OCCURRENCE OF TRITERPENOIDS AND POLYSACCHARIDES ON *GANODERMA TROPICUM* WITH *GANODERMA LUCIDUM* AS REFERENCE

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Abstract

A local strain of *Ganoderma tropicum* has been analyzed for the appearance of triterpenoids and polysaccharides, with *Ganoderma lucidum* as reference. These two compounds are reported to have medicinal value in *G. lucidum*. *Ganoderma tropicum* was isolated from a *Delonix regia* tree, an ornamental tree locally known as Flamboyant, in December 1999. The fungus is parasitic to the tree and is quite aggressive, killing the tree within one to five years. The fruiting bodies of both *G. tropicum* and *G. lucidum* were obtained by growing them in sawdust after three months' incubation. Extraction for triterpenoids was conducted in wash benzene and ethanol, while extraction for polysaccharides was done in hot water. Analysis for triterpenoids was conducted by using silica gel-thin layer chromatography (TLC) with eluent of chloroform:methanol (10:1) for the first extract and dichloromethane for the second extract. Detection of triterpenoids was done by spraying the plate with Carr-Price as well as Lieberman-Burchard reagents then observing under UV light (366 nm). The same principle, except for the eluent n-buthanol : acetic acid : eter : water (9:6:3:1) and reagent of aniline ftalate, was used for detecting polysaccharides in the form of their monomers (glucose, galactose, xylose and rhamnose) under normal light. Both triterpenes and polysaccharides were detected on *G. tropicum* and *G. lucidum*, suggesting that the local strain of *G. tropicum* also possesses medicinal value.

Keywords: *Ganoderma tropicum*, *Ganoderma lucidum*, triterpenes, polysaccharides, medicinal mushroom.

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Introduction

Ganoderma mushrooms, particularly *G. lucidum*, have some medicinal properties including antitumor (Kishida *et al.* 1988, Zhang *et al.* 2000), antivirus (Eo *et al.* 2000), anti-inflammatory (Patocka 1999), lowering blood pressure (Morigiwa *et al.* 1986) and increased immune system (Kino *et al.* 1989, Tomoda *et al.* 1986, Yu *et al.* 1997). Other species such as *G. tsugae*, *G. chinense* and *G. applanatum* also possess medicinal properties. Extensive research has been done on *G. lucidum* and other species, but there is very little information available for *G. tropicum*. Jun & Shuqin (1987) reported that *G. tropicum* has been used in China for curing coronary heart disease and also inhibited sarcoma cells up to 70 per cent.

The two main substances in the fruiting body of *G. lucidum* which are considered to possess medicinal properties are polysaccharides and triterpenoids (Min *et al.* 2000). A polysaccharide isolated from spores of *G. lucidum* was found to be a complex glucan (Bao *et al.* 2001). Other reports stated that *G. lucidum* contains polysaccharides called GLA (galactan) with monomers of mainly galactose and others such as glucose, rhamnose and xylose and GLB (glucan) with monomers of mainly glucose and others such as rhamnose and xylose (Qing-Yao *et al.* 1994). Triterpenoids contained in *Ganoderma* mushrooms are classified as ganoderic acid (C30) and lusidenic acid (C27) (Mizuno 1984). Indonesia has mega-diversity of natural resources including its mycoflora, yet lacks information available on chemical composition of mushroom isolates.

The aim of this study was to investigate whether triterpenoids and polysaccharide are present in *G. tropicum* as they are in *G. lucidum*.

Materials and Methods

Isolate of *G. lucidum* and *G. tropicum*

Ganoderm lucidum was obtained from Fungi Perfecti, U.S.A. *Ganoderm tropicum* was isolated, by using tissue culture techniques, from a fresh fruiting body collected from a *Delonix regia* tree (Fig. 1) in Bandung during 1999. The flesh edge part of the mushroom was cut into small pieces with a scalpel aseptically, then cultured onto PDA before incubation at room temperature. Young hyphae growing on the plate were cut and transferred into fresh slant PDA as stock culture.

Fruiting body production

Cereal substratum consisting of corn cob, rice bran and red rice was inoculated with seven-day-old mycelium growing on plate then incubated for 20 days at room temperature. Mycelium growing from this substratum (spawn) was inoculated into log substratum made mainly of sawdust and rice bran. Four weeks' incubation at room temperature was required to complete the colonization of the log before the log was exposed to fresh air in a humid condition (RH 85%). A total of three months was required to obtain mature fruiting bodies.

Analysis for Triterpenoids

Extraction. A fresh fruiting body was washed, then sliced into small pieces and dried in an oven at 50°C before being blended to granules. Twenty-five grams of dried granules were then extracted with 250 ml wash benzene in soxhlet for 24 hours. Another extraction was done by using 250 ml ethanol. Both extracts were concentrated by vacuum evaporation and heating evaporation at 70°C in a water bath before running the Thin Layer Chromatography.

Thin Layer Chromatography (TLC). Detection was conducted by spotting the extract with a micro pipette onto a silica gel plate (60 F 254) as a stationary phase and developing solvent of dichloromethane for the first extract and chloroform : methanol (10:1) for the second extract. After developing then drying the plate, spraying with Carr-Price reagent (20% antimon solution chloroform) at 100°C for 10 minutes and Lieberman Burchard (1 ml H_2SO_4 conc. 20 ml acetic anhydride and 50 ml $CHCl_3$) at 85–95° for 15 minutes were done before observing under UV light (366 nm).

Analysis for polysaccharides

Extraction. Ten (10) grams of granules of fruiting body were soaked in 100 ml of hot distilled water (90–95°C) over 48 hours. The extract was then hydrolyzed with H_2SO_4 1M at 100°C over 8–16 hours then neutralized with $Ba(OH)_2$. Precipitation of $BaSO_4$ was then removed after centrifugation. Ultimately, the filtrate was concentrated by evaporating at 70°C on a water bath before running TLC.

Thin Layer Chromatography (TLC). The same principle as used for detecting triterpenoids was used for detecting polysaccharides, except for the developing solvent (n-buthanol : acetic acid : ether : water 9:6:3:1) and reagent (aniline hydrogen ftalat) at 100°C for 10 minutes were applied before observing under UV light (366 nm).

Results and Discussion

Isolation of *G. tropicum*. Incubation over four days was required for hyphae to develop from tissue pieces before transferring into slant agar. No purification was required since the mycelium growing from tissue culture was found to be axenic. This can be achieved by careful handling when opening the fresh fruiting body before cutting the inner part into pieces. Opening should start from the center towards the edge of the fruiting body. The knife should be used to just open the fruiting body slightly then continued by hands without touching the inner part of the fruiting body to avoid contamination. This way was proven to be mostly successful in tissue culture isolation.

Fruiting body production of *G. tropicum* and *G. lucidum*. Both *G. tropicum* and *G. lucidum* were grown on the same sawdust substratum to obtain fruiting bodies (Fig. 2). At first, the fruiting initials for both species appeared after 1.5–2 months, then maturation took place in 1–1.5 months. *G. lucidum* grew faster than *G. tropicum* both in mycelium running and fruiting initiation. As can be seen on Fig. 2, the fruiting body of *G. lucidum* is bigger than that of *G. tropicum* since the formula was initially developed for producing *G. lucidum* commercially. The fruiting body of *G. tropicum* produced from cultivation was different from the fruiting body obtained from the field (Figs 1 & 2). The environmental condition, which is different between field and indoor condition, can explain this phenomenon. In addition, the substratum used in producing fruiting

bodies in 1,5 L log is also different from the natural substratum of a live tree. The size of fruiting bodies in the field may reach 40 cm in diameter on a big tree. According to our observations, the more substratum available (as on the big tree) the bigger size of fruiting bodies produced.

Occurrence of triterpenoids. First extraction using wash benzene, a non-polar solvent, was able to extract non-polar substances. TLC pattern for non-polar triterpenoids without applying any reagent can be seen in Fig. 3. Without reacting agent Carr Price or Lieberman Burchard, only four spots for *G. lucidum* and five spots for *G. tropicum* were observed under UV light (Fig. 3). After applying Carr Price reagent, both *G. lucidum* and *G. tropicum* gave 11 spots (Table 1). However, when applying Lieberman Burchard to the TLC plate less spots were produced, eight spots for *G. lucidum* and 10 spots for *G. tropicum* (Table 2). This means that Carr Price reagent is better for detecting triterpenes diversity as also stated by Harborne (1996). Some of the spots have the same value of retention factor (Rf) and colour under UV light indicating the same substance (Tables 1 and 2). This phenomenon may explain that both species possess medicinal value which have been traditionally used for medicinal herbs in Asia. From Table 2 it can also be noticed that *G. tropicum* produces more spots than *G. lucidum*, suggesting that *G. tropicum* contains more types of triterpenoids.

Table 1. TLC spots of *G. lucidum* and *G. tropicum* (wash benzene extract) run on dichloromethane after applying Carr Price reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	G. lucidum	*G. tropicum*
1	0.051 (White fluorescent)	0.051 (White fluorescent)
2	0.204 (White fluorescent)	0.204 (White fluorescent)
3	0.264 (Red fluorescent)	0.264 (Red fluorescent)
4	0.381 (White fluorescent)	0.381 (White fluorescent)
5	0.457 (White fluorescent)	0.457 (White fluorescent)
6	0.498 (White fluorescent)	0.498 (White fluorescent)
7	0.572 (White fluorescent)	0.558 (White fluorescent)
8	0.660 (White fluorescent)	0.660 (White fluorescent)
9	0.762 (White fluorescent)	0.742 (White fluorescent)
10	0.871 (White fluorescent)	0.863 (White fluorescent)
11	0.901 (White fluorescent)	0.888 (White fluorescent)

Table 2. TLC spots of *G. lucidum* and *G. tropicum* (wash benzene extract) run on dichloromethane after applying Lieberman Burchard reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	G. lucidum	*G. tropicum*
1	0.204 (White fluorescent)	0.204 (White fluorescent)
2	0.264 (Red fluorescent)	0.264 (Red fluorescent)
3	0.457 (White fluorescent)	0.335 (White fluorescent)
4	0.498 (White fluorescent)	0.381 (White fluorescent)
5	0.559 (White fluorescent)	0.457 (White fluorescent)
6	0.711 (White fluorescent)	0.498 (White fluorescent)
7	0.863 (White fluorescent)	0.558 (White fluorescent)
8	0.888 (White fluorescent)	0.660 (White fluorescent)
9	-	0.863 (White fluorescent)
10	-	0.888 (White fluorescent)

Two spots are very strong *i.e.* Rf: 0,204 (white fluorescent) and Rf: 0,264 (red fluorescent) under UV (yellow and blue) under normal light after spraying with both reagents. These two spots indicate the presence of steroids, one group of triterpenes (Harborne 1996). This may explain how some people become awake and feel fresh even working until late after drinking crude water extract of *Ganoderma* fruiting body (personal experience).

Ethanol extraction is able to separate the polar compounds including glycosidic triterpenoid. Nishitoba *et al.* (1987) discovered that some triterpenoids of *G. lucidum* can be obtained from ethanol extraction. TLC plate after spraying with Carr Price reagents gave 11 spots for *G. lucidum* and 12 spots for *G. tropicum* (Table 3). However, Lieberman Burchard reagent gave less spots *i.e.* eight spots for *G. lucidum* and nine spots for *G. tropicum* (Table 4). Both reagents can react with triterpenoids producing various fluorescent colour ranging

from blue, purple and red fluorescent under UV light 366 nm (Harborne 1996). All spots observed from TLC plate were white fluorescent, ranging from weak to strongly fluorescent. Weak fluorescence indicates less quantity of the metabolites and strong fluorescence indicates more quantity. Again more spots are observed on the treatment with Carr Price reagent indicating that Carr Price is more sensitive than Lieberman Burchard to react with triterpenoid metabolites. As was observed on previous extract, *G. tropicum* contains more types of metabolites than *G. lucidum*.

The metabolite production is also influenced by the way the culture is maintained (Garraway & Robert 1984). *Ganoderma lucidum* was first obtained from Fungi Perfecti in the early 1990s. For more than 10 years the isolate was subcultured routinely on synthetic medium. On the other hand, *G. tropicum* was isolated in 1999 from a fruiting body collected from nature, in other words, it is only two years since the first isolation. Theoretically, its genetic diversity is more than the isolate maintained for more than 10 years in the laboratory. This may explain why more types of metabolite were obtained from the *G. tropicum* extract than from the *G. lucidum* extract. Another explanation is that *G. tropicum* naturally (genetically) may produce more metabolites than *G. lucidum*.

Table 3. TLC spots of *G. lucidum* and *G. tropicum* (Ethanol extract) run on Chloroform:Methahnol (10:1) after applying Carr Price reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	G. lucidum	*G. tropicum*
1	0,022 (White fluorescent)	0,058 (White fluorescent)
2	0,058 (White fluorescent)	0,134 (White fluorescent)
3	0,089 (White fluorescent)	0,301 (White fluorescent)
4	0,301 (White fluorescent)	0,334 (White fluorescent)
5	0,357 (White fluorescent)	0,368 (White fluorescent)
6	0,435 (White fluorescent)	0,446 (White fluorescent)
7	0,479 (White fluorescent)	0,479 (White fluorescent)
8	0,535 (White fluorescent)	0,580 (White fluorescent)
9	0,569 (White fluorescent)	0,624 (White fluorescent)
10	0,624 (White fluorescent)	0,680 (White fluorescent)
11	0,736 (White fluorescent)	0,769 (White fluorescent)
12	-	0,814 (White fluorescent)

Table 4. TLC spots of *G. lucidum* and *G. tropicum* (Ethanol extract) run with Chloroform:Methanol (10:1) after applying Lieberman Burchard reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	G. lucidum	*G. tropicum*
1	0,203 (White fluorescent)	0,220 (White fluorescent)
2	0,305 (White fluorescent)	0,305 (White fluorescent)
3	0,339 (White fluorescent)	0,339 (White fluorescent)
4	0,424 (White fluorescent)	0,424 (White fluorescent)
5	0,636 (White fluorescent)	0,534 (White fluorescent)
6	0,721 (White fluorescent)	0,636 (White fluorescent)
7	0,778 (White fluorescent)	0,721 (White fluorescent)
8	0,825 (White fluorescent)	0,778 (White fluorescent)
9	-	0,835 (White fluorescent)

TLC is not a very precise technique for detecting chemical compounds of any natural products. Nevertheless, Carr Price and Lieberman Burchard are the most popular reagents for detecting triterpenes (Harborne 1996). The further steps of using HPLC technique for quantitative analysis or even GCMS and NMR for determining chemical structure of the compounds are required. With the last technique, Kleinwachter *et al.* (2001) were able to determine seven new triterpenoids metabolites (colossalactones 1-7) from *Ganoderma colossum*. Meanwhile, Su *et al.* (2001) divided *Ganoderma* mushroom into 18 groups based on triterpenoids patterns analyzed with HPLC technique. This grouping was also confirmed with morphological data and infertility testing by di-monokaryotic mating.

Overall, triterpenoids from *G. tropicum* and *G. lucidum* are quite similar, based on the Rf values and colour reaction with both Carr Price and Lieberman Burchard. *Ganoderma lucidum* has been extensively studied for

active compounds, including triterpenoid metabolites which are mainly reported as ganoderic acid and lusidenic acid (Komoda *et al.* 1985, Nishitoba *et al.*1987). Nevertheless, overall TLC patterns for triterpenoids of both species are not exactly the same. This tells that *G. tropicum* is a different group from *G. lucidum* (Moncalvo *et al.* 1994).

Figure 1. *Ganoderma tropicum* fruiting body parasitizing a *Delonix regia* tree in nature.

Occurrence of polysaccharides based on their monomer forms. TLC results of polysaccharides analysis are presented in Table 5. It can be seen that there are four spots produced after running the extract on a TLC plate with spots 1 and 2 having the same Rf value. Based on the standard, these four spots are glucose, galactose, xylose and rhamnose. Spots 1 and 2 (Rf = 0.333) belong to glucose and galactose. This may happen owing to the similarity of the chemical structures of both sugars. Previous findings have reported that *G. lucidum* can produce GLA which consists of glucose, galactose, xylose and rhamnose. The same pattern of TLC spots was observed from *G. lucidum* and *G. tropicum*, suggesting both species produce the same polysaccharides. All colours of spots are not the same contrast, (intensity) indicating different quantity of each monomer.

Table 5. TLC pattern of monomer forms of polysaccharides of *G. lucidum* and *G. tropicum* (hot water extract) run on n-buthanol : acetic acid : ether : water (9:6:3:1) and spraying with Aniline Ftalat

No of Spot	Rf value and colour after spraying with Aniline Ftalat	
	G. lucidum	***G. tropicum***
1	0,333 Glucose (Light brown)	0,333 Glucose (Light brown)
2	0,333 Galactose (Light brown)	0,333 Galactose (Light brown)
3	0,444 Xylose (Red)	0,444 Xylose (Red)
4	0,555 Rhamnose (Yellow)	0,555 Rhamnose (Yellow)

In this study, only the occurrence of four sugar monomers was observed. It is not conclusive as to whether the monomers belong to GLA or GLB since both polysaccharides were not separated before running TLC. Qing-Yao *et al.* (1994) determined the Mol ratio of GLA and GLB monomer forms based on their retention time of the peak and the areas of HPLC analysis. They found that GLA consists of glucose, galactose, xylose and rhamnose with Mol ratio of 3.2:2.7:1.8:1.0, whilst GLB consists of glucose, xylose and rhamnose with Mol ratio of 6.8:2.0:1.0.

Conclusion

From this preliminary examination of the fruiting bodies of *G. tropicum,* it can be concluded that *G. tropicum* contains triterpenoids and polysaccharides as contained in *G. lucidum*. TLC pattern for triterpenoids of both isolates is not exactly the same, indicating they are different groups of *Ganoderma*.

A.

B.

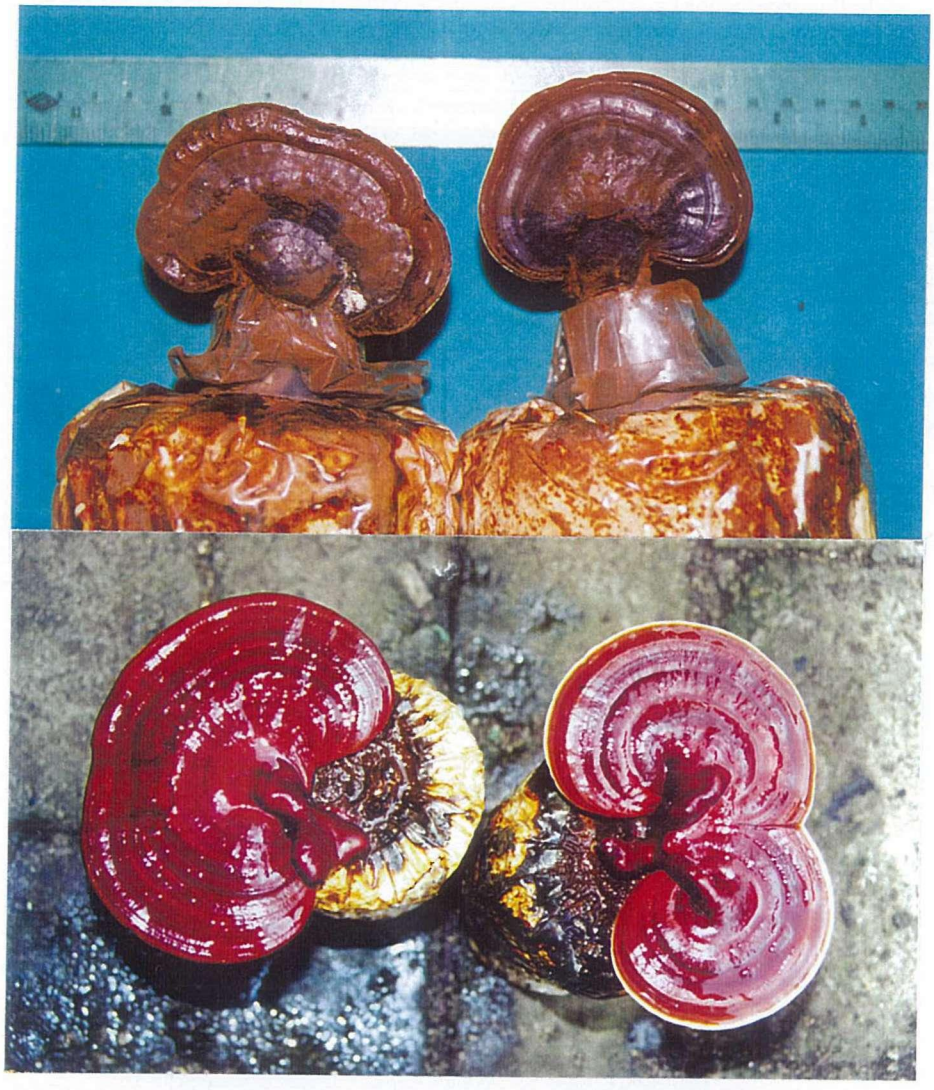

Figure 2. A. *Ganoderma tropicum* and B. *Ganoderma lucidum* fruiting bodies grown on sawdust based substratum.

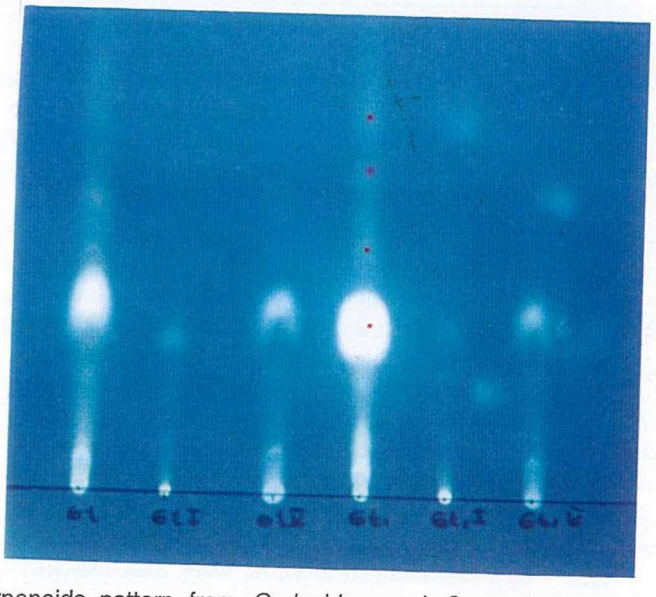

Figure 3. TLC of triterpenoids pattern from *G. lucidum* and *G. tropicum* (wash benzene extract) run on dichloromethane observed with UV light 366 nm.

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