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Ganoderma boninense mycelia for phytochemicals and secondary metabolites with antibacterial activity[§]

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Antiplasmodial nortriterpenes with 3,4-*seco*-27-norlanostane skeletons, almost entirely obtained from fruiting bodies, represent the main evidential source for bioactive secondary metabolites derived from a relatively unexplored phytopathogenic fungus, *Ganoderma boninense*. Currently lacking is convincing evidence for antimicrobial secondary metabolites in this pathogen, excluding that obtained from commonly observed phytochemicals in the plants. Herein, we aimed to demonstrate an efficient analytical approach for the production of antibacterial secondary metabolites using the mycelial extract of *G. boninense*. Three experimental cultures were prepared from fruiting bodies (GBFB), mycelium cultured on potato dextrose agar (PDA) media (GBMA), and liquid broth (GBMB). Through solvent extraction, culture type-dependent phytochemical distributions were diversely exhibited. Water-extracted GBMB produced the highest yield (31.21 ± 0.61%, $p < 0.05$), but both GBFB and GBMA elicited remarkably higher yields than GBMB when polar-organic solvent extraction was employed. Greater quantities of phytochemicals were also obtained from GBFB and GBMA, in sharp contrast to those gleaned from GBMB. However, the highest antibacterial activity was observed in chloroform-extracted GBMA against all tested bacteria. From liquid-liquid extractions (LLE), it was seen that mycelia extraction with combined chloroform-methanol-water at a ratio of 1:1:1 was superior at detecting antibacterial activities with the most significant quantities of antibacterial compounds. The data demonstrate a novel means of assessing antibacterial compounds with mycelia by LLE which avoids the shortcomings of standardized methodologies. Additionally, the antibacterial extract from the mycelia demonstrate that previously unknown bioactive secondary metabolites of the less studied subsets of *Ganoderma* may serve as active and potent antimicrobial compounds.

***Keywords*:** antibacterial activity, disc diffusion assay, *Ganoderma boninense*, *Ganoderma* mycelium, liquid-liquid extraction, phytochemical analysis, solvent extraction

Introduction

Ganoderma boninense is a bracket fungi species belonging to a genus of polypore fungi *Ganoderma* in the family Ganodermataceae, of the class Agaricomycetes (Peng *et al.*, 2014; Richter *et al.*, 2015). The white rot basidiomycetes *G. boninense* has been identified as a double-walled basidiospore and wood-decay/phytopathogenic hemibiotroph, with the morphological characteristics of fruiting bodies and mycelia (Pilotti *et al.*, 2002; Rakib *et al.*, 2015). As the most virulent and causative agent of basal stem rot, *G. boninense* is specifically phytopathogenic to *Elaeis guineensis* and/or *E. oleifera* (Ho and Tan, 2015). At present there is little evidence that *G. boninense* might possess potentially useful bioactive molecules. Instead, due to their status as non-pathogens, species such as *Ganoderma* spp. have attracted far more attention, with the latter's crude extracts now being relatively well-documented and currently in use in treatments for human diseases (Baby *et al.*, 2015). However, *G. lucidum* has managed to recently become a focus of research, with the potential utilization of its triterpenes in therapeutic anticancer agents being recently highlighted (González *et al.*, 2020). Considering the emerging literature supporting the viability of species other than non-pathogenic *Ganoderma*, it has been noted that there is still a lack of detailed experimental protocols and supporting data concerning bioactive secondary metabolites in the pathogenic species *G. boninense*.

Ganoderma boninense exhibits behaviors in common with other pathogenic species of this genus, including *G. tornatum*, *G. miniatocinctum*, and *G. zonatum* (González *et al.*, 2020). At present most of the descriptions of bioactive phytochemicals/secondary metabolites have predominantly been restricted to lanostane-type triterpenoids and their derivatives in both *G. boninense* and the genus of *Ganoderma* (Gong *et al.*, 2019). The common norlanostanes from *G. boninense* are 3,4-*seco*-27-norlanostane triterpenes including, 3 ganoboninketals and 6 ganoboninones, with tetradecahydrobenzo-[4,5]indeno[1,7a-c]furan backbone (Ma *et al.*, 2014, 2015). The known ganoboninketals, 3,4-*seco*-27-norlanostane triterpenes, exhibit anti-plasmodial activity against the malaria parasite *Plasmodium falciparum* and/or agonistic activity to liver X receptor beta transcription factor (lxrβ). Other bio-

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activities of these compounds include them being cytotoxic against alveolar basal epithelial A549 and cervical carcinoma HeLa cells. However, both ganoboninones and ganobinketals are only partially defined within the context of lanostane-type triterpenoids (Baby *et al.*, 2015), with their chemical structures being similar to four other types of 3,4-*seco*-27-norlanostane triterpenes obtained from the pathogenic *Ganoderma* species, *G. orbiforme* (Li *et al.*, 2018).

We have previously observed the antibacterial activities of *G. boninense* cultures against food borne and skin disease bacterial pathogens, and suggested there might be nine kinds of potential antimicrobial compound present (Ismail *et al.*, 2014). Based on this finding, two anti-methicillin-resistant *Staphylococcus aureus* (MRSA) compounds are hypothesized as being ergosta-5,7,22-trien-3β-ol and ganoboninketal, with the latter compound also belonging to the family of 3,4-*seco*-27-norlanostane triterpenes (Abdullah *et al.*, 2018b). Only a few studies, including our own, support the notion of biologically active secondary metabolites from the fruiting bodies of *G. boninense* or *G. orbiforme*. Importantly, studies on *G. boninense* are still predominantly data limited to fruiting bodies and other pathogenic and non-pathogenic species of *Ganoderma* (Baby *et al.*, 2015). This tendency reflects the absence of convincing proven delicate and detailed antimicrobial experimental set-ups for evaluating unknown secondary metabolites.

Thus, for the provision of efficient quantitative analysis methods pivotal for the isolation of phytochemicals/secondary metabolites, this study has aimed at establishing a standardized antibacterial experimental set-up which is dependent of the specific characteristics of the experimental materials. To ensure the procedure employed met these criteria, our laboratory trials intensively extended the experimental bioactive materials employed to rarely investigated mycelia metabolites combined with liquid-liquid extraction (LLE) by solvent extraction using five types of (non)organic and/or (non)-polar solvents. To confirm our hypothesis that pathogenic mycelia might commonly be in possession of useful antibacterial compounds, *G. boninense* was the focus of solvent-extraction and testing for highly selective mycelium-derived extracts. Therefore, based on LLE, our data provide a clear framework and robust methodology for the future analysis of a multitude of antibacterial mixtures, using the relatively unknown plant-pathogenic fungus *G. boninense* as a template.

Materials and Methods

Mycelia and fruiting body collection

The mycelia and fruiting bodies were obtained from oil palm (*Elaeis guineensis* Jacq.) infected by *G. boninense*. The infection score of the selected palm trees was assessed by methods previously reported (Chong *et al.*, 2014). In accordance with the disease severity index of *G. boninense*-infected oil palms, the *G. boninense* collection was performed at Langkon Plantation, Sawit Kinabalu, Sabah, Malaysia.

Strains and culture conditions

In this study, three culture type-dependent *G. boninense* were used, including fruiting bodies (GBFB), mycelia cultured on potato dextrose agar (PDA) plates (GBMA) and potato dextrose broths (PDB, GBMB). *G. boninense* was grown to collect mycelia by means described previously with minor modifications (Ismail *et al.*, 2014). In the case of GBMA and GBMB, the *G. boninense* mycelia was inoculated on PDA agar media and grown at 28°C for 14 days. The fully grown mycelial plugs were respectively transferred and grown onto PDA agar plates and PDB liquid media for further biochemical experiments and antibacterial preparation. Specifically, the resulting mycelial plugs were loaded onto PDA agar plates and cultured for 7 days at 28°C. In addition, approximately five mycelial plugs were inoculated into PDB liquid media and cultured for 7 days at 28°C at 120 rpm (SHKE8000, Thermo Scientific). Despite the use of different culture media by experiment, unless otherwise stated, each strain for all biochemical experiments was routinely cultured in nutrient broth (NB; Merck) at 37°C. The resulting cultured mycelia and fruiting bodies were harvested by centrifugation at 10,000 × *g* for 5 min, washed with 200 mM phosphate-buffered saline (PBS), pH 7.0, and resuspended in 30% glycerol solution for further experimentation.

For the antibacterial activity assay, six bacterial strains were used. These pathogenic bacterial strains included Gram-positive bacteria (i.e., *S. aureus* American Type Culture Collection [ATCC] 25923, multidrug-resistant *Staphylococcus aureus* [MRSA] National Collection of Type Culture [NCTC] 11939, and *Streptococcus pyogenes* ATCC 19615) and Gram-negarive bacteria (i.e., *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 1705, and *Escherichia coli* ATCC 35218). In this study, ATCC bacterial strains and MRSA (NCTC) 11939 were purchased from Sigma-Aldrich and Oxoid Ltd., respectively. Prior to antibacterial activity assays using solvent-extracted *G. boninense* samples, the exponential growth phase of each pathogenic bacterial strain was observed using previously detailed methods (Brannan, 2006).

Solvent extraction

To obtain antibacterial extracts derived from *G. boninense* cultures, three culture type-dependents of GBFB, GBMA, and GBMB were utilized for organic and/or non-organic solvent extraction for evaluating compound extraction yield efficiency as previously described elsewhere (Ng *et al.*, 2013). Approximately 10 g of samples from GBFB and GBMA was rinsed twice with triple-distilled water (TDW), lyophilized by a freeze-dryer for 24 h, and recovered in a powdered form. In the case of GBMG preparation, 5 L of culture media-containing GBMB sample was extensively evaporated and lyophilized under low pressure conditions, ranging from 768 mmHg to 7 mmHg, at 25°C using a Rota Vapor™ (BUCHI), to obtain a powdered form. Following this procedure, 10 g of powdered samples were used for the solvent extractions.

To extract the secondary metabolites and phytochemical constituents of each culture type-dependent *G. boninense* sample, approximately 10 g of *G. boninense* culture was soaked in 50 ml water and organic solvent (i.e., methanol, acetone, chloroform, and hexane), and shaken at 150 rpm (LabCompanion™), 28°C for 24 h. The resulting extracts were re-dissolved in the same solvents to a final concentration of 1 mg/µl.

Phytochemical quantification

In this study, biochemical analysis was mainly employed to quantify *G, boninense* phytochemicals, including alkaloids, glycosides, phenolic compounds, flavonoids, tannins, terpenoids, and saponins. Also, carbohydrates and proteins were examined and their content determined to be primary metabolite in nature.

Experiments for phytochemical detection and quantification were conducted as follows: The magnesium and HCl reduction test, Dragendorff, Mayer, and Wagner assay, and Keller-Killani test were performed to detect a number of phytochemicals, including flavonoids/flavones, alkaloids, and glycosides, as has been described elsewhere (Archana *et al.*, 2012). To monitor phytosterols/steroids, phenolic compounds, terpenoids, and saponins, the Liebermann-Burchard test, $FeCl_3$ test, Salkowski test, and Frothing test were carried out, respectively, as previously described (Raaman, 2006; Ayoola *et al.*, 2008). In addition, the carbohydrates and proteins in each sample were calculated to predict the amount of primary metabolites in three different *G. boninense* samples, using Benedict's qualitative reagent and the biuret method, respectively (Raaman, 2006).

LLE

To determine the efficacy of solvent extraction in detail, LLE was employed using both chloroform and methanol as previously described, with modifications (Bucar *et al.*, 2013). A 14-day-cultured mycelia, GBMA, was freeze-dried for 24 h. From a chloroform : methanol ratio of 1:1, water was added to the mixture at an equal ratio, creating a 1:1:1 (chloroform : methanol : water) mixture. Approximately 100 g of lyophilized and powdered GBMA was added to 50 ml of chloroform, and then, 100 ml of methanol and water at a ratio of 1:1 was added to the mixture, after which it was sonicated at 10,000 Hz for 5 min. The mixture was kept for at least 12 h to complete the separation prior to fractionation. The yield for each fraction was calculated, and the dried form for each fraction was kept at 4 ± 2°C for further use.

In addition, after LLE was completed, the samples were divided into upper and lower fractions. The upper fraction consisted of a fraction with water and methanol extraction and was designated as WM. The lower fraction, designated as CM, consisted of a fraction with extraction agent within the chloroform and methanol combination.

Antibacterial assays using extracts of *G. boninense* obtained via LLE

The antibacterial activities of the WM and CM fractions were evaluated using disc diffusion assay in accordance with the guidelines of the Clinical & Laboratory Standards Institute (CLSI) (Olowe *et al.*, 2013). Sterilized Whatman® No. 3 paper discs (6 mm diameter) were used for the assay. Before loading 20 µl (100 µg, 5 µ/µl concentration) of the *Ganoderma* extracts, the concentration of each sample extract was set to 5 mg/ml. The lyophilized extracts of the *Ganoderma* samples, including WM and CM, were dissolved in 10% dimethyl sulfoxide (DMSO) by diluting with triple-distilled water, except for the water-extracted samples.

For the positive control experiment, 30 µl of 10% DMSO was added to the discs and supplemented with tetracycline, chloramphenicol, or ampicillin; chloroform was used as a negative control. The diameter of the zone of inhibition (mm) around the disc was measured after cultivation as described above and was compared with control experiments.

Antibacterial activity assays

A comparative study using antibacterial activity-based disc diffusion assay experiment was conducted by using bacterial culture media, including tryptic soy agar (TSA), Muller-Hinton agar, nutrient agar, and Luria-Bertani agar (LBA). All bacterial cultures were incubated at 37°C until the exponential phase of cell growth. To estimate the potent antibacterial materials, antibacterial activities were examined using conventional disc diffusion assay as proposed by the guidelines of the Clinical & Laboratory Standards Institute (CLSI) (Olowe *et al.*, 2013).

Approximately 0.1 ml of each bacterial culture was adjusted to 1×10^8 CFU/ml, before seed inoculation onto the nutrient agar plates. Sterilized Whatman® No. 3 paper with 6 mm diameter was used for the antibacterial assays. Prior to loading 50 µl (5 mg) of each *Ganoderma* extract, the corresponding content of each sample was adjusted to 100 mg/ml. All lyophilized-extracts of *G. boninense* samples were dissolved in 10% dimethyl sulfoxide (DMSO) by serially diluting with TDW, except for *G. boninense* extracts with water extraction.

For the positive control experiment, 50 µl of 10% DMSO was added to the discs with the treatment of antibiotics, including tetracycline, chloramphenicol, or ampicillin. In addition, chloroform was used as a negative control in this study. The resulting diameter of the inhibition zone (mm) was measured by the means described above and compared with the control.

Statistical analysis

Data are expressed as means ± standard deviation (SD) of at least three independent experiments. The statistical significance of the differences was preliminarily evaluated by Student's t-test in Microsoft Office Excel 2016. To evaluate the statistical significance of experimental parameters, data were mainly compared using one-way analysis of variance (ANOVA), followed by conducting post hoc tests in accordance with Tukey's, Duncan, and least significance difference (LSD) protocols. The level of significance was set at $p = 0.05$ and statistical analysis was performed using SPSS software, ver. 21 (SPSS Inc.).

Results

High extraction yields from GBFB and GBMA by polar-organic solvents

The limited experimental approaches from the phytochemical extraction strategies solely with fruiting bodies prompted us to study the significantly less performed experiments of secondary metabolites derived from *G. boninense* mycelium. To examine the antibacterial activities, the extraction yield of *G. boninense* mycelia was preferentially compared with that of fruiting bodies. The assumed antibacterial two *G. boni-*

Table 1. Extraction yield efficiency of *G. boninense* extracts (e.g., fruiting body, mycelia, and broths) using five organic solvents

Solvent[1]	Culture type[2]	Yield (%)[3]
Water	GBFB	4.41 ± 0.22[e]
	GBMA	3.89 ± 0.18[d]
	GBMB	31.21 ± 0.61[i]
Methanol	GBFB	8.61 ± 0.33[h]
	GBMA	7.74 ± 0.28[g]
	GBMB	3.11 ± 0.16[c]
Acetone	GBFB	6.73 ± 0.41[f]
	GBMA	6.26 ± 0.33[f]
	GBMB	3.87 ± 0.15[d]
Chloroform	GBFB	6.55 ± 0.45[f]
	GBMA	6.01 ± 0.25[f]
	GBMB	1.33 ± 0.11[b]
Hexane	GBFB	1.12 ± 0.10[b]
	GBMA	1.07 ± 0.14[b]
	GBMB	0.35 ± 0.03[a]

[1] Analytical-grade extraction solvents were purchased.
[2] GBFB, *G. boninense* fruiting body; GBMA, *G. boninense* mycelia cultured on PDA plates; GBMB, *G. boninense* mycelia cultured in PDB cultures. The extraction yield, designated as %/extracted matter from plant samples, are presented as mean ± standard deviation. Values denoted with the same letters are not significantly different, $p > 0.05$.
[3] Data are presented as mean ± standard error of the mean from three independent experiments.

nense mycelial cultures, including GBMA (Supplementary data Fig. S1A) and GBMB (Supplementary data Fig. S1B), were designed to compare with GBFB from fruiting bodies. A typical morphology representing the properties of *G. boninense* fruiting body was observed with brown-reddish colored and waved-margin patterns of the pileus (Supplementary data Fig. S2). The extraction yield for metabolite content obtained from mycelial culture GBMA was shown to be highly efficacious, as was that from GBFB by polar-organic methanol. Meanwhile, to a lesser degree, the total extraction yield of GBMA and GBMB was shown to be relatively lower when using absolute polar water, slightly polar-organic chloroform, and non-polar hexane (Table 1). The extraction yield with hexane was significantly lower in all tested experiments, in sharp contrast to materials with other solvent extractions. The use of polar-organic and slightly polar-organic solvents resulted in a higher extraction yield efficiency in both GBFB and GBMA, in contrast to that observed in GBMB. The result was convincing evidence for high total extraction yields by methanol extraction in GBFB and GBMA, with GBFB and GBMA content from methanol extraction being approximately 8.61 ± 0.33 and 7.74 ± 0.28% ($p < 0.05$), respectively. Despite being slightly lower than these latter figures, the extraction efficiency of GBFB and GBMA were comparable by acetone and chloroform; 6.73 ± 0.41 and 6.26 ± 0.33% for acetone, respectively; and 6.55 ± 0.45 and 6.01 ± 0.25% for chloroform, respectively. The results indicated the outstanding resolving capability of polar-organic solvents in contrast to non-polar solvents during the extraction using *G. boninense*.

High contents of phytochemicals in GBFB and GBMA

Based on the high extraction yield efficiency in GBFB and GBMA using polar-organic solvents (Table 1), phytochemi-

Table 2. Biochemical analysis for the detection of phytochemicals in *Ganoderma* extracts from fruiting bodies and mycelia

Phytochemical analysis		Solvent[1] and culture type[2]															
		Water			Methanol			Acetone			Chloroform			Hexane		
		GBFB	GBMA	GBMB	GBFB	GBMA	GBMB	GBFB	GBMA	GBMB	GBFB	GBMA	GBMB	GBFB	GBMA	GBMB
Alkaloids[3]	Dragendorff's test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Wagner's test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Mayer's test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phenolic compounds[3]	$FeCl_3$ test	+++	+++	+++	+++	+++	+++	+++	+++	+++						
Steroids[3]	Liebermann-Burchard Test	–	–	–	+++	+++	–	+++	+++	–	+++	+++	+++	+++	+++	+++
Terpenoids[3]	Salkowski test	–	–	–	–	–	–	–	–	–	+++	+++	+++	+++	+++	–
Saponins[3]	Frothing test	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–	–
Glycosides[3]	Keller-Kiliani test	–	–	+++	+++	+++	–	+++	+++	–	+++	+++	–	–	–	–
Flavonoids[3]	Magnesium-HCl reduction	–	–	–	+++	+++	–	+++	+++	+++	+++	+++	–	–	–	–
Proteins[3]	Benedict test	–	–	+++	–	–	–	–	–	–	+++	+++	–	–	–	–
Carbohydrates[3]	Biuret test	+++	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–

[1] All *G. boninense* extracts were in the crude form, and their concentration was standardized to 5 mg/ml.
[2] GBFB, *G. boninense* fruiting body; GBMA, *G. boninense* mycelia cultured on PDA plates; GBMB, *G. boninense* mycelia cultured in PDB culture.
[3] Symbols of (+) and (-) indicate positive and negative results respectively. All tests were independently conducted in triplicates as indicated by the number of (+) or (-). Intensities of colors indicate the extent of the given change in the presence of compounds.

cals were extensively observed using *G. boninense* extracts, additionally including primary metabolites (i.e., carbohydrates and proteins) (Table 2). carbohydrates were detected by extraction, solely using water and methanol, in all types of experimental sets. Protein detection was only observable in water-extracted GBMB and chloroform-extracted GBFB and GBMA. Alkaloids were not detected in all *Ganoderma* extracts, despite efforts to observe the biosynthesis of these metabolites. To a greater or lesser degree, extraction experiments commonly showed similarly high or moderate amounts of phytochemical distribution, in the case of using the same solvents in GBFB and GBMA. Except for proteins and glycosides, the metabolite distribution of GBMB was relatively moderate, low, or non-existent, compared to that of GBFB and GBMA, despite showing a high amount of glycosides in GBMB with water extraction. When using polar-organic solvents (including methanol, acetone, and chloroform), phenolic compounds were not observable in the chloroform-extracted materials. Except for primary compounds, including carbohydrates and proteins, phenols, saponins, or glycosides were solely seen in water-extracted materials. Moreover, water extraction allowed fewer kinds of metabolite extractions, in a sharp contrast to other polar-organic methanol and acetone extractions, and slightly polar-organic chloroform extraction. The results in Table 2 suggested that both significantly similar yields and metabolite distributions between GBFB and GBMA were typically revealed by all the used solvents, including methanol, acetone, and chloroform. Thus, higher extraction efficiencies were commonly obtained in GBFB and GBMA with polar-organic solvents than with water and hexane in both experiments (Tables 1 and 2).

Potent antibacterial activity in the mycelium-derived GBMA

To guarantee highly selective and efficient metabolite screening for the antibacterial experimental set-up, the antibacterial activity of each solvent-extracted sample was comparatively examined using disc diffusion assays (Table 3). Preferentially, we monitored the exponential phase of cell growth in all bacterial strains to confirm reference experiments for further antibacterial evaluation (Supplementary data Fig. S3 and Table S1). In disc diffusion assays, based on total yield efficiency and phytochemical contents through the combined culture type-dependent solvent extraction, we observed higher antibacterial activities in both GBFB and GBMA in a sharp contrast to GBMB. The corresponding antibacterial activities were typically seen through the extractions with methanol, acetone, and chloroform (Table 3). Compared with GBFB, the methanol-extracted GBMA exhibited higher and broader ranges of antibacterial activity against MRSA. Moreover, methanol- and acetone-extracted GBFB and GBMA demonstrated lower antibacterial activity than chloroform-extracted

Table 3. Antibacterial activity of extracts from three experimental cultures tested
The activity was measured using disc diffusion assays with the size of the inhibition zone (mm).

Strain[1]	Extracts obtained from GBFB[2]					Positive control[3]			Negative control[4]
	Water	Methanol[6]	Acetone	Chloroform	Hexane	Tetracycline	Chloramphenicol	Ampicillin	Chloroform
S. aureus	[5]N.D.	7.67 ± 0.58^{cd7}	7.67 ± 0.58^{cd}	10.67 ± 0.58^{h}	7.33 ± 0.58^{bc}	19.67 ± 1.53	22.67 ± 1.53	18.67 ± 1.53	N.D.
S. pyogenes	N.D.	7.67 ± 0.58^{cd}	7.67 ± 0.58^{cd}	9.33 ± 0.58^{fg}	N.D.	24.33 ± 1.53	25.33 ± 1.53	18.33 ± 1.53	N.D.
MRSA	N.D.	N.D.	N.D.	9.33 ± 0.58^{fg}	N.D.	17.00 ± 1.00	17.00 ± 1.00	7.00 ± 1.00	N.D.
P. aeruginosa	N.D.	7.33 ± 0.58^{bc}	8.00 ± 0.00^{de}	8.00 ± 0.00^{de}	N.D.	17.67 ± 1.53	17.67 ± 1.53	17.67 ± 1.53	N.D.
K. pneumoniae	N.D.	7.67 ± 0.58^{cd}	N.D.	7.67 ± 0.58^{cd}	N.D.	17.33 ± 0.58	18.33 ± 0.58	17.33 ± 0.58	N.D.
E. coli	N.D.	8.00 ± 0.00^{de}	N.D.	8.00 ± 0.00^{de}	N.D.	19.33 ± 1.53	18.67 ± 1.53	19.33 ± 1.53	N.D.
Strain[1]	Extracts obtained from GBMA[2]					Positive control[3]			Negative control[4]
	Water	Methanol	Acetone	Chloroform	Hexane	Tetracycline	Chloramphenicol	Ampicillin	Chloroform
S. aureus	N.D.	8.00 ± 0.00^{de}	7.67 ± 0.58^{cd}	11.33 ± 0.58^{i}	N.D.	17.67 ± 0.58	22.67 ± 1.53	18.00 ± 0.05	N.D.
S. pyogenes	N.D.	7.33 ± 0.58^{bc}	8.00 ± 0.00^{de}	9.67 ± 0.58^{gh}	N.D.	23.67 ± 1.53	24.33 ± 1.53	19.67 ± 1.53	N.D.
MRSA	N.D.	7.33 ± 0.58^{bc}	N.D.	9.33 ± 0.58^{fg}	N.D.	17.00 ± 0.58	18.00 ± 1.00		N.D.
P. aeruginosa	N.D.	7.33 ± 0.58^{bc}	7.33 ± 0.58^{bc}	8.00 ± 0.00^{ab}	N.D.	17.67 ± 1.53	18.67 ± 1.53	16.67 ± 1.53	N.D.
K. pneumoniae	N.D.	7.67 ± 0.58^{cd}	N.D.	7.33 ± 0.58^{bc}	N.D.	18.33 ± 1.53	18.67 ± 0.58	17.33 ± 0.58	N.D.
E. coli	N.D.	8.00 ± 0.00^{de}	8.00 ± 0.00^{de}	8.00 ± 0.00^{ab}	N.D.	18.33 ± 1.53	18.67 ± 1.53	19.67 ± 1.53	N.D.
Strain[1]	Extracts obtained from GBMB[2]					Positive control[3]			Negative control[4]
	Water	Methanol	Acetone	Chloroform	Hexane	Tetracycline	Chloramphenicol	Ampicillin	Chloroform
S. aureus	N.D.	N.D.	N.D.	N.D.	N.D.	17.33 ± 0.58	22.33 ± 1.53	19.33 ± 1.53	N.D.
S. pyogenes	N.D.	7.00 ± 0.00^{ab}	7.33 ± 0.58^{bc}	7.33 ± 0.58^{bc}	N.D.	23.67 ± 1.53	22.33 ± 1.53	19.33 ± 1.53	N.D.
MRSA	N.D.	N.D.	N.D.	N.D.	N.D.	16.88 ± 1.00	17.33 ± 0.58	7.33 ± 1.00	N.D.
P. aeruginosa	N.D.	N.D.	7.00 ± 0.00^{ab}	7.33 ± 0.58^{bc}	N.D.	17.67 ± 1.53	17.67 ± 1.53	17.67 ± 1.53	N.D.
K. pneumoniae	N.D.	N.D.	N.D.	N.D.	N.D.	17.67 ± 1.53	18.67 ± 1.53	16.33 ± 0.58	N.D.
E. coli	N.D.	7.33 ± 0.58^{bc}	N.D.	7.33 ± 0.58^{bc}	N.D.	19.33 ± 1.53	18.33 ± 0.58	18.33 ± 1.53	N.D.

[1] Bacterial strains: *S. aureus* ATCC 25923; *S. pyogenes* ATCC 19615; MRSA NCTC 11939; *P. aeruginosa* ATCC 9027; *K. pneumoniae* ATCC 1705; *E. coli* ATCC 35218.
[2] All extracts are in crude form; concentrations and the extracts amount loaded into the disc were standardized to 5 mg.
[3] Standard antibiotics were used as positive controls. Total amount of antibiotics loaded for each assay was 30 μg with a final concentration of 1 mg/ml.
[4] Chloroform was used as a negative control.
[5] N.D. means that no antibacterial activity was detected. No inhibition was observed on negative control disc containing 10% dimethyl sulfoxide. Values denoted with the same letters are not significantly different, $p > 0.05$. Unless otherwise stated, all data shown represent triplicate mean ± standard deviation values.
[6] The values represent the average of three independent experiments ± standard errors expressed as inhibitory zone diamters (mm) in three types of *Ganoderma* extracts.
[7] For each sample, mean values with different superscript letters across each row were significantly different at $p < 0.05$ according to HSD Tukey's post hoc test.

GBMA. GBMB did not exhibit any antibacterial activity against *S. aureus*, MRSA, and *K. pneumoniae*. The antibacterial activity of chloroform-extracted GBMA was observed with inhibition zones of 11.33 ± 0.58, 9.67 ± 0.58, and 9.33 ± 0.58 mm, against *S. aureus* ATCC 25923, *S. pyogenes* ATCC 19615, and MRSA NCTC11939, respectively. Chloroform-extracted GBFB elicited slightly lower antibacterial activities, approximately 10.67 ± 0.58, 9.33 ± 0.58, and 9.33 ± 0.58 mm inhibition zone, against *S. aureus* ATCC 25923, *S. pyogenes* ATCC 19615, and MRSA NCTC11939, respectively. These results demonstrated that the chloroform-extracted GBMA displayed a potent and broad spectrum of antibacterial activity among the tested samples. Hexane-extracted GBFB was excluded as one of aims of this study was to address the most potent *G. boninense* culture conditions combined with the most effective solvent selection for the efficient isolation of antibacterial metabolites.

Antibacterial experimental model using chloroform-extracted GBMA

To ensure convincing evidence for antibacterial compounds, positive controls under the same experimental conditions with solvent-extracted cultures were tested using three standard antibiotics (tetracycline, chloramphenicol, and ampicillin), utilizing agar-based methods described elsewhere (Saïdana *et al.*, 2008; Jenkins and Schuetz, 2012). To control the sensitivity of the tested bacterial strains, the inhibition rate was defined as being resistant or susceptible to the previously evaluated individual antibiotics (Qiao *et al.*, 2017). A negative control, involving the presence of microorganisms without test material, was used. When chloroform was also used as a negative control, no inhibitory effect was detected in any experimental trials by disc diffusion assay. In these experiments, chloramphenicol was observed to be the most significantly effective against all tested bacteria (Table 3). Although all bacterial strains were susceptible to tetracycline, the inhibition zone against *S. aureus* was the higher for chloramphenicol treatments compared to those of tetracycline. The bacterial pathogens were susceptible to the other antibiotics, with the exception of MRSA, which was resistant against ampicillin, ranging from 7.00 ± 1.00 to 7.33 ± 0.58 ($p < 0.05$). Our results suggested that chloramphenicol was the best positive control for the comparison of antibacterial activities with chloroform-extracted GBMA. In addition, we assessed antibacterial activity under different culture media (i.e., TSA, Muller-Hinton agar, nutrient agar, and LBA), both with antibiotics and *G. boninense* extracts. A comparative experiment using the confirmed chloroform-extracted GBMA resulted in higher antibacterial activity on nutrient agar media with both the positive control and chloroform-extracted GBMA (Fig. 1). The positive control employed in the antibacterial assay was inconclusive, as were the other three media that were not significantly different. Although the effect of chloramphenicol treatments on the other three media was not significantly different, the antibacterial activity with nutrient agar in chloroform-extracted GBMA was remarkably higher, and this activity pattern was also observed with at least one other medium,

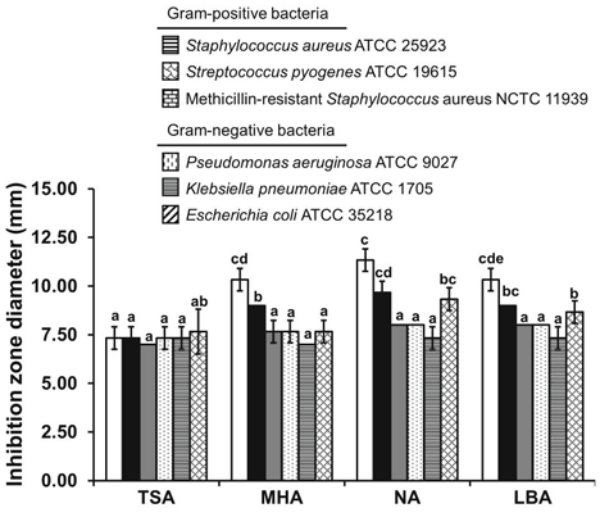

Fig. 1. The bacterial growth-inhibitory effects of the chloroform-based *Ganoderma* extracts in different media (mm). Inhibitory effect against the growth of bacteria by the chloroform-based extraction in different media as measured by inhibition zone diameter (mm). All experiments were performed in triplicate and were repeated at least three times independently. The bars denoted with the same letters are not significantly different ($p > 0.05$). Representative experiments or means ± standard deviations are shown. Error bars represent standard deviation from the mean of triplicates.

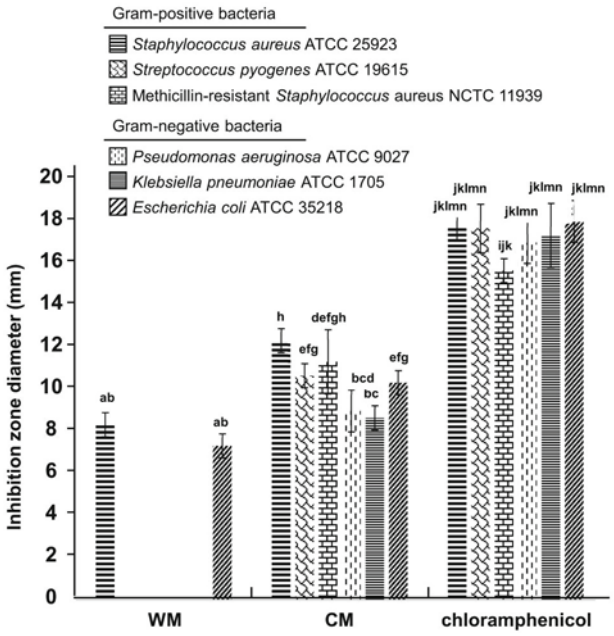

Fig. 2. Antibacterial activity of two fractions, including water-methanol (WM) and chloroform-methanol (CM), of *G. boninense* extract. The bars denoted with the same letters are not significantly different ($p > 0.05$). Representative experiments or means ± standard deviation are shown. Error bars represent standard deviation from the mean of triplicates. Data are expressed as means ± standard deviation (SD) of at least three independent experiments. For each extract, mean values were significantly different at $P < 0.05$ according to HSD Tukey's post hoc test. TSA, tryptic soy agar; MHA, Muller-Hinton agar; NA, nutrient agar; LBA, Luria-Bertani agar. Bacterial strains were as follows: *S. aureus* ATCC 25923, *S. pyogenes* ATCC 19615, MRSA NCTC 11939, *P. aeruginosa* ATCC 9027, *K. pneumoniae* ATCC 1705, *E. coli* ATCC 35218. SA, *S. aureus*; SP, *S. pyogenes*; MRSA, methicillin-resistant *S. aureus*; PA= *P. aeruginosa*; KP, *K. pneumoniae*; EC, *E. coli*.

except for tryptic soy agar. The antibacterial activities against *S. aureus* ATCC 25923 and *S. pyogenes* ATCC 19615 were also significantly higher in Muller-Hinton agar and Luria-Bertani agar media compared with that of other bacterial strains with chloroform-extracted GBMA. A slightly higher antibacterial activity was similarly seen against *E. coli* ATCC 35218 in nutrient agar and Luria-Bertani agar media compared with that in tryptic soy agar. Our data suggested nutrient agar media was ideal as a highly selective culture medium for subsequent investigations by enhancing the inhibitory effects of solvent-extracted *G. boninense* cultures against antibiotics.

Efficient extraction yield using LLE and the corresponding antibacterial activity

Another LLE for detection of antibacterial compounds was performed on *G. boninense* to enhance the extraction efficiency of the antibacterial compounds. Two fractions were obtained, and the upper and lower fractions were respectively designated as WM and CM, which consisted of water-methanol and chloroform-methanol fractions. A relatively higher yield was observed in WM (10.75 ± 1.33 g) as compared to CM (8.52 ± 0.8 g). The data suggested that the secondary metabolite profile composition is a key factor for determining extraction yields when using a specific solvent system. Higher quantities of polar compounds such as carbohydrate moieties and acidic/basic derivatives (e.g., carboxylic acid, short chain fatty acids, and proteins) have been suggested to contribute to greater yields in more polar solvents (Seyd *et al.*, 2012). Thus, the slightly higher yield of WM in the LLE extract indicated a composition made up of polar compounds.

Despite the lower yield, the antibacterial activity of CM was significantly higher than that of WM (Fig. 2). CM was seen to exert antibacterial activity against all tested bacterial samples, in contrast to WM. The combination of water and methanol exerted antibacterial activity solely against *S. aureus* and *E. coli*. The highest inhibition was shown in the CM against *S. aureus* ATCC 25923 (12.33 ± 0.58 mm), followed by MRSA NCTC 11939 (11.33 ± 1.53 mm), and *S. pyogenes* ATCC 19615 (10.67 ± 0.58 mm). A relatively lower antibacterial activity with inhibition zones was particularly exhibited in Gram-negative bacteria, with the lowest observed inhibitory effect in *K. pneumonia* ATCC 1705 (8.67 ± 0.58 mm) and *P. aeruginosa* ATCC 9027 (9.00 ± 1.00 mm). The observed antibacterial activity in WM and CM indicated that CM seemed to contain more potent antibacterial compounds.

Discussion

There are two pivotal reasons *G. boninense* mycelia, and mycelia from similar pathogenic species, need more in depth exploration. First, when reviewing related research, there is a distinct lack of works concerning the antibacterial roles of these mycelia and their bioactive compounds. Specifically, while secondary metabolite compounds in non-pathogenic *Ganoderma* species have been examined (Baby *et al.*, 2015), *G. boninense* mycelia metabolites, and metabolites in other species of pathogenic *Ganoderma*, have not been extensively studied. Also, the prevalent majority of metabolite screening strategies for phytochemical and other compound analysis have utilized fruiting bodies in *Ganoderma* species (Baby *et al.*, 2015; Alexander *et al.*, 2017; Abdullah *et al.*, 2018a). This focus on fruiting body-dependent experimental trials has meant that secondary metabolites derived from *G. boninense* mycelia are significantly less researched than those derived from fruiting bodies. In consideration of the need for experimental evidence supporting plant material extraction protocols suitable for employment with a wider scope of materials, this study has extended the commonly performed experimental methodologies previously used with fruiting bodies to the more rarely used and less studied mycelia. Using this methodology we have evaluated *G. boninense* mycelial cultures for potentially potent antibacterial secondary metabolites.

The second reason for a warranted exploration of *G. boninense* mycelia is that lanostane-type triterpenoids have until now been the only known types of compounds seen in this pathogen. Compounds within a homologous series commonly exhibit a fixed set of functional groups, and display similar chemical and physical properties. The isolation of these compounds sometimes employs extraction methods using organic and/or non-organic solvents singularly, but meanwhile utilizing quite similar experimental techniques. Tendencies for using homologous compounds with fruiting bodies and similar experimental protocols have been observed with other *Ganoderma* species (Baby *et al.*, 2015).

This lack of more varied research also extends to species selected in the literature. For example, in one study 34 types of secondary metabolites were noted among 240 kinds of lanostane-type triterpenoids in *G. lucidum* mycelia (Xia *et al.*, 2014). In the latter study, interestingly, 431 compounds belonging to the lanostane homologous series were isolated from a mere 23 *Ganoderma* species, where over 300 known species of this genus have been characterized, with most (91%) of these compounds being identified solely from the 5 species of *G. lucidum*, *G. applanatum*, *G. sinense*, *G. amboinense*, and *G. colossum* (Baby *et al.*, 2015; Sharma *et al.*, 2019). Moreover, of more than 316 triterpenes which were isolated from the fruiting bodies, spores, gills, and mycelia; 240 kinds of lanostane-type triterpenoids were isolated from *G. lucidum* alone, including C30 ganoderic acids, C30 lanostanes, C27 lucidenic acids, C27 lanostanes, C24 lanostanes, and meroterpenoid (Boh *et al.*, 2007; Li *et al.*, 2013; Baby *et al.*, 2015; Matos Lopes *et al.*, 2015; Zhou *et al.*, 2015; Sharma *et al.*, 2019). The limited number of studies concerning the mycelia of the *Ganoderma* species suggest that extensive medicinal and/or pharmacotherapeutic uses of *G. boninense* and its extracts may exist that have simply yet to be revealed. Hence, in this study, methodologies were extended to mycelia, so as to re-evaluate whether mycelium-derived cultures provide a useful means of more efficiently selecting for antibacterial bioactivities.

When examining the broader picture, yeasts, phycomycetes, and slime molds are seen to be weak producers of bioactive secondary metabolites (Abdel-Razek *et al.*, 2020). Therefore, this study considered previously elucidated *Ganoderma* spp. compounds (Boh *et al.*, 2007; Ng *et al.*, 2013; Baby *et al.*, 2015; Abdullah *et al.*, 2018b; Sim *et al.*, 2019); where the secondary metabolites are hypothesized to exhibit a variety of biologi-

cally and physiologically active substances with structural and functional diversity (Mohammed *et al.*, 2014). To verify this concept, three sets of *G. boninense* cultures, namely GBFB, GBMA (Supplementary data Fig. S1A), and GBMB (Supplementary data Fig. S1B), were preferentially prepared and solvent-extracted to assess whether mycelium-derived cultures may indeed provide us with a useful method for efficiently selecting for antibacterial bioactivities. Among three culture type-dependent *G. boninense*, including fruiting bodies and mycelia cultured on potato dextrose agar (PDA) plates and potato dextrose broth (PDB), respectively, the GBMA, the mycelium cultured on PDA, has been seen to exhibit the most significant antibacterial activity in this study. Based on this finding, a combination of chloroform and methanol produced appropriate and ideal solubility strength in extracting the possible antibacterial compounds from *G. boninense* (Fig. 2). In contrast to WM, due to its nature, CM was noticeably a more non-polar organic solvent, suggesting that the antibacterial compounds may either be non-polar or slightly polar in nature. This hypothesis was supported by some non-polar metabolites exhibiting meaningful antimicrobial properties (Daouda *et al.*, 2014; Zengin and Baysal, 2014), even though these non-polar metabolites are less abundant in nature (Gao *et al.*, 2015).

An example of a typical fruiting body morphology with brown-reddish colored-/waved-margin patterns of the pileus, representing the morphological properties of *G. boninense* is presented in Supplementary data Fig. S2 for reference. Table 1 illustrates the highly-yielded secondary metabolites of GBFB and GBMA by extraction with organic-polar methanol, and their slightly lower yield with acetone and chloroform. The solvent extraction efficiency reflects the metabolite content of GBFB and GBMA cultures extracted by polar-organic (i.e., methanol and acetone) and slightly polar-organic (i.e., chloroform) solvents. The results suggest a higher extraction yield for both polar-organic and slightly polar-organic solvents than that of the more absolute polar (e.g., water) and non-polar solvents (e.g., hexane). The resulting yield from hexane-extracted materials was seen to be considerably lower than samples for the extraction of other solvents. We also noticed a higher yield efficiency of polar-organic and slightly polar-organic solvents in both GBFB and GBMA compared with GBMB. Despite the similar or slightly lower extraction yields for both acetone- and chloroform-extracted GBFB and GBMA, this tendency was evident in the yield data obtained from both methanol-extracted GBFB and GBMA. Our findings suggest an outstanding resolving capacity for polar-organic solvents, as compared with non-polar solvents in *G. boninense*. This result aligns well with our previous works which indicated that the high yield for secondary metabolite extraction in GBFB and GBMA presumably owes much to their solvent-supported structures derived from static structures altered by the stimulation of solvent-specific interactions (Dwyer *et al.*, 2017). The data also indicated that the molecular strength of non-polar solvents for resolving polar compounds is extremely low and/or even absent, with polar solvents being more proper for resolving polar metabolites (Otsuka, 2006). This explains why GBMB with water extraction yields a high content of metabolites, and is thus the most appropriate experimental material for water-soluble metabolites. The effect of each complex concentration on the resolving power of the solution in polar and non-polar solvents is illustrated in Table 2. Primary metabolite distribution with identical solvents in GBFB and GBMA allowed polar or slightly polar substance releases that could cause selective proteinaeous and/or glycosidic metabolites' detection in type-dependent cultures. Experiments using water and methanol ensure the presence of the primary building blocks of physiological metabolism necessary for mycelial growth and fruiting body formation (Wang *et al.*, 2017; Abdullah *et al.*, 2018b). Our findings can be supported by previous works regarding the intermolecular hydrophobic interactions between water and non-polar compounds, which decrease in soluble secondary metabolites (Jones and Kinghorn, 2006). This indicates that specific solvent-extraction from *Ganoderma* mycelia requires a highly selective experimental model for obtaining antibacterial metabolites. Supporting evidence for extraction efficiency using various solvents suggest metabolite distribution patterns involving phenolics, saponins, and glycosides, in *G. lucidum* or *G. boninense*; molecules commonly detected in any extract when using water, methanol, and/or acetone (Baby *et al.*, 2015; Cör *et al.*, 2017; Abdullah *et al.*, 2018b). However, we speculated as to why terpenoids and steroids were not detected in organic solvents such as water-, methanol-, and/or acetone-extracted samples. Also, although the exact mechanism is not known, chloroform- and hexane-based extraction revealed large amounts of steroids and terpenoids, as compared to extraction with strong polar and/or hydrophilic solvents. The typical yield pattern regarding phytochemical extracts from both chloroform and hexane also seemed to correspond with a higher yield from the GBFB and GBMA than in GBMB when using polar solvents (i.e., water, methanol, and acetone). Our data shows that the resolving power of non-polar hydrocarbon compounds (e.g., lipids, fats, and waxes) is lower than that of non-polar-organic solvents. However, the resolving capability of such compounds may be considerably stronger than that of water due to the interchangeable dipole moment between solvent molecules that allows polar-organic solvents to adopt a partial non-polar state (Jones and Kinghorn, 2006). Interestingly, chloroform-extracted GBMA, in the highly selective antibacterial mixture, was revealed to be an antibacterial fraction from the *G. boninense* mycelium. Table 3 illustrates how the chloroform-extracted GBMA commonly exhibited slightly higher antibacterial activities than GBFB. However, this result was not consistent with that of total extraction yields and metabolite content in GBFB and GBMA (Tables 1 and 2). Our results suggested that phytochemical or secondary metabolites do not always reflect antibacterial activities in *G. boninense*. A combination between less-studied mycelia and chloroform extraction could help rectify the paucity of literature regarding secondary metabolites in mycelium extracts.

The distribution and/or localization of antibacterial secondary metabolites derived from *G. boninense* are thought to result from the role of endogenously or exogenously produced metabolites in intra- and intercellular communication and/or activity via complex biological events (Jensen and Keasling, 2015). As a representative example, Ascomycetes *Penicillium* sp. produce penicillin antibiotics via biosynthesis

triggering a series of physiological changes that exogenously employ the counter-defensive arsenal of the fungi (Okada and Seyedsayamdost, 2017). Meanwhile, *G. boninense* utilizes defense mechanisms endogenously by non-competitive fungal growth dependent on wood-decaying mechanisms to maintain cellular physiology and homeostasis (Halbwachs *et al.*, 2016; Ulyshen, 2016). This hypothesis is supported by our data regarding extracts from GBFB and GBMA that can select endogenous metabolites owing to their natural physicochemical properties in each solvent extraction, while solvent extraction of GBMB might differently fractionate exogenous metabolites. The characteristics of extracts from *Ganoderma* fruiting bodies is thought to reflect their bioactive antisaprophytic, anti-phytopathogenic, anti-phytofungal, anti-phytobacterial, anti-phytoviral, mosquito larvicidal, and nematicidal activities (Radhajeyalakshmi *et al.*, 2012; Mukherjee, 2015; Sivanandhan *et al.*, 2017). For this reason, the antibacterial activities of chloroform-extracted GBMA were the most appropriate model for evaluating potent antibacterial agents, along with use of GBFB for chloroform-extracted fruiting bodies, due to the endogenous defense systems of *G. boninense*. Based on these findings, for extraction yield efficiency, a quantitative relationship between metabolite amounts is dependent on the interaction between culture media and solvents.

However, experimental evidence for the pharmaceutical use of *G. boninense* and/or its extracts are elusive. The pharmacological activities of the bioactive metabolites of *G. boninense* have been highlighted with a focus on the urgent need for new antibiotics. In addition to *G. lucidum,* other *Ganoderma* species, including *G. tsugae*, *G. applanatum*, and *G. multipileum,* have been investigated for their medicinal potential. To develop a strategy for the pharmaceutical use of *G. boninense* and its extracts, we suggest a combination method of dereplication and classical/hyphenated (on-line) approaches via *in silico* and qualitative structure-activity relationship (QSAR) for the prediction of the drug mode of action. The dereplication methods, including bioassay, separation science, spectroscopic methods, and database searching, are regarded as chemical or biological screening processes. *In silico* methods, such as QSAR, are helpful in deciding and simulating each aspect of drug discovery and development.

Conclusion

Despite its notorious reputation, *G. boninense* and its medicinal properties are yet to be sufficiently examined, with most studies having focused on its pathogenicity in oil palms, rather than exploring its potential in antimicrobial applications. *G. boninense* provided an excellent experimental model by both emphasizing attention on culture type-dependent metabolite purification and the development of analytical methods. These are required for the metabolite profiling of unknown metabolites, by controlling delicate culturing conditions and producing bioactive constituents for downstream applications. This study also emphasizes the significance of potent bioactive compounds with antibacterial activity by comparative measurements. This is important for establishing *G. boninense in vitro* data. We optimized the culture procedures, including culture sources, culture media, and solvent extraction strategies. Solvents with a high polarity range, including methanol, acetone, and chloroform, exhibited a higher yield in both GBFB and GBMA. Water-based extraction was the only method to give a relatively high yield in GBMB. Chloroform-extracted GBFB and GBMA exhibited significantly higher antibacterial activity in all six bacterial strains, than other extracts from cultured materials. Therefore, GBMA was the most potent source of antibacterials and/or phytochemicals due to its relatively higher phytochemical and secondary metabolite distribution and broader antibacterial activities, as compared with GBFB. To link between the experimental design and future studies, it should be noted that *G. boninense* produced phytochemicals endogenously, rather than exogenously releasing secondary metabolites. Additionally, chloramphenicol was determined to be the most effective positive control antibiotic. Based on our findings, we concluded that mycelium-derived GBMA exerts stronger antibacterial activities than GBMB.

Acknowledgments

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Conflict of Interest

The authors declare that there are no competing interests associated with the manuscript.

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