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Research Article

SCREENING FOR POTENTIAL ANTIMCROBIAL COMPOUNDS FROM *GANODERMABONINENSE* AGAINST SELECTED FOOD BORNE AND SKIN DISEASE PATHOGENS

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ABSTRACT

Objective: The present study aims to investigate the potential antimicrobial compounds from the fungi against selected foodborne and skin disease pathogens.

Methods: In this study, four different types of solvents (hexane, chloroform, dichloromethane and methanol) were used to screen the potential antimicrobial compounds from the *G. boninense* fruiting bodies. The antimicrobial activity of the *G. boninense* crude extract was studied against some common food borne and skin diseases bacterial pathogens such as *Escherichia coli, Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa, Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus aureus and Klebsiella spp*. using disc diffusion assay.

Results: Crude extraction showed methanolic extraction produced the highest yield (2.61%) followed by chloroform (1.36%) and dichloromethane (0.50%). The lowest yield obtained was from hexane extraction (0.17%). Antimicrobial study revealed that methanol and chloroform extracts showed broad spectrum activity to all tested pathogens with inhibition ranging from7.8-11.3 ± 0.0-1.0 mm and 6.8-8.3 ± 0.0-1.0 mm respectively. A clear inhibition zone where *Aspergillusniger* failed to developed on TLC platesdipped in chloroform: ethyl acetate (95:5, v/v) at R_f 0.33, 0.40 and 0.69 was also observed using chloroform crude extract. GC-MS results confirmed *G. boninense* contains bioactive compoundssuch as dodecanoic acid, cyclododecane, octadecanoic acid, 9-octadecenoic acid, hexadecanoic acid, methyl tetradecanoate, 9, 12-octadecadienoic acid, dodecyl acrylate and hexadecanoic acid.

Conclusion: *G. boninense*contains many bioactive compounds which are potential to be further explored as an antimicrobial agent against food borne and skin diseases pathogens.

Keywords: *Ganodermaboninense*, Crude extract, Antimicrobial activity, Food borne and Skin disease pathogens.

INTRODUCTION

The emergence of infectious diseases caused by antibiotic resistant pathogens have raised awareness among the medical practitioners regarding the importance on developing more effective drugs in tackling the event. Among the pathogens involved in the development, food borne and skin disease pathogens are getting more attention as these pathogensfrequently reported to be less susceptible to antibiotic treatment, while the contagiousness is rapid since they can be transmitted through food and air [1,2]. In Malaysia, the number of cases reported on food borne diseases rose from 6,930 to 17,320 cases between 2006 and 2008 [3]. Antibiotics have become so broadly used clinically and at the same time many microorganisms have developed antibiotic resistance especially for some clinical pathogens. Search for the new antimicrobial drugs have become one of the major interest in medical research. Previous studies have shown bioactive compounds from certain fungal mycelia contain antimicrobial properties which able to inhibit the growth of drug resistance bacteria [4]. For this reason, pharmacological research on the bioactive compounds of medicinal fungi has becomes more important for the development of new potential antibiotics.

Ganodermaboninense is a white rot fungus which can be found in oil palm estates. It is one of the most devastating pathogens causing major losses in oil palm yield in oil palm industry [5,6]. Although *G. boninense* is the devastating pathogen of oil palm in Malaysia, other genus has been known all over the world for their high medicinal purposes[7,8].Various reports suggest that *Ganoderma* species can be used as antitumor, antibacterial, anti-inflammatory and antiviral agent [9].Traditional Chinese medicine used*Ganoderma* for several treatments in China and other countries of the Orient such as for treatment of chronic hepatopathy, hypertension, bronchitis, arthritis, neurasthenia and neoplasin [10]. Most current researches on *G.boninense*solely focusing on its pathogenicity and the effect on palm oil [6,11,12], rather than exploring its potential in medicine.However, one of the current findings has found that the annoyance *G. boninense*fruiting bodies might become a precious source of antimicrobial drugs from their abundant potential resources, which the study had reported the effect of *G. boninense*fruiting body extract against *Candida albicans* [9]. However, there is no information on antimicrobial activities of *G. boninense* against other common pathogens in food borne and skin disease pathogens. Hence, this study was carried out to extract potential bioactive compounds from *G. boninense* fruiting bodies, determine the antimicrobial activity from the crude extract of *G. boninense* against the growth of some common skin and food borne pathogens, purify the potential bioactive compounds using Thin Layer Chromatography (TLC) and identify the potential bioactive compounds using GC-MS.

MATERIALS AND METHODS

G. boninense Fruiting Bodies Collection

G.boninense fruiting bodies (1 kg) werecollected from oil palm plantations at Langkon, Kota Marudu, Sabah. Fresh fruiting bodies werewashed thoroughly with tap water. The fruiting bodies later were further rinsed with freshwater and brushed using a soft brush to remove any impurities before oven-dried at 40°C for two weeks. The dried fruiting bodies werecut into smaller pieces and ground to powder using a mechanical blender (Waring ® Commercial Blender).

Serial exhaustive extraction

Polar solvents (methanol and dichloromethane) and non-polar solvents (hexane and chloroform) were used for extraction of natural compounds from *G. boninense* fruiting bodies. This method involves successive extraction withsolvents of increasing polarity from non-polar (hexane and chloroform) to more polar solvents (dichloromethane and methanol) to ensure a wide polarity range of compound could be extracted. The crude extract was used to study the profile of secondary metabolites via direct Thin Layer Chromatography bioautography and disc diffusion methods.

Powdered sample (225 g) was extracted by dissolving them into a conical flask containing 1000 mL each of the following solvent: hexane, chloroform, dichloromethane and methanol. The mixture was left for two days. The extractwas filtered throughWhatman No. 1 filter paper, evaporated and concentrated under reduced pressure using a Rota Vapor™ (BUCHI).The bathing temperature was set at 27°C with rotary speed at 150 rpm. The aliquot was weighed and kept at -20°C in air tight containers for further investigations. Extraction yield was determined using the following formula:

$$\text{Yield (\%)} = \frac{\text{Dry weigth of extract}}{\text{Dry weight of plant powder}} \times 100$$

Test Microorganisms

Bacteria used in this study were *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Klebsiella sp.* and *Streptococcus pneumoniae* which obtained from Queen Elizabeth Hospital, Kota Kinabalu, Sabah. The microbial cultures were preserved in 30% glycerol stock solution at -85°C. Prior to the antimicrobial activity study, the test microorganisms were sub-cultured on Nutrient Agar (NA), incubated at 37°C for 24 hours before the inoculums of the test microorganisms being transferred into Nutrient Broth (NB). The final concentration was adjusted according to Mac Farlands Standard to achieve approximately 1 x 10^8 CFU/ml before introduced into the test media.

Antimicrobial activity study

The antimicrobial test was done using the disc diffusion technique as described previously [13]where *G. boninense* fruiting bodies extract (GBFBE) was pipette into a 5 mm round disc. Fresh bacterial culture was spread over the plate using cotton bud. Filter paper discs were impregnated with crude extract of *G. boninense* fruiting bodies (10 mg/disc), 0.05mg/disc chloramphenicol was used as a positive control and solvents used forextraction served as a negative controls.GBFBE was pipetted few times(10 µL each time) onto the discuntil the final concentration was 10 mg of GBFBE/disc. The discs were overlaid on NA and incubated at 37°C for 24 h. Thetest was done in triplicates and the mean values were calculated. Clear zones of inhibition were measured in mm, including the diameter of disc.

Thin Layer Chromatography (TLC) of *Ganodermaboninense* Fruiting Bodies Extract (GBFBE)

Crude extracts with strong antimicrobial activity was separated using TLC. Origin with 1 cm in length was drawn on a silica gel pre-coated plate(Merck Kieselgel 60 F_{254} silica gel). Crude extract was spotted on the TLC plate with capillary tube. The plate was developed in a suitable solvent system. Different solvent systems were used for different classes of compounds based on the polarity of the organic solvents. Solvent system was started with the least polar solvent such chloroform: ethyl acetate (95:5, v/v for chloroform crude extract) and hexane: ethyl acetate (70:30, v/v for methanol crude extract). When the solvent reached 16 cm from the origin, the plates were takenout, air-dried and retention factor (R_f) were calculated. The chromatogram was examined under UV light, 366 nm wavelength to observe any presence of fluorescence band and adequate TLC reagent such as 50% sulphuric acid was used to detect the phytoconstituents. Retention factor (R_f) for all bioactive and reactive bands were calculatedand recorded.

TLC Bioautography

TLC bioautography was conducted with spraying conidia suspensions of *Aspergillusniger*on the plate to screen potential compounds from the extracts. All chromatogramswere incubated in a moist chamber for 4 days at 25°C in darkness. Any inhibition zones on the plate after this period was recorded and its retention factor (R_f) value was calculated.

Purification of Bioactive Compounds from GBFBE

Bands containing the potential compounds were marked using pencil and the silica was scrappedoff using a clean razor blade and deposited into Eppendorf tubes 1.5mL. The potential compounds were repeatedly eluted from the silica gel using chloroform. The samples were then centrifuged (13,000 x g for 15 min to spin off the silica gel and the fractions were combined. The supernatants were dried using nitrogen gas and 1 mL of n-hexane was added. Samples later were subjected to GC-MS identification.

Analysis of Bioactive Compounds

Gas Chromatography Mass Spectrometry (GC-MS), model: Agilent, 5975C. GC-MS analysis of this extract was performed using a Perkin Elmer GC Clarus500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with a Elite-1 fused silica capillary column (30 m x 0.25 mm ID x 1 iMdf, composed of 100% Dimethyl poly siloxane). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min and an injection volume of 2µL was employed (split ratio of 10:1). Injector temperature was set to 250°C with Ion-source temperature of 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min. The relative percentage of each component was calculated by comparing its average peak area to the total area. Software adopted to handle mass spectra and chromatograms was TurboMass Ver5.2.0.

RESULTS

Extraction Yield from Various Solvents

Different solvents have different ability to extract different compounds based on the polarity of the solvents and also compounds which presence in *G. boninense*fruiting bodies in these different extraction yield is shown in Table 1. From the extraction results, methanol soluble fraction of the extracts was more in quantity (2.61%), followed by chloroform (1.36%), then dichloromethane (0.50%) and lastly hexane (0.17%).

Table 1: Percentage extraction yields of *G. boninense* fruiting bodies with different types of solvents.

Solvent	Yield of extracts in (%)* (w/w)
Hexane	0.17
Chloroform	1.36
Dichloromethane	0.50
Methanol	2.62

*Percentage of yield extracts was calculated based on weight

Antimicrobial Activity of GBFBE

Both chloroform and methanol extracts shown broad spectrum of antimicrobial activity. Both chloroform and methanol extracts showed significant activity toward almost all tested bacteria with the size of inhibition ranging from 6.8 ± 0.3 mm to 8.3 ± 0.6 mm for chloroform extract and 7.8 ± 0.8 mm to 11.3 ± 0.6 mm for methanol extract (Figures 1 and 2). The highest activity was observed against *P. aeruginosa* and *S. aureus* (8.3 ± 0.6 mm) for chloroform extract while *S. aureus* (11.3 ± 0.6 mm) for methanol extract. The lowest activity was observed against *B. cereus* (6.8 ± 0.3 mm) for chloroform extract while *B. subtilis* (7.8 ± 0.8 mm) for methanol extract. *Klebsiella sp*. was only sensitive to methanol extract. Meanwhile, hexane and dichloromethane extract showed no sign of inhibition to the tested bacteria. While, inhibition that produced by commercial antibiotic disc (chloramphenicol) was around 10.0 ± 0.6 mm to 13.7 ± 0.6 mm with the concentration of 1 mg mL^{-1}(not shown). There was no zone of inhibition observed in the disc impregnated with chloroform and methanol solvents (negative controls).

TLC and Bioautography

The extracts that showed promising inhibitory activity against tested bacteria (chloroform and methanol extracts) were screened for activity against *A. niger*. Table 2 shows the solvents system used in TLC-bioautography which gave the best separation. Clear

inhibition zones were only observed at R_f 0.33, 0.4 and 0.69 of chloroform extract when bioautography was conducted with *A. niger* (Figure 3), but not in methanol extract (Data not shown). Compounds at these R_f values were further identified using GCMS.

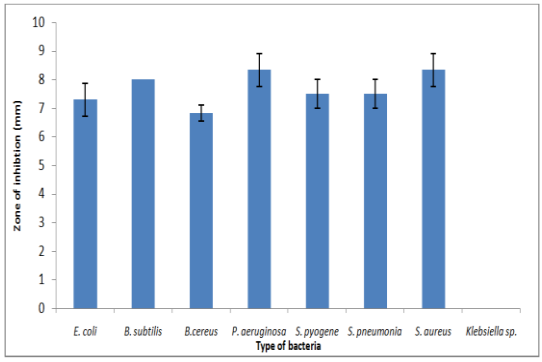

Fig. 1: Antimicrobial activity of chloroform extract of *G. boninense* fruiting bodies against tested bacteria.

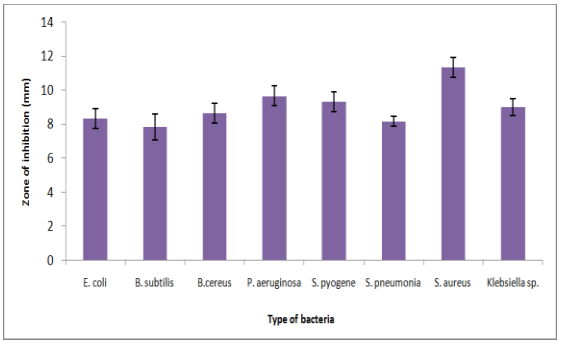

Fig. 2: Antimicrobial activity of methanol extract of *G. boninense* fruiting bodies against tested bacteria.

Table 2: Solvent systems for extraction of the *G. boninense* fruiting bodies

Fraction	Solvent System	Number of bands	R_f value
Chloroform	Chloroform: ethyl acetate (95:5)	4	0.33, 0.4 and0.69
Methanol	Hexane: ethyl acetate (70:30)	8	0.25, 0.28, 0.40, 0.47, 0.53, 0.7, 0.8 and 0.98

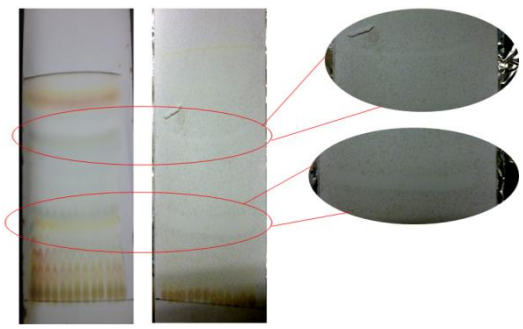

Fig. 3: Chromatograms of chloroform crude extract of *G. boninense* fruiting body extracts developed in chloroform: ethyl acetate (95: 5, v/v). TLC plate was sprayed with suspension of *A. niger*.

GC-MS analysis Compound of Interest from GBFBE

Based on the GC-MS analysis, there were four major compounds presence in the chloroform extract such as dodecanoic acid, methyl ester (R_T 8.719; 17.761%), cyclododecane (R_T 8.091; 15.038%), octadecanoic acid, butyl ester (R_T 19.790; 13.624%), and 9-octadecenoic acid, methyl ester, (E)- (R_T 15.753; 11.059%). Five other compounds constituting 34.725% of the total were hexadecanoic acid, methyl ester (R_T 13.423; 8.296%), methyl tetradecanoate (R_T 11.053; 8.160%), 9, 12-octadecadienoic acid (z,z)-, methyl ester (R_T 15.679; 6.837%), dodecyl acrylate (R_T 10.702; 6.355%) and hexadecanoic acid, butyl ester (R_T 16.914; 5.077%) (Figures 4 and 5).

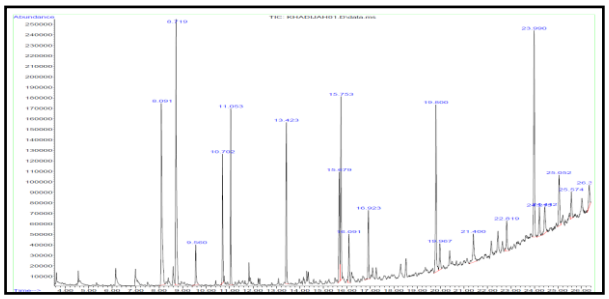

Fig. 4: The GCMS profiles of the *G. boninense* fruiting bodies at R_f value 0.33 and 0.40.

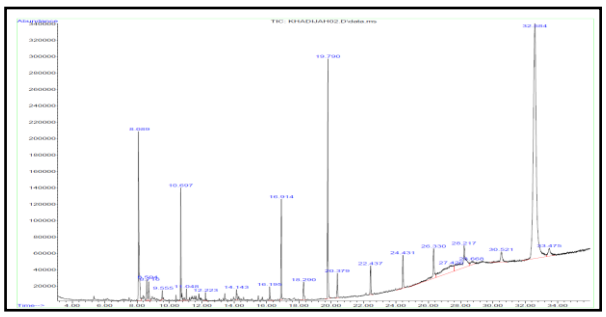

Fig. 5: The GCMS profileof the *G. boninense* fruiting bodies at R_f value 0.69.

DISCUSSION

In this study a serial of exhaustive extraction was chosen to extract bioactive compounds from *G. boninense*fruiting bodies as this extraction method involved successive extraction with solvents of increasing polarity that begin from non-polar solvent such as hexane to more polar solvent like methanol. The technique is very crucial to ensure a wide polarity range of compounds could be extracted from *G. boninense* fruiting bodies.The successful determination of biologically active compound from plant material is largely depends on the type of solvent used in the extraction[13]. In the present study, most of the *G. boninense* fruiting bodies from different solvent extraction showed some degree of inhibition to both Gram negative and Gram positive bacteria. The GBFBE, fully inhibit the Gram positive bacteria but only 66.7% for Gram negative bacteria. The presence of lipopolysaccharide in their outer wall of Gram negative bacteria may help them to become more resistance to GBFBE [14]. Previous studies have demonstrated that gram positive bacteria are more susceptible towards plants extracts as compared to Gram negative bacteria [13,15]. These differences may be reasoned to the fact that the cell wall in the Gram positive bacteria is composed of a single layer whereas the Gram negative cell wall is multilayered structure [16]. Previous studies reported an important characteristic of plant extracts and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membranes and also mitochondria, disturbing the cell structures and rendering them more permeable [17]. Thus, extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death. GC-MS revealed the presence of some pharmacologically active compounds in the chloroform extract. There are several

compounds such as hexadecanoic acid, hexadecanoic acid ethyl ester and linoleic acid were found in leaf extract of spirulina and reported to possess antimicrobial activity [18]. The identification of the components in the extracts after scrapped off from TLC plate was based on their retention times and mass spectra fragmentation patterns with those stored in the MS library and also with the published literatures. National Institute Standard and Technology (NIST) is the library sources which used for matching the identified components from GBFBE. The interpretation on mass spectrum GC-MS was conducted using the database of NIST that composed of more than 62,000 patterns. GC-MS spectrum of the unknown component was compared with the spectrum of the known components which stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained through this mean [19].More than 60% of the total amount of plant volatiles constituents studied are dodecyl acrylate and cyclododecane[20] which may have varied antioxidant and antimicrobial [21]properties, and very similar with the constituents found in the *G. boninense* fruiting bodies based on the GC-MS profile. Most compounds are fatty acids which are known to have antibacterial and antifungal properties [22]. Lipids can kill microorganisms by disrupting the cellular membrane of bacteria, fungi and yeasts [23]. This is because they can penetrate the extensive meshwork of peptidoglycan in the cell wall without visible changes and reach the bacterial membrane leading to its disintegration. Dodecanoic acid, tetradecanoic acid, hexadecanoic acid, octadecanoic acid and oleic acids are among the fatty acids which known to have potential antibacterial and antifungal activity [24,25]. Previous study have reported that oleic acid has been found to be fungistatic against a wide spectrum of moulds and yeasts such as delaying the germination of fungal spores up to 6-8 at concentration as low as low as 0.7% v/v [25]. Moreover, it has also been proposed that these fatty acids have potential antibacterial and antifungal principle for clinical application [26]. In this study, fatty acids from GBFBE contain tetradecanoic, hexadecanoic and oleic acids as well as long chain unsaturated alcohol would be expected to exercise potent antibacterial and antifungal which contribute to the to the inhibition to the tested pathogens.

CONCLUSION

G. boninense has a good potential as an antimicrobial agent against Food borne and skin diseases pathogens based on its antimicrobial activity and identity of the potential compounds.However, further work is needed to purify these potential compounds using other methods of purification and confirm the mode of action of these fatty acids and alcohol.

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