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RESEARCH ARTICLE OPEN ACCESS

Antimicrobial Activity and Metabolite Analysis of *Ganoderma boninense* Fruiting Body

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Abstract

***Ganoderma*, a genus of polypore white rot fungus with some species such as *G. lucidum* and *G. sinense* have been reported with remarkable biological activities and used as traditional medicine for long. On the other hand, *G. boninense* is recognized as an important oil palm pathogen which causes intensive losses in oil palm industry. Hence, this study reveals the potential of antimicrobial properties of *G. boninense* fruiting bodies extract using different solvents. Ethyl acetate extract demonstrated a broad spectrum pathogens inhibition activity, followed by hot water > acetone > methanol > ethanol > chloroform. Ethyl acetate extract also shown the strongest growth inhibition against *Proteus mirabilis* (14.20 ± 0.40 mm). Lowest MIC (0.625 mg/ml) was observed in methanol extract against *Coagulase-Negative Staphylococci*. LC-MS analysis identified the extracts of *G. boninense* putatively contains natural occurring derivatives of alkaloid, fatty acid, heterocyclic compounds and glycosides. *G. boninense* may potential as the future drug. However, further investigation is required to understand this fungus in depth.**

Keywords: *Ganoderma boninense*, antimicrobial, natural compounds, pathogenic bacteria

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INTRODUCTION

New drug discovery has become a hot research area due to the emergence of antibiotic resistance bacteria. Presently, some clinical isolates such as Methicillin Resistant *Staphylococcus aureus* (MRSA), Penicillin Resistant *Streptococcus pneumoniae* (PRSP), Vancomycin Resistant *Enterococci* (VRE) which have become resistant to certain commonly used antibiotics are easily found in the environment[1,2]. The report of Global Antimicrobial Resistance Surveillance System (GLASS) rises the alarm of the seriousness in worldwide antibiotic resistance's issue with more than 500,000 isolates showing non-susceptible to one or more of the conventional antibiotics[3]. According to National Surveillance of Antibiotic Resistance (NSAR) report, most of the notorious antibiotic resistant bacterial has risen since 2008 to 2016 in Malaysia[4].

Hence, discovery and development of new alternative drugs to combat with the multidrug resistant bacteria remain as a challenge. Naturally occurring molecules derived from secondary metabolites formed by living organisms that are chemically and structurally diverse could further contribute knowledge and strategy to overcome such issue[5]. Secondary metabolites are synthesized as biochemical compounds in defense against stressful environments and invasion of predators for self-healing and rejuvenating to assist survival[6]. Currently, extraction of the natural compounds with antimicrobial properties such as saponins, alkaloids, sesquiterpenes, terpenoids, glycopeptide and others were used as prescription drug in numerous developed countries[7,8].

Macro fungi, a group of wood-decaying fungi of the Phylum Basidomycetes have been exploited as new alternative in development of novel antibiotics due to the bioactive compounds which shown strong antimicrobial activity. In particular, *Ganoderma* species such as *lucidum*, showed good inhibition against different bacterial strains[9]. *Ganoderma* triterpenes (GTs) and polysaccharides (PS) derivatives are reported as the most common antimicrobial compounds from *Ganoderma* sp.[10].

G. boninense is a polypore fungus in the family of Ganodermataceae. This species belongs to *Ganoderma* genus, which are well-known as medicinal mushrooms. On the other hand, *G. boninense* is a pathogen of basal stem rot (BSR) disease, a devastating disease of oil palm trees[11]. In the current work, different solvents were used to extract the active compounds from fruiting bodies of *G. boninense* to investigate the potential of antimicrobial properties.

MATERIALS AND METHODS

Fungal Material

Forty-two fruiting bodies of *Ganoderma* were collected from infected oil palm trees in Sawit Kinabalu Oil Palm Plantation at Langkon, Sabah Malaysia. Fresh fruiting bodies were cleaned under running tap water and freeze dried using freeze dryer (ScanVac CoolSafe 110-4, Denmark). Dried fruiting bodies were stored at -80°C for further use.

Molecular Identification of *G. boninense Fruiting Bodies*

DNA extraction

Approximately, 100 mg sliced tissue of *G. boninense* fruiting bodies were powdered and homogenized with liquid nitrogen using a mortar and pestle. After that, the DNA of fruiting bodies were extracted using DNeasy plant mini kit (Qiagen, Germany) with slight modifications as described by Chong *et al*.[12].

PCR amplification

Amplification of the fungal DNA was done on the 5.8S gene using ITS1: 5'- TCC GTA GGT GAA CCT GCG G-3' as forward primer and ITS4: 5'- TCC GCT TAT TGA TAT GC -3' as reverse primer. The PCR reagents (Vivantis, Malaysia) were prepared in concentration of 1 x PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM forward and reverse primers, one unit of *Taq polymerase* and 1 μl of DNA template. The thermalcycler (GeneAtlas, Astec, Japan) was programmed for initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 40 s and extension at 72°C for 50 s. The final extension was set at 72 °C for 10 min. The PCR product was detected by agarose gel electrophoresis with 1.5% TAE agarose gel, 70 V for 40 min.

DNA sequencing and analysis

The DNA sequencing was done by Apical Scientific Sdn Bhd at Selangor, Malaysia. The obtained sequence was trimmed and later BLAST searched for the closest matches in NCBI GenBank. Phylogenetic tree was constructed using Maximum

Likelihood method with 1000 bootstraps replicate to show the relationship among sequences.

Preparation of *G. boninense* Extracts

Identified fruiting bodies of *G. boninense* were grounded using mortar and pestle with the aid of liquid nitrogen. The mortar and pestle were autoclaved, cooled and swabbed with 70% ethanol prior used. The extraction of ground powder was prepared by using maceration method. Briefly, 10 g of fine powders were soaked into 400 ml of different absolute solvents (chloroform, acetone, methanol, ethanol, ethyl acetate) at room temperature (±26°C) and shaken with 150 rpm for five days. Meanwhile, hot water extraction was prepared using water bath incubator at 90°C and 100 rpm. The supernatant was filtered through Whatman no.1 filter paper. The filtrates were then evaporated under reduced pressure using rotary evaporator (Buchi-Rotavapor R-300, Germany). Crude extracts were kept in -20°C for further use.

Microbial Culture and Inoculums Preparation

The clinical isolates were obtained from the stock culture of Genetic Laboratory, Faculty of Science and Natural Resources, Universiti Malaysia Sabah. The tested bacterial strains include eight Gram-negative bacteria (*Escherichia coli, Enterobacter* sp, *Pseudomonas aeruginosa, Proteus mirabilis, Salmonella* sp, *Acinetobacter* sp, *Klebsiella pneumoniae,* and *Serratia marcescens*) and four Gram-positive bacteria (*Streptococcus pyogenes, S. faecalis, Coagulase negative Staphylococci,* and Methicillin Resistant *Staphylococcus aureus*). All the bacteria were revived from glycerol stock by sub-cultured on nutrient agar (NA) and incubated overnight at 37°C. For inoculum preparation, a loopful of culture was taken from a single colony and further grown in nutrient broth (NB). The bacterial suspensions were standardized following the CLSI guidelines by adjusting the turbidity equivalent to 0.5 McFarland standard or approximately 1-2 x 10^8 CFU/ml[13].

Antimicrobial Assay of *G. boninense* Fruiting Bodies Extracts

The disc diffusion method was performed to test the antimicrobial activity of each solvent extracts[14]. Firstly, 100 µl of bacterial inoculums containing 1-2 x 10^8 CFU/ml were spread over the Mueller Hinton agar (MHA) plate by sterile cotton swab. Extracts were re-dissolved in DMSO and loaded over sterile filter paper discs (6 mm in diameter) to obtain final concentration of 20mg/disc. Then, discs were placed on the top of inoculated MHA. Two control discs were used containing Chloramphenical (30 µg) and DMSO as positive and negative control respectively. The diameters of growth inhibition zones were measured after incubated for 24 h at 37°C. The experiments were performed in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

The microdilution method was used to determine the minimum inhibitory concentration (MIC) according to CLSI guidelines[13]. MICs were evaluated using 96 well microtiter plates. Stock solutions of the extracts were diluted in a serial of two-fold dilution, resulting in concentration ranging from 0.625-20 mg ml^{-1}. The final concentration of bacterial suspension was adjusted to 5 x 10^5 CFU/ml with 50 µl of bacterial inoculum supplemented 100 µl of NB in each well. The microtiter plate was included a set of 3 controls: a column with Chloramphenical (30 µg) as positive control; a column with all solutions except the extracts; and a column of solution with DMSO as negative control. The plates were incubated at 37°C for 24 h. The absorbance was determined at 590 nm. All tests were performed in triplicate.

Metabolites Analysis of the *G. boninense* Crude Extracts by LC-QTOF-MS

Chromatographic separation was performed using Vanquish™ Horizon UHPLC system (Thermo Fisher Scientific, USA) coupled with electrospray ionization Impact II QTOF-mass spectrometry system (Bruker Daltonics, Germany). Crude extracts (20 µL) was injected into Kinetex F5 LC column (2.1 mm x 100 mm, 2.6 µm; USA) and maintained at 30°C with flow rate of 1.0 ml min^{-1} with mobile phase consisted of acetonitrile (A) and 0.2% formic acid in water (B). Detection wavelength was set at 254 nm for acquiring chromatograms. The gradient elution program was used as follows: 0 min (20% A); 8 min (29% A); 25 min (29% A); 55 min (30% A); 65 min (30% A); 75 min (31% A); 90 min (65% A); 110 min (90% A); 135 min (90% A)[15].

The ESI source used was negative ionization mode and deployed at 4200 V. The mass data acquisition was set to nebulizer flow

at 5.0 bar, gas temperature of 300°C, drying gas flow at 12 L min^{-1}, scan range of *m/z* 50-1500. The acquired data were converted into netCDFdata format (*.cdf) using Bruker Compass DataAnalysis 4.1.

Data Preprocessing and Analysis

The mass data in netCDF files were processed using an open source software tool MZmine 2.0. Typically, this software allowed raw data processing by removes of noise background. Then, peak detection was done by centroid algorithm to detect all specified data points as m/z peaks into chromatograms. After that, local minimum search was chosen for deconvolution. Prior alignment and peaks were grouped by m/z and retention time by isotope grouper. The m/z value and retention time of the fragmented ions were utilized in comparing with those from compound database, namely PubChem.

RESULTS AND DISCUSSION

Identification of the Collected Fruiting Bodies

DNA extracted from the fruiting bodies were amplified using ITS1 and ITS4 primers which generates approximately 650 bp of the 5.8S rDNA gene as shown in Fig. 1. The primer pair was intended to detect the intraspecies variability in the highly reserved internal transcribed spacers (ITS) regions of fungi and basidiomycetes[16]. PCR produced similar size of DNA copies, nevertheless the order of nucleotide was not determined.

Sequences obtained were subjected to BLAST search for the similarity of nucleotide

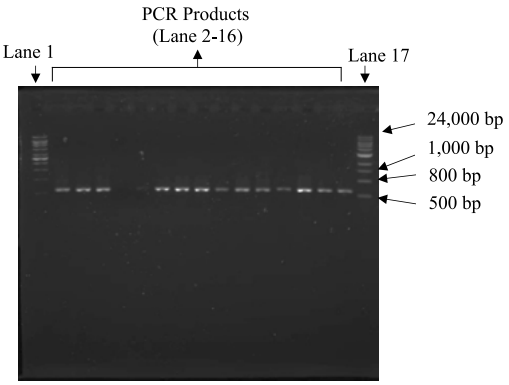

Fig. 1. Amplification of *Ganoderma* DNA isolates which produced approximately 650 bp size of PCR product using primers ITS1 and ITS4. Lane 1 and 17: 1kb DNA ladder, lane 2-16: PCR products.

Table 1. BLAST search results of fruiting bodies collected from Sawit Kinabalu Oil Palm Plantation shown 99% sequence identity to *Ganoderma sp.* BRIUMSa with highest score following by *Ganoderma sp.* BRIUMSb and *Ganoderma boninense* strain GBLS

Accession	Descriptions	Max score	Total score	Query cover (%)	E-value	Percent identity(%)
JN234427.1	Ganoderma sp. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1134	1134	97	0	99
JN234428.1	Ganoderma sp. BRIUMSb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1129	1129	97	0	99
KF164430.1	Ganoderma boninense strain GBLS internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1118	1118	97	0	99
JN400511.1	Ganoderma sp. BL-94 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1112	1112	97	0	99
JN400510.1	Ganoderma sp. BL-9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1112	1112	97	0	99

sequences in NCBI database. Phylogenetic tree was constructed to study the evolutionary relationship within taxa. Table 1 showed the possible identity of *Ganoderma* fruiting bodies collected from field. BLAST results demonstrated the fruiting bodies possess 99% sequence similarity with *Ganoderma* species. The inserted nucleotide sequence showed closest homology with *Ganoderma* sp. BRIUMSa with highest maximum score followed by *Ganoderma* sp. BRIUMSb and *Ganoderma boninense* strain GBLS. Therefore, the closest species suggested by NCBI database matches list was *G. boninense*. *Ganoderma* sp. BRIUMSa, BRIUMSb, and BRIUMSc were belongs to the genus of *Ganoderma* and deposited into NCBI after isolated from Sabah, East Malaysia[17] meanwhile *Ganoderma boninense* strain GBLS was isolated from University of Nottingham Malaysia campus, located at West Malaysia. Kok and colleagues[18] suggested that the intra-species variation in fungi's genetic could be due to the different geographical locations. Therefore, the sequence variation of basidiomata collected from field in this study possibly having lesser genetic variation with *Ganoderma* sp. BRIUMS compare to stain GBLS. Fig. 2 has shown that the collected basidiomata was sharing same clade with *Ganoderma* sp. BRIUMSa and forming a sisterly clade with *Ganoderma* sp. BRIUMSb with 98% bootstrap value. Both clades were closely clustered with *Ganoderma boninense* strain GBLS branch node with 94% bootstrap value. Hence, we can assume that *Ganoderma* sp. BRIUMS and collected basidiomata are mostly homologous and evolved from *Ganoderma boninense* strain as shown in the phylogenetic tree.

Antimicrobial Activity of *G. boninense* Extracts

The zone of inhibition was recorded in Table 2. The results revealed that the fruiting bodies of *G. boninense* were potentially effective in suppressing microbial growth of tested pathogens with variable potency depending on the types of solvent used for extraction purpose. Solvent extraction is likely to extract the active compounds with similar polarity[19]. Ethyl acetate extract showed a broad variable antimicrobial properties against both Gram positive and negative bacterial strains (*Enterobacter* sp*, P. aeruginosa, P. mirabilis, Acinetobacter* sp*, K. pneumoniae, S. marcescens*, *S. pyogenes, Coagulase negative Staphylococci,* and *MRSA*) followed by hot water > acetone > methanol > ethanol and chloroform extract exhibited least antimicrobial activity. Ethyl acetate extract also exhibited the strongest growth inhibition against *P. mirabilis* (14.20 $\pm$ 0.40 mm). The results indicating solvent extracts of *G. boninense* fruiting bodies exerted antimicrobial properties were in agreement with previous works on other *Ganoderma* species. For example, solvent and aqueous extracts of *G. lucidum* fruiting bodies exhibited inhibitory effect against *S. aureus*, *B. subilis*, *P. aeruginosa*, *V. cholera* and

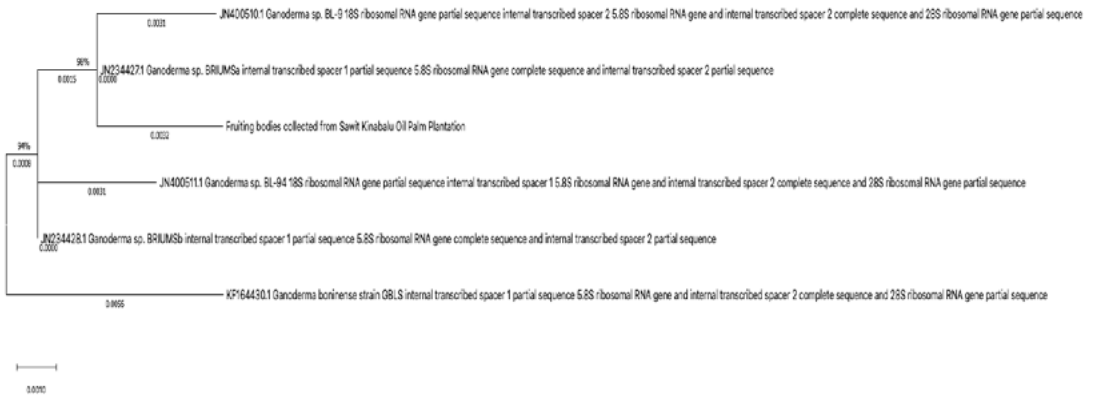

Fig. 2. Relatedness relationship of the collected fruiting bodies from the Sawit Kinabalu oil palm plantation with the five closest sequence retrieved from NCBI database. The topology was inferred by maximum likelihood. The bootstrap value is expressed in percentage and displayed next to each node. The branch length is drawn in scale with nucleotide change in unit of number of base substitutions per site.

Table 2. Inhibition zone of different solvent extracts of *G. boninense* fruiting bodies (20 mg/disc) against several clinical isolate pathogens. Data expressed as meann ± SD, *n* = 3. Different lowercase alphabets within rows represent statistically significant difference with the different solvent extracts within same bacterial strain at p < 0.05 by One Way ANOVA Tukey's test. Different uppercase alphabets within columns represent statistically significant difference within same solvent extracts with different bacterial strain at p < 0.05 by One Way ANOVA Tukey's test

	Inhibition zones (mm)						
Bacterial strains	Chloramphenical (30 µg)	Chloroform	Ethyl Acetate	Acetone	Methanol	Ethanol	Hot water
Gram negative pathogens							
E. coli	22.03 ± 0.05^{aAF}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bG}	0.00 ± 0.00^{bF}	0.00 ± 0.00^{bC}	0.00 ± 0.00^{bD}	0.00 ± 0.00^{b}
E. coli (urine sample)	22.03 ± 0.18^{aAG}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{cG}	0.00 ± 0.00^{cF}	0.00 ± 0.00^{cC}	0.00 ± 0.00^{cD}	7.50 ± 0.10^{bBCDEFG}
Enterobacter sp	23.60 ± 0.08^{aAC}	0.00 ± 0.00^{dB}	11.68 ± 0.10^{bAC}	10.63 ± 0.06^{bA}	0.00 ± 0.00^{dC}	0.00 ± 0.00^{dD}	7.67 ± 0.06^{cBCDEFG}
Pseudomonas aeruginosa	21.40 ± 0.17^{aBCDEFG}	0.00 ± 0.00^{dB}	12.60 ± 0.05^{bAB}	8.37 ± 0.05^{cAE}	11.70 ± 0.09^{bA}	13.50 ± 0.09^{bA}	8.33 ± 0.14^{cAF}
Proteus mirabilis	21.30 ± 0.05^{aBCDEFG}	0.00 ± 0.00^{dB}	14.20 ± 0.40^{bA}	9.20 ± 0.20^{cAB}	0.00 ± 0.00^{dC}	0.00 ± 0.00^{dD}	8.67 ± 0.08^{cAC}
Salmonella sp	24.97 ± 0.15^{aA}	0.00 ± 0.00^{bB}	0.00 ± 0.00bG	0.00 ± 0.00^{bF}	0.00 ± 0.00^{bC}	0.00 ± 0.00^{bD}	0.00 ± 0.00^{b}
Acinetobacter sp	9.97 ± 0.07^{aH}	0.00 ± 0.00^{bB}	9.83 ± 0.16^{aBCDEF}	7.67 ± 0.16^{aBCDE}	8.87 ± 0.08^{aB}	0.00 ± 0.00^{bD}	9.27 ± 0.06^{aAB}
Klebsiella pneumoniae	23.47 ± 0.10^{aAD}	0.00 ± 0.00^{cB}	10.73 ± 0.10^{bAE}	0.00 ± 0.00^{cF}	0.00 ± 0.00^{cC}	0.00 ± 0.00^{cD}	10.07 ± 0.11^{bA}
Serratia marcescens	23.47 ± 0.08^{aAE}	0.00 ± 0.00^{dB}	9.77 ± 0.04^{bBCDEF}	0.00 ± 0.00^{dF}	0.00 ± 0.00^{dC}	0.00 ± 0.00^{dD}	8.20 ± 0.03^{cAG}
Gram positive pathogens							
Streptococcus pyogenes	18.63 ± 0.26^{aFG}	7.93 ± 0.04^{cA}	11.67 ± 0.13^{bAD}	8.60 ± 0.08^{bcAD}	0.00 ± 0.00^{dC}	0.00 ± 0.00^{dD}	8.67 ± 0.12^{bcAD}
Streptococcus faecalis	25.43 ± 0.13^{aA}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bG}	0.00 ± 0.00^{bF}	0.00 ± 0.00^{bC}	0.00 ± 0.00^{bD}	0.00 ± 0.00^{b}
Coagulase neg staphylococci	19.27 ± 0.04^{aFG}	0.00 ± 0.00^{dB}	9.17 ± 0.12^{cBCDEF}	8.77 ± 0.07^{cACD}	13.43 ± 0.18^{bA}	9.67 ± 0.06^{cB}	7.77 ± 0.06^{cBCDEFG}
MRSA	24.60 ± 0.12^{aAB}	0.00 ± 0.00^{dB}	10.23 ± 0.07^{bAF}	8.20 ± 0.17^{cBCDE}	8.80 ± 0.04^{bcB}	7.70 ± 0.07^{cC}	8.47 ± 0.06^{cAE}

Table 3. Minimum inhibitory concentration (MIC) of the different solvent extracts of *G. boninense* fruiting bodies against selected bacterial strains

Bacterial strains	Type of solvents	MIC ($mgml^{-1}$)
Gram negative pathogen		
E.coli (Urine sample)	Hot water	5.000
Enterobacter sp	Ethyl acetate	1.250
Pseudomonas aeruginosa	Ethanol	1.250
Proteus mirabilis	Ethyl acetate	1.250
Acinetobacter sp	Ethyl acetate	2.500
Klebsiella pneumoniae	Ethyl acetate	2.500
Serratia marcescens	Ethyl acetate	2.500
Gram positive pathogen		
Streptococcus pyogenes	Ethyl acetate	1.250
Coagulase neg staphylococci	Methanol	0.625
MRSA	Ethyl acetate	1.250

S. typhi[20]. Moreover, extracts of *G. lucidum* mycelia demonstrated wide variation of bacteria inhibition for chloroform, acetone and aqueous extracts except methanol extract showed no inhibition activity against *P. mirabilis*, *K. pneumoniae* and *P. aeuroginosa*[21]. Crude extracts of *G. applanatum* and *G. australe* also exerted broad spectrum of bacteria inhibition except ethanol and water extracts of *G. australe* showed no inhibition activity against *P.mirabilis*, *Actinomyces* sp and *P. aeruginase* and *K. pneumoniae* respectively[22]. Besides *Ganoderma* species, various cultivated or wild macrofungi were reported for their antimicrobial activity[23,24]. Effect of different solvents extract of *G. boninense* on antimicrobial activity in this study was similar with some literatures. Wild fruiting bodies of *Ganoderma* collected from *Causurina equiestifolia* and *Morinda tinctoria* hard wood tree in India possess maximum and minimum inhibition activity against tested bacteria by ethyl acetate and chloroform extracts respectively[25]. Ameri *et al*.[26]

Table 4. Putative compounds identification of ethyl acetate crude extract of *G. boninense* from PubChem by HPLC coupled with electrospray ionization mass spectrometry system

Putative Identification	Retention time	m/z	Peak intensity	Compound nature	Reference
2-amino-4-chloro-3-hydroxybutanoic acid	0.62	153.0211	1.7E6	Fatty acid	Isogai et al.[41]
Cryptopleurine	0.65	377.2027	4.5E5	Alkaloid	Al-Shamma et al.[42]
6-ethoxy-2-(2-phenylethenyl)quinoline	9.16	275.1291	2.0E5	Alkaloid	Singh et al.[43]
Americine	30.30	545.3056	1.5E5	Alkaloid	Li et al.[44]
Pyroaconitine	42.38	585.2982	5.4E5	Alkaloid	Borcsa et al.[45]
(4-(Bis(2-chloroethyl)amino)phenyl) methyl hexadecanoate	46.71	485.2834	1.5E5	Fatty acid	Chandrasekaran et al.[46]
Lythranine	73.42	527.2918	2.8E5	Alkaloid	Al-Snai.[47]
Catuabine C	77.79	357.1644	3.0E5	Alkaloid	Aguiar et al.[48]
Cyano-cribrostatin IV	77.79	715.3347	1.7E5	Alkaloid	Pettit et al.[49]
4-Acetyl-4-guanidino-6-methyl(propyl) carboxamide-4,5-dihydro-2H-pyran-2-carboxylic acid	79.32	341.1697	5.2E5	Phenol	Srinivas et al.[50]
2-[(1R)-3-[di(propan-2-yl)amino]-1-phenylpropyl]-4-(hydroxymethyl) phenol; heptanoic acid	86.46	471.3392	2.1E5	Fatty acid	Ara et al.[51]
Sodium; hexadecenoic acid	94.76	279.2276	1.5E6	Fatty acid	Abubakar et al.[52]
Levofloxacin monohydrate	94.92	379.1510	3.6E5	Quinolone	Noel[53]
3-decyl-N,N-dimethyl-2H-imidazole-1-carboxamide	99.33	281.2432	1.6E6	Heterocyclic compound	Gupta et al.[54]
Dioctyltin(2+); tetradecanoate	121.65	800.5725	1.1E5	Fatty acid	Sales[55]

Table 5. Putative compounds identification of ethanol crude extract of *G. boninense* from PubChem by HPLC coupled with electrospray ionization mass spectrometry system

Putative identification	Retention time	m/z	Peak intensity	Compound nature	Reference
Nitrooxadiazole	0.60	114.9991	3.5E5	Heterocyclic compound	Prajapati et al.[56]
2-methylidenebutanedioic acid	1.33	153.0149	4.6E6	Dicarboxylic acid	Cordes et al.[57]
Auramycin G	5.23	783.3414	3.5E5	Glycoside	Fujiwara et al.[58]
Butyric acid, 2-bromo-4-[4-nitrophenyl]-, methyl ester	5.66	300.9923	3.8E5	Fatty acid	Fafal et al.[58]
Desoxyharringtonin	6.10	515.2540	9.1E5	Alkaloid	Mikolajczak et al.[60]
Ergovaline	6.41	533.2642	3.1E5	Alkaloid	Panaccione, et al.[61]
Deoxyharringtonine	6.77	515.2536	4.7E5	Alkaloid	Mikolajczak et al.[60]
N-[(3-cyclopentyloxyphenyl)methyl] -2-(2-propan-2-yloxy-5-propylphenoxy) ethanamine; oxalic acid	7.18	501.2750	3.1E5	Fatty acid	Kwak et al.[62]
Deoxyharringtonin	11.91	515.2542	1.4E6	Alkaloid	Mikolajczak et al.[60]
Ergocornine	13.13	561.2948	7.0E5	Alkaloid	Panaccione, et al.[61]
Leu-Nle-Uracil polyoxin C	17.38	513.2382	2.7E5	Peptide	Smith et al.[63]
Pyroaconitine	46.14	585.2931	5.0E5	Alkaloid	Borcsa, et al.[45]
amikacin	46.38	585.2934	4.7E5	Aminogly-coside	Yoshikawa et al.[64]
N-epsilon-(Octanoyl)lysyl-uracil polyoxin C	56.60	541.2690	3.0E5	Glycoside	Smith et al.[63]
Catuabine C	78.22	357.1642	3.6E5	Alkaloid	Aguiar et al.[48]
Ambruticin VS3	90.82	501.3487	3.0E5	Heterocyclic compound	Vetcher et al.[65]
Hexadecenoic acid; linoleate	94.78	279.2284	2.8E5	Fatty acid	Canli et al.[66]
Levofloxacin monohydrate	94.81	379.1514	2.9E5	Glycoside	Noel[53]
Isooctadecanoic acid, 1,1'-(dibutylstannylene)ester	121.83	800.5696	1.9E5	Fatty acid	Fan et al.[67]

also reported ethyl acetate extract of *G. lucidum*, *G. praelongum* and *G. resinaceum* exhibited maximum inhibition activity against 30 strains of MRSA while chloroform extract was shown weak activity. However, numerous studies have shown inverse findings. Samarokoon *et al*.[27] revealed that the chloroform fraction of *G. lucidum* extract from Sri Lankan showed profound antibacterial activity. Non-polar fractions of antimicrobial compounds isolated from *G. lucidum* were related to ergonstan-type steriods and lanostan-type triterprnoid[28]. All *G. boninense* extracts were not sensitive to *E. coli, Salmonella* sp (Gram negative bacteria) and *S. faecalis* (Gram positive bacteria). Klaus and Niksic[29] also reported that aqueous extract of some *G. lucidum* strains showed no inhibition against *Salmonella* sp, *E. coli*, and *S. aureus*. Difference sensitivity of bacteria toward extracts could be related to morphological differences of bacteria cell wall. The characteristics of the cell wall and cell surface could be attributed to the permeability of the extract[30]. For instance, Gram positive bacterial pathogens have high resistant to treatment due to the ability of biofilm formation that creating a barrier for the penetration of extract compounds[31].

Minimum Inhibitory Concentration (MIC) of the Effective Extracts

Results of MIC were recorded in Table 3. The MIC values obtained ranged between 0.625 mg ml^{-1} to 5.000 mg ml^{-1} with methanol extract demonstrating the highest antimicrobial activity against *Coagulase neg staphylococci* while hot water extracts exhibited with the lowest antimicrobial activity. Comparatively moderate activities were observed in ethyl acetate extracts exhibited MIC values of 1.250 mg ml^{-1} (*Enterobacter*

Table 6. Putative compounds identification of hot water crude extract of *G. boninense* from PubChem by HPLC coupled with electrospray ionization mass spectrometry system

Putative identification	Retention time	m/z	Peak intensity	Compound nature	Reference
2-Furanmethanethiol, 5-methyl-	0.56	128.0322	3.8E5	Heteroaromatic compound	Chen et al.[68]
Formic acid	0.61	115.0003	1.3E6	Carboxylic acid	Kovanda et al.[69]
1-benzothiophene	0.61	133.0111	4.0E6	Heterocyclic compound	Aganagowda et al.[70]
Thiazolo[5,4-d]pyrimidine-7(6H)-thione	0.64	168.9781	3.6E5	Heterocyclic compound	Habib et al.[71]
Octanoic acid	2.65	187.0946	3.2E5	Fatty acid	Huang et al.[72]
Ergovaline	5.35	533.2653	3.6E5	Alkaloid	Panaccione, et al.[61]
2-hydroxypropane-1,2,3-tricarboxylic acid	8.70	517.2709	7.6E5	Carboxylic acid	Hawkins[73]
Desoxyharringtonin	11.51	515.2552	1.6E5	Alkaloid	Mikolajczak et al.[60]
Ergocornine	13.17	561.2957	2.9E5	Alkaloid	Panaccione, et al.[61]

sp, *P. aeruginosa, P. mirabilis, S. pyogenes*) and 2.500 mg ml^{-1} (*Acinetobacter* sp, *K. pneumoniae, S. marcescens*) against tested pathogens. Various degree of inhibitory concentration was observed for the *G. boninense* extracts could be due to the polarity of solvent. The extractability of secondary metabolites mainly depend on the type of solvents and methods that is used during

Fig. 3. Extracted ion chromatogram (XIC) of ethyl acetate crude extract of *G. boninense* after peak deconvolution by local minimum search. Each individual recognized peak within the chromatogram is indicated in different colour with peak intensity and m/z value.

Fig. 4. Extracted ion chromatogram (XIC) of ethanol crude extract of *G. boninense* after peak deconvolution by local minimum search. Each individual recognized peak within the chromatogram is indicated in different colour with peak intensity and m/z value.

Fig. 5. Extracted ion chromatogram (XIC) of hot water crude extract of *G. boninense* after peak deconvolution by local minimum search. Each individual recognized peak within the chromatogram is indicated in different colour with peak intensity and m/z value

extraction of sample. Ethyl acetate is a semi polar solvent that could dissolve broad spectrum of compounds, varying from polar to nonpolar compounds. Results of ethyl acetate extract of *G. boninense* showed growth inhibition of Gram positive and Gram negative pathogens indicating that the extract contains broad spectrum of active compounds and suggesting that the antimicrobial compounds are leaning toward polar-soluble. These were supported by few reports showed numerous strains of *Ganoderma species* and edible mushrooms exhibited broad spectrum of antibacterial properties in higher polarity extracts like ethyl acetate and methanol compare with lower polarity extracts like hexane[32-34]. However, the existence of low polar-soluble compounds in *Ganoderma* species or edible mushrooms are undeniable[35,24]. Preliminary phytochemical has shown that ethyl acetate soluble fraction of *G. lucidum* possess compounds like alkaloid, carbohydrate and glycoside, phenol, sterol, terpenoid[36,37]. Strong potent of inhibition zones and MIC values were observed in ethyl acetate extract of wild *G. lucidum* fruiting bodies against selected bacterial strains and study suggested that the antibacterial activity of solvent extracts were related to the high occurrence of total phenolics, flavonoids, alkaloids, terpenoids and glycosides compounds[38]. Antimicrobial compounds isolated from *Ganoderma* species by difference solvent polarity were reviewed by Basnet and co-workers[32]. Antimicrobial activity of mushrooms were reported in varied levels. Apart from solubility in solvents and extraction methods, other factors could be due to sensitivity and tolerance of the bacterial strains to mushroom crude extracts. Moreover, the production of secondary metabolites by natural resources and bioactive compositions may varied upon different environmental conditions.

Crude polysaccharide of difference *Ganoderma* species were reported to exert broad spectrum of antimicrobial activity covering both Gram positive and Gram negative bacterial[39]. However, polysaccharide compounds could limit the antimicrobial activity because carbohydrates are element that help the growth of microorganism[40]. Further investigation on the active compound that responsible for the inhibition is necessary as crude extract contains numerous of active compounds.

Identification of the Compounds from Crude Extracts of *G. boninense*

Extracts with good antimicrobial activity were identified using HPLC-MS. As the result, a list of m/z chromatographic peaks were obtained and illustrated in Fig. 3 (Ethyl acetate extract), Fig. 4 (Ethanol extract) and Fig. 5 (Hot water extract).

Based on the PubChem database search, a total of 15 compounds for ethyl acetate crude extract of *G. boninense* were putatively identified with previously reported with antimicrobial activity. Presented compounds presumably represented by alkaloid, quinoline, fatty acid, carboxylic acids derivatives. On the other hand, compounds of ethanol crude extract were putatively categorized into several groups, majority represented by fatty acid, alkaloid, heterocyclic compound with a few of peptide and glycoside derivative compounds as listed in Table 5. Hot water crude extract has shown the least number of compounds with only 9 compounds reported in previous studies on antimicrobial activity. Those putative identified compounds which may contribute to antimicrobial activity of *G. boninense* were tabulated in Table 4-6 with references.

Numerous synthetic alkaloids using quinoline or pyridine as scaffold for development of antimicrobial drug has been exploited[74,75]. Nonetheless, several natural occurring alkaloid derivatives like quinoline, indole, piperazine, aptamine have been reported for their antibacterial activity[76]. For example, GC-MS analysis of crude extract from *Plumeria alba* flowers revealed compounds contributed to antimicrobial activity were alkaloid derivatives of pyridine, isoquinoline, indole, vinca and reserpine classes[77]. According to Ismail *et al.*[78], fatty acids such as octadecanoic acid, dodecanoic acid, methyl ester, hexadecenoic acid were identified from chloroform extract of *G. boninense* fruiting bodies and exhibited growth inhibition properties against *P. aeruginosa* and *S. aureus*. Natural occurring of fatty acids such as methyl ester, tetradecenoic, hexadecenoic, octadeceadienoic extracted from *G. resinaceum* possess antimicrobial effects against *Fusarium oxysporum*, *C. albicans*, and *S. aureus*[79]. The presence of the compounds from three different

solvent extracts of *G. boninense* may have contributed to the antimicrobial activity presented in the current work.

CONCLUSION

Different solvents were used to extract bioactive compounds of *G. boninense* fruiting body and their antimicrobial properties were examined. Ethyl acetate is a potential solvent for *G. boninense* extraction because of the remarkable broad spectrum of antimicrobial activity toward Gram positive and Gram negative pathogens. The extract gave the strongest inhibition to the growth of *P. mirabilis*. LC-MS analysis confirmed the crude extracts of *G. boninense* contains numerous compounds such as alkaloid, fatty acid, heterocyclic compound, peptide, and glycoside derivatives. The results obtained from this study revealed that *G. boninense* possess great potential with antimicrobial activities. Hence, this fungus deserves more attention in discovering their medicinal values in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors participated in design of the experiments. YS conducted the experiments and drafted the manuscript. KP supervised the project and reviewed the manuscript.

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ETHICS STATEMENT

Not applicable.

DATA AVAILABILITY

All data generated and analyzed for current study were presented in form of tables and Figs. and included in this article.

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