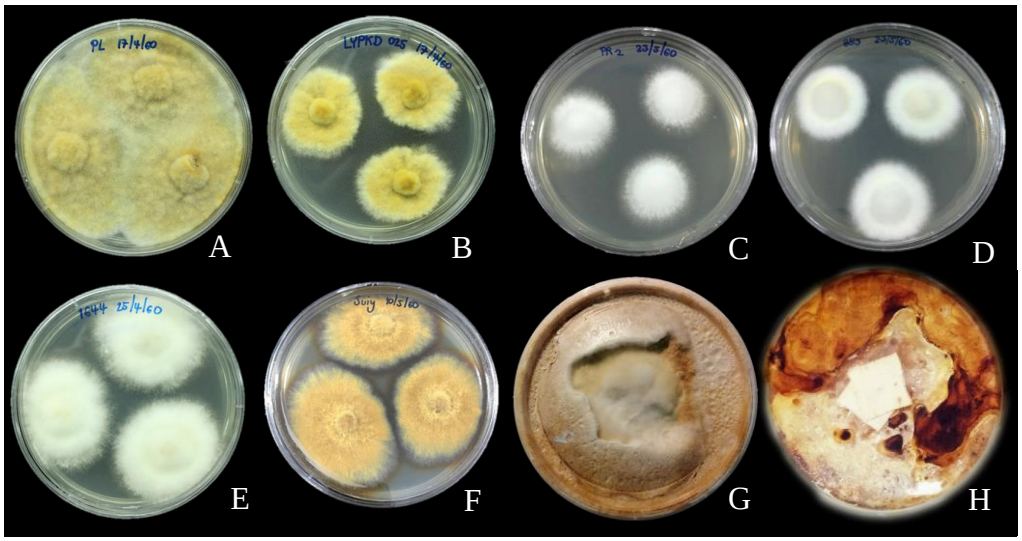


Figure 4. Colonies of six different species of *Phellinus* grown on PDA for 10 days. *Phellinus linteus* (A), PKD025 (B), *Phellinus n*oxius (C), PKD853 (D), PKD644 (E), and SYI002 (F) G, A colony of PL grown on grain medium for 20 days. H, And a colony of PKD025 grown on grain medium for 30 days.

After PL and PN were cultured with 2 liquid medium recipe (PDB and PDBx). The result by focusing on the growth was not different, but when comparing the dry weight at 20 days of culture, PDBx gave more important qualities. And when the recipe was changed to a cereal, the grain medium contented of PDBx gave a fast growth of fiber at a rate 5.5 mm per day that is faster than culture in a recipe without nitrogen source about 1.4 times. As a result, the proper medium for culturing all Phellinus and extract in 3 polar solvents gave the different solutions after evaporation such as net weight, color and smell. In this regard, SYI002 gave the most total weight of the extract when culturing in both medium (liquid and grain).

Test of separation by using bioassay and bioautographic method

Table 3 Antimicrobial activity result

			antimicrobial activity						
medium	**Isolated**	**Conc.**	**Ethylacetate**			**Ethanol**		**Water**	
type		**µg/ml**		*B. subtilist*	*S. aureus*	*B. subtilist*	*S. aureus*	*B. subtilist*	*S. aureus*	% antibiotic
			Bioassay	**Bioautography**						
PDBx	*P. linteus*	*50*	++	1	-	+	-	-	-	31.78
	P.noxius	*50*	+++	2	+	-	-	-	-	18.25
	PKD644	100	++	2	-	-	-	-	-	36.26
	PKD853	100	+++	1	+	+	-	-	-	15.31
	SYI002	100	++	1	-	-	-	-	-	8.76
	PKD025	100	++	1	-	-	-	-	-	7.63
Grain	*P. linteus*	*50*	++++	1	++	+	-	-	-	23.42
	P. noxius	*50*	+++	2	+	-	-	-	-	17.28

Bioassay result

The extraction by using 3 solvents and preliminary testing antibiotic against bacteria have the result that show on table 3. Most of antibiotics against *B. subtilis* were found from the extraction with ethyl acetate, and some were against *S. aureus.* Where extract from PL contains the most abundant substances with streptomycin 400 ppm.

Separation result

They were selected and separated by some solvents, S2-S5, in TLC method (Table 1). S2 is not found the water mixture and has high polarity, but the most substances are separated and closed to a solvent front. While S3

cannot use solvents to clearly separate the effect substances. Otherwise, S5 has a clearly separate between high polarity (at baseline) and none polarity (at solvent front) substances. Thus, S4 is the best solvent to separate the effect substance, and also use in mobile phase for performance confirmation in bioautography as well.

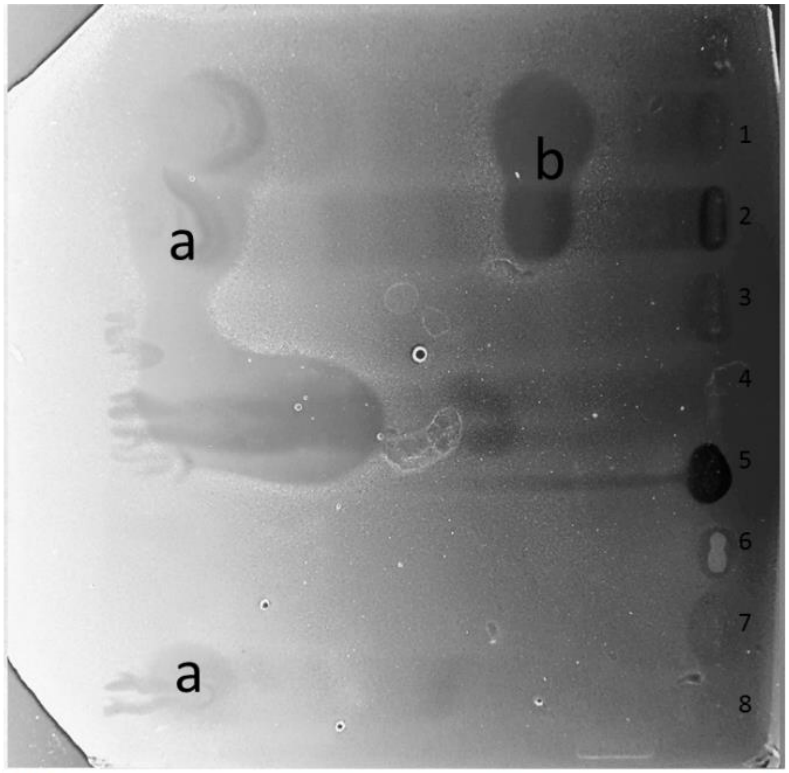
1D, 2D TLC - Bioautographic result

As a TLC method by using S4 solvent that found, PL with PKD025 has only low polarity antibiotic group, and PN with PKD644 has a very clearly different polarity antibiotic (Table 3) (Figure 5). Because of the one-dimension TLC cannot confirm the form of active complex agent. If it uses column chromatography for solvent separation; as a result, the active agent will divide until no activity. Therefor, the next method is 2D TLC. The extraction from PL found the most of antibiotic that is single and complex compound. A solvent separation between none polarity by S1 and derivative compound by using bioautographic method; as result, antibiotic in PL and PKD025 found 1 and 2 groups of co-activity (Figure 6). and it will get %yield of antibiotic from Phellinus (Table 3). According to the quantitative test of both groups of antibiotics substances by HPTLC, the results are as follows. (Table 4).

Table 4 %yield from each type of antibiotic result

medium type	Isolated	Antibiotic type	antibiotic %
PDBx	*P. linteus*	A	31.78
Grain	*P. linteus*	A	23.42
PDBx	PKD644	A	24.97
		B	11.30
PDBx	*P.noxius*	A	5.57
		B	12.67
PDBx	PKD025	A	7.63

A



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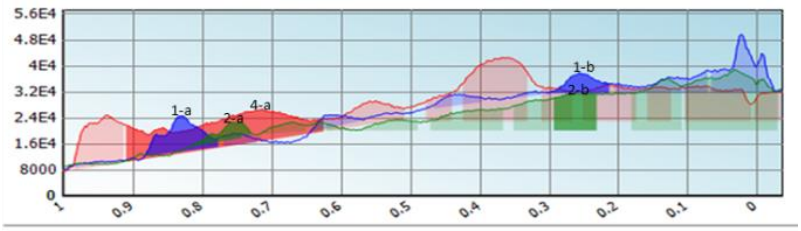
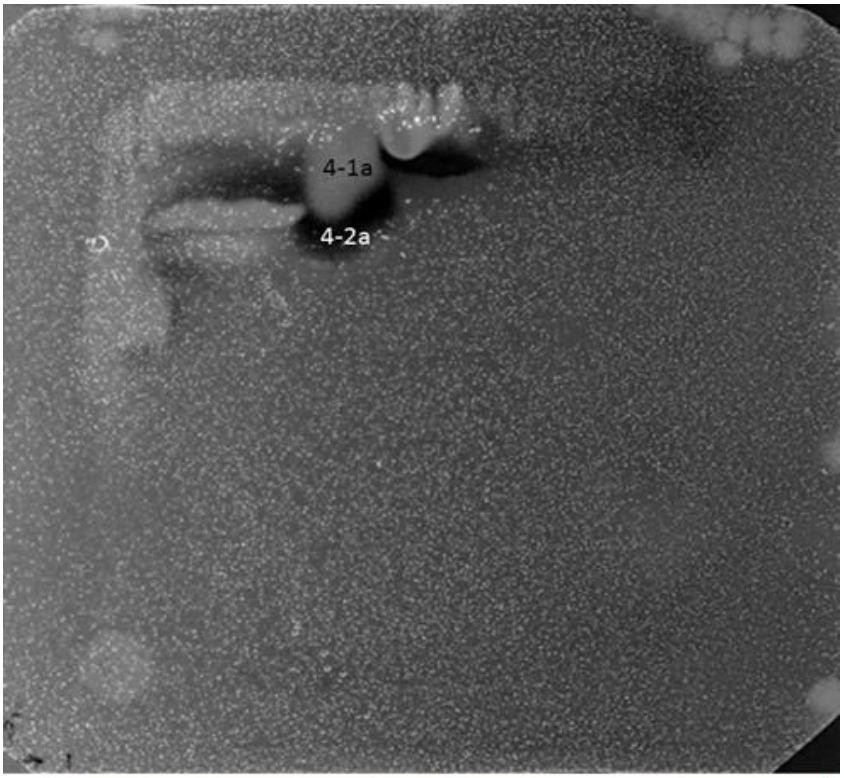


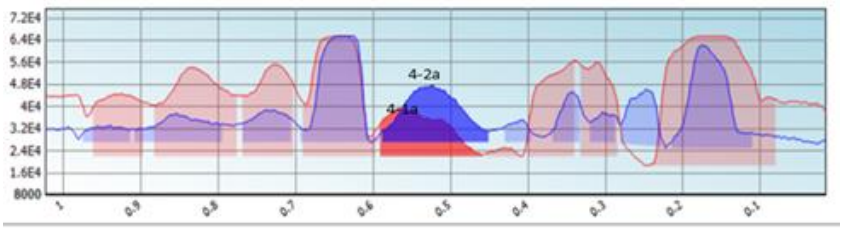
Y-axis: intensity, X-axis : R*f*

Figure 5. (A) 1D-TLC Bioautographic chromatogram of antibiotic ethyl acetate extracts from Phellinus isolated. Appear to inhibit zone and found 2 types of antibiotic groups. And Phellinus extracted from lens no. 1; PKD644, 2; PN 3; PKD025, 4; PL grain medium, 8; PL liquid medium respectively. Lens 5 -7 is an ethanolic extract, not an inhibit zone (B) Densitogram of the separation of compound (a' and b') from each kind of phellinus extracted appear peaks (1-a' *Rf* = 0.82 and 1-b' *Rf* =0.25 from PKD644, 2-a' *Rf* = 0.77 and 2-b' *R*f=0.26 from PN, 4-a' *Rf* = 0.71 from PL, elution). But bands with a' was not complete visible absorption with the applied wavelength (254 nm).

A



B



Y-axis: intensity, X-axis : R*f*

Figure. 6. (A) 2D-TLC Bioautographic chromatograms of antibiotic ethyl acetate extracts from *P. linteus* to detect derivative compound. The program of eluent for second dimension development is presented S4 in Table 1 (B) Densitogram of the separation of compound (a') from PL extracted (4-1a' (red) *Rf* = 0.54 and 4-2a' *Rf*=0.52 (blue) may are derivative co-activity compound represent 7.7% and 18.35 respectively). Peaks with 4-1a' were not visible absorption completely with the applied wavelength (254 nm). Peaks with low *Rf* values, not marked on the densitogram, were appear inhibit zone but not visible with the naked eye.

Column chromatography- Bioautographic result

After column separation and fraction validation by S4 for finding derivative compound, each fraction was tested by bioautography that found the activity of high and low polarity of antibiotic from PKD025. They clearly divided in 2 groups that are F3-5 and F9-18 (fig 3). The most purification of active agent is F3 and F15 at 75.8% and 33.7% respectively. Column chromatography is the method for finding the purification substance, but it is no need for a group of mushrooms in term of supplementary. Otherwise, it can use for a pharmaceutical or finding a toxic compound such as lignin or active phenol compound by using the similar solvents, and it will get highest purity of antibiotic from PL as 75.86% from Fraction 3

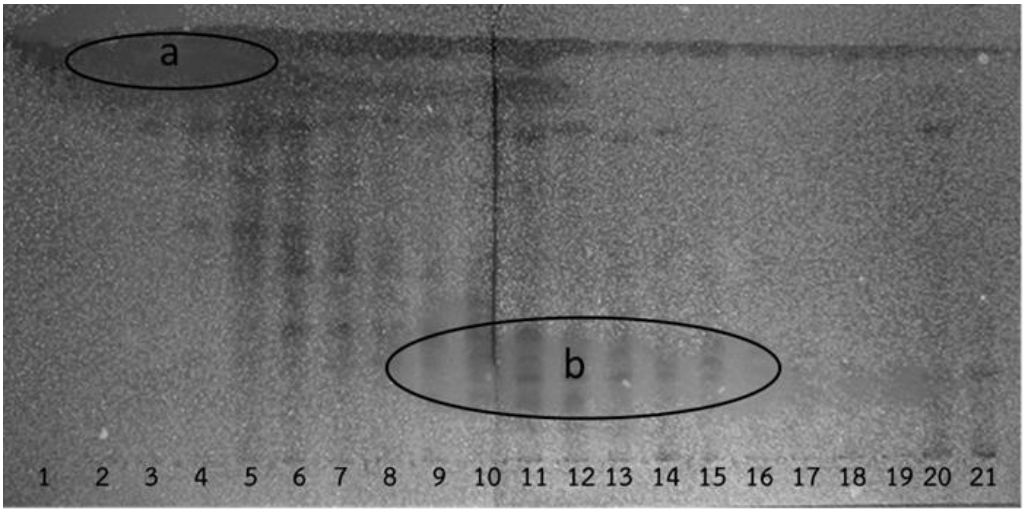


Figure. 7: The 1D-TLC bioautographic chromatograms from column separation of PKD644 crude extracted. The mobile phase see in table 1; S1, S4 and S6 respectively. Appear to inhibit zone and found 2 types of antibiotic groups (a' and b')

Qualitative phytochemical analysis and antioxidant

From the result found Tannins, Saponins, phytosterols, coumarins and Phenolic compounds. It may predict that the mycelium of PL in grain culture can produce the compound, but cannot prove to qualitative analysis.

Table 5 Antioxidative result

Sample	Radiation dose (kGy)	IC_{50}* (mg/ml)	FRAP** ($\mu molFeSO_4/g$)	Total phenolics (mg GAE/g)
medium	0	12.35	37.67	7.39
	10	12.20	32.67	6.81
mycelium	0	9.98	32.21	10.99
	10	10.94	29.02	12.19

*IC50 less value is better than high value
*FRAP is the ability of ferric reduction

Inhibitory concentration 50 (IC50) is the value of antioxidant concentration that reduce free radicals by 50%.

In vitro anticancer result

Table 6 Anticancer activities

			Cell servival ratio (%) and IC50 (ug/ml)					
medium	**Isolated**	**Conc.**	**Ethylacetate**			**Ethanol**		
type		**ug/ml**	**Chago-k1**	**HT-29**	**Hep-G2**	**Chago-k1**	**HT-29**	**Hep-G2**
	P. linteus	***50***	13	23	7, 8 (3.48)			
	P.noxius	***50***	64,68 (>20)	81	70			
Liquid	**PKD644**	**100**	50	87, 77 (>100)	38			
	PKD853	**100**				113	83, 98 (>100)	110
	SYI002	**100**				118	101	111, 112 (>200)
	PKD025	**100**	68	94	89, 85 (>100)	92	91, 92	87
Grain	***P. linteus***	***50***	*90*	87, 96 (>100)	78			
	P. noxius	***50***	96	100	86			

Mushroom has a protein inhibitor, but in each specie provides a clearly different type for a cancer cell prevention. The activity of cancer cells found that PL has the effect of inhibiting cancer cells of, HT-29 and Chago-k1 as %survival 23 and 13. And apoptosis effect with Hep-G2 as %survival 7.5. PKD644 has inhibitory effect on Hep-G2 and Chago-k1 cancer cells as %survival 38 and 50 respectively. With an average of IC_{50} at 3.48 and 20 µg/ml (Table 6).

The finding analysis of hispidin protein inhibitor, by culturing PKD644 and PL in a liquid medium, the hispidin was found at all stage and the most in the last. Moreover, the finding with PKD644 found hispidin the most in 20 days, and the percentage is 38. While PL had a stability of hispidin from 5 days after the average raising at 30 percent.

The results revealed that the cultivation in liquid or grain medium is able to produce hispidin, therefore the synthetic pathway was completed.

Discussion

Based on the results of in *vitro* cancer cell testing, Ethyl acetate extracts are able to apoptosis HT-29 cell, not different form Jeon *et al*., (2012). However, the results of each cell type specific to each mushroom (Table 6). Which indicates the same extract can make active in a different cells types. Moreover, it also has activity with both, rising liquid and grain medium, that contained nitrogen source. In Thailand, there is no report showing bio-activity details of this genus.

The results of various types of cancer cell inhibition has the efficiency not less than natural mushroom extracts (compared with the research of Konno *et al*., **(**2015**)**).

However, the results of antibiotic separation by chromatograms and bioautograhy have been no reports of saparation of antibiotics compound from this mushroom obtained from culture as well. Also from review report by Hsieh *et al*., (2013) has found a compound showed selectively antimicrobial activity against *B.subtilis* at a concentration of 10 mg/ml, which is the result as closey to this research**.**

According to research by Jayashree et al. (2017), the study with various extracted solvents and the samples in the nature found the substance that was similar to the culture in the research except alkaloids group. Moreover, the relation between mushroom and tree exchanged some chemical transduction for stimulation certain substances among each other. ; as a result, it may synthesized a saponins.

From the research, "Kratinphiman" has a variety of medicinal properties that found for use and treat in traditional Thai medicine, and some species cannot find at present. Moreover, it was discovered the culture solution such as recipes and the effect of antibiotic. In addition, it can reduce a natural storage and respond a demand; as a result; it will help to keep a balancing of mushroom diversity. In pharmaceutical, the research will guide and develop to a new product. This is a sign to jump from the tradition to modern medicine. At present, a request of registration is based on the traditional formula. Mostly, the references were distorted because the traditional Thai medicine text was destroyed; as a result, it is difficult to use which the correct method are.

Furthermore, it is also a guideline for the public health organization to raise the important of mushroom in this group, and develop herbal medicine as a traditional medicine as well.

From the study found a variety of medicinal property of mushroom, Kratin Phiman. In each species, it was used and confirm about to retain in traditional Thai medicine. Now, we found the way to culture such as a formula for finding a specific substance. As a result, the natural harvesting will decrease and also responds to demand in a market, and will help the balancing of various mushroom species to remain in a nature. Especially, the confirmation of cultural process will help to develop products to a pilot scale. Moreover, the analysing and quality method will be a pharmaceutical approach for connecting between herb to a modern medicine in a future.

Due to a current medicine registration request is based on traditional medicine formula in a text, but many texts were destroyed or the formula would be distorted; as a result, it cannot use safely enough.

In addition, it will be a guideline for the public health organization to raise the importance level of mushrooms and biological resources from the herbal medicines to traditional medicine. Moreover, the rest of mushroom group was registered as a medicine and supplement as well.

Acknowledgement

This research work was partially supported by Biotransformatics Co., Ltd. and KKF Frontier Co., Ltd., Sakaerat Environmental Research Station Sakaerat Biosphere Reserves, For areas in ecological studies. The authors are grateful to members of the Medicinal mushroom and Natural products Association Thailand and the Scientific and Technological Research Equipment Centre Laboratory, Chulalongkorn University for their laboratory, equipment and Cancer cell line in an experiment. Dr.Surachai Techaoei, Thai Traditional Medicine College, Rajamangala University of Technology Thanyaburi, For site station working.

References

Balandaykin, E.M. and Zmitrovich, V.I. (2015) Review on Chaga Medicinal Mushroom, *Inonotus obliquus* (Higher Basidiomycetes): Realm of Medicinal Applications and Approaches on Estimating its Resource Potential. International Journal of Medicinal Mushrooms, 17(2): 95-104.

Basyuni, M., Sagami, H., Baba, S., Oku, H. (2017) Distribution, occurrence, and cluster analysis of new polyprenyl acetones and other polyisoprenoids from North Sumatran mangroves. dendrobiology. 78; 18-31.

Blagodatski, A., Yatsunskaya, M., Mikhailova, V., Tiasto, V., Kagansky, A. and Katanaev, V. L. (2018). Medicinal mushrooms as an attractive new source of natural compounds for future cancer therapy. Rev. Oncotarge. 9(49): 29259-29274.

Chen, H, Tian, T., Miao, H. and Zhao, Y-Y. (2016). Traditional uses, fermentation, phytochemistry and pharmacology of *Phellinus linteus*: Rev. Fitoterapia. 113: 6–26.

Department of Thai traditional and alternative Medicine, Ministry of Public Health. (2011) "Mushroom Guide is a medicine for health." ISBN : 978-616-11-0641-6

Glamoclija, J., Ciric, A., Nikolic, M., Fernandes A., Barros L., Calhelha, C. R., Ferreira, R., Sokovic, M. and Griensven, V. (2014). Chemical characterization and biological activity of Chaga (*Inonotus obliquus*), a medicinal "mushroom". Ethnopharmacology.

Han, S.B., Lee, C.W., Jeon, Y.J., Hong, N.D., Yoo, I.D., Yang, K.H., Kim, H.M. (1999.) The inhibitory effect of polysaccharides isolated from *Phellinus linteus* on tumor growth and metastasis. Immunopharmacology. 41(2): 157-64.

Hsieh, P.-W., J.-B. Wu and Y.-C. Wu. (2013). Chemistry and biology of *Phellinus linteus*. Bio-Medicine. 3(3): 106-113.

Huang, Y., J. Wang, G. Li, Z. Zheng and W. Su. (2001). Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants Taxus mairei, Cephalataxus fortune and Torreya grandis. FEMS Immunology and Medical Microbiology. 31(2): 163-167.

Jeon, T.-I., C.-H. Jung, J.-Y. Cho, D. K. Park and J.-H. Moon. (2013). Identification of an anticancer compound against HT-29 cells from *Phellinus linteus* grown on germinated brown rice. Asian Pacific Journal of Tropical Biomedicine. 3(10): 785-789.

Kedare, S.B. and Singh, R.P. (2011) Genesis and development of DPPH method of antioxidant assay. Journal of Food Science and Technology. 48(4):412–422.

Kim, S. E., B. S. Hwang, J. G. Song, S. W. Lee, I. K. Lee and B. S. Yun. (2013). New bioactive compounds from Korean native mushrooms. Mycobiology. 41(4): 171-176.

Kodiyalmath, J.K. and Krishnappa, M. (2017) Evaluation of antimicrobial activity of *Phellinus linteus* (Berk. & M.A. Curtis.) with their wild collections from Western Ghats of India. Tropical plant Research an International Journal. 4(2): 351–357.

Konno, S., Chua, K., Feuer, N., Phillips, J. and Choudhury, M. (2015). Potent anticancer effects of bioactive mushroom extracts (*Phellinus linteus*) on a variety of human cancer cells. Clinical Medicinal Research. 7(2): 76-82.

Masaladol, V. and Makariya, T. (1995). "Chinese medicine book" 1:23–33.

Panphut W. (1997). Bioactive compounds from endophytic fungi of Thai medicinal plants. M.Sc. (Micorbiology) Thesis, Faculty of ScienceMahidol University,Bangkok Thailand.

Raghava-Rao1, K.V., Mani, P., Satyanarayana1, B. and Raghava-Rao1, T. (2017) Purification and structural elucidation of three bioactive compounds isolated from Streptomyces coelicoflavus BC01 and their biological activity. Biotech. 7:24.

Reis, F.S., Barreira, J.C.M., Calhelha R.C., Griensven, J.I.D., Ćirić, A., Glamočlija, J., Soković, M.S and Ferreira, I.C.F.R. (2014). Chemical characterization of the medicinal mushroom *Phellinus linteus* (Berkeley & Curtis) Teng and contribution of different fractions to its bioactivity. LWT - Food Science and Technology. 58(2): 478-485.

Rizzo, D.M., Gieser, P.T. and Burdsall, H.H. (2003). Phellinus coronadensis: a new species from southern Arizona, USA. Mycologia, 95(1): 74–79.

Sonawane, H., Bhosle, S. and Garad, S. (2012). Antimicrobial activity of some species of phellinus and ganoderma sample from western Ghats of india. Journal of Pharmaceutical Sciences and Research. 3(6): 1795-1799.

Sharon, F.B., Kalidass, S and Daniel, R.R. (2013) Qualitative analysis of antimicrobial compound by High performance thin layer chromatography method. Asian Journal of Pharmaceutical and Clinical Research, 6(4): 117-120.

Shahverdi, A.R., Abdolpour, F., Monsef-Esfahani, H.R. and Farsam, H. (2007) A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. Journal of Chromatography B. 850: 528–530

Shetty, P.R., Buddana, S.K., Tatipamula, V.B., Naga, Y-V.V. and Ahmad, J. (2014) Production of polypeptide antibiotic from Streptomyces parvulus and its antibacterial activity. Brazilian Journal of Microbiology 45(1): 303-312.

Sliva, D. (2010). Medicinal mushroom *Phellinus linteus* as an alternative cancer therapy. Experimental and Therapeutic Medicine 1(3): 407-411.

Silva, M.T-G., Simas, S.M., Batista, T., Cardarelli, P. and Tomassini, T.C-B., (2005) Studies on antimicrobial activity, in vitro, of *Physalis angulata* L.(Solanaceae) fraction and physalin B bringing out the importance of assay determination. Memórias do Instituto Oswaldo Cruz. 100(7): 779-782.

Siriwattanametanon, W., Kanchanarach, W., Thiwthong, R. and Dodgson, J.L.A. (2014) Culture filtrates from laboratory grown Phellinus mushrooms for use as antibacterial agents. Chiang Mai Journal of Science. 41(1): 243-247.