

Anti-Staphylococcal and Antioxidant Properties of Crude Ethanolic Extracts of Macrofungi Collected from the Philippines

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ABSTRACT

Introduction: Macrofungi have been used in the Philippines as source of food and traditional medicines. However, these macrofungi in the Philippines have not yet been studied for different biological activities. Thus, this research determined the potential antibacterial and antioxidant activities of crude ethanolic extracts of seven macrofungi collected in Bataan, Philippines. **Methods:** Kirby-Bauer disk diffusion assay and broth microdilution method were used to screen for the antibacterial activity and DPPH scavenging assay for the determination of antioxidant activity. **Results:** *F. rosea*, *G. applanatum*, *G. lucidum* and *P. pinisitus* exhibited zones of inhibition ranging from 6.55 ± 0.23 mm to 7.43 ± 0.29 mm against *S. aureus*, *D. confragosa*, *F. rosea*, *G. lucidum*, *M. xanthopus* and *P. pinisitus* showed antimicrobial activities against *S. aureus* with an MIC₅₀ ranging from 1250 µg/mL to 10000 µg/mL. *F. rosea*, *G. applanatum*, *G. lucidum*, *M. xanthopus* exhibited excellent antioxidant activity with *F. rosea* having the highest antioxidant activity among all the extracts tested (3.0 µg/mL). **Conclusion:** Based on the results, these Philippine macrofungi showed antistaphylococcal activity independent of the antioxidant activity. These can be further studied as potential sources of antibacterial and antioxidant compounds.

Key words: Antimicrobial, Antioxidant, Mushrooms, Philippines, Staphylococcus aureus.

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History

- Submission Date: 26-09-2017;
- Review completed: 27-10-2017;
- Accepted Date: 02-11-2017

DOI : 10.5530/pj.2018.1.19

Article Available online

<http://www.phcogj.com/v10/i1>

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INTRODUCTION

Macrofungi have been used for a long time in traditional medicine especially in Asian countries.¹ This indicated the potential presence of mycochemicals that are responsible for the health benefits of macrofungi. It has been previously reported that constituent compounds of macrofungi have cytotoxic, antineoplastic, and immunomodulatory properties.^{2,3,4}

Alternative antibacterials and antioxidants have been found in compounds from the environment. The growing potentials of mushrooms have also encompassed such properties as it has been confirmed that its chemical components also have antibacterial⁵ and antioxidant activities by radical and metal-scavenging.⁶ Despite these literatures, there are not much published work on the antibacterial and antioxidant activities of the mushrooms in the Philippines as there is a lack of documentation on Philippine local mushrooms in general.⁷

The aim of this study was to determine the antibacterial and antioxidant activities of crude ethanolic extracts of *Daedaleopsis confragosa* (Bolton) J. Schrot., *Fomitopsis rosea* (Alb. & Schwein.) P. Karst., *G. applanatum* (Persoon) Patouillard, *Ganoderma lucidum* (Curtis) P. Karst., *Lenzites elegans* (Spreng.) Pat., *Microporus xanthopus* (Fr.) Kuntze, and *Polyporus pinisitus*. *In vitro* methods of antibacterial evaluation against *Staphylococcus aureus* and antioxidant assessment via

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging were performed using the mushroom extracts to determine the species with potential bioactive molecules of pharmaceutical importance.

MATERIALS AND METHODS

Collection of mushroom species

The mushroom species were collected from three sites Sitio Pastolan, Hermosa from July to December of 2016. The map coordinate of the study site is 14°50'26.29"N and 120°20'47.17"E. Collected macrofungi were wrapped in newspaper. Each were labeled accordingly and voucher specimen were submitted to the National Museum of the Philippines for taxonomic identification.

Preparation of ethanol mushroom extracts

Macrofungi were weighed separately using a top-loading balance. They were dried in the oven overnight at 40°C and were air-dried after for seven days. They were washed with distilled water and cut into small pieces. About 150 grams of each macrofungus were soaked in 500 mL of 95% ethanol for five days. Filtration was done using Whatman filter paper No. 1. The supernatant was concentrated by a rotary evaporator at 40°C. Stock solutions were prepared by dissolving the dried extracts in 1% DMSO to a

Cite this article: Gaylan CM, Estebal JC, Tantengco OAG, Ragragio EM. Anti-Staphylococcal and Antioxidant Properties of Crude Ethanolic Extracts of Macrofungi Collected from the Philippines. Pharmacogn J. 2018;10(1):106-9.



concentration of 100mg/mL after collection. The extracts were stored at 4°C for subsequent use.

Test microorganism

Staphylococcus aureus (ATCC 25923) was retrieved from the Department of Medical Microbiology, College of Public Health in the University of the Philippines Manila.

Kirby-bauer disk diffusion assay

A 24 h culture of *S. aureus* was prepared prior to the methods of disk diffusion assay. It was inoculated in sterile Brain Heart Infusion broth with the turbidity adjusted to 0.5 McFarland standard of approximately 1.5×10^8 CFU/mL. A volume of the inoculum was swabbed on Mueller-Hinton agar plate using a sterile cotton swab within 15 minutes after standardization. Twenty microliters (20µL) of the crude ethanolic macrofungal extract were loaded on sterile 6 mm paper disks and were placed on the agar plates. Ciprofloxacin (5 µg) was used as the positive control while 95% ethanol and 1% DMSO were used as negative controls. Incubation of the plates were done at $35 \pm 2^\circ\text{C}$ for 16 to 24 h. The setup was done in triplicates and the diameters of zone of inhibition were measured in millimeters using a Vernier caliper.

Broth microdilution method

The minimum inhibitory concentration at which 50% of the bacteria were inhibited (MIC_{50}) of the macrofungi was determined by broth microdilution method according to Clinical Laboratory Standard Institute M07-A9 (2012). Two-fold dilution of the macrofungal extracts with a starting concentration of 100 mg/mL was done using Mueller-Hinton broth in Wasserman tubes generating concentrations from 19.5 µg/mL to 10000 µg/mL. One hundred microliters (100 µL) of each macrofungal extract concentration was transferred to its designated plates on the 96-well micro plate.

The inoculum was prepared as described in the Kirby-Bauer disk diffusion method. A 1:20 dilution of the suspension was made using MHB as the diluent. Ten microliters (10µL) of the inoculum was added to each of the wells. Media were mixed and the trays were incubated at $35 \pm 2^\circ\text{C}$ for 16 to 24 h. Reading of absorbance values was done using the ELISA micro plate reader. Controls include a reference drug control and a negative control blank containing no bacterial inoculum. Extracts with 1% DMSO, 95% ethanol and MHB were also used as controls. Determining the MIC_{50} required the use of the formula:

$$\text{MIC}_{50} = [(Ac - As)/Ab] \times 100$$

Where *Ac* is the absorbance of the blank control, *As* is the absorbance with the extract and *Ab* is the absorbance of the MHB control. The lowest concentration which had a percentage of at most 50% served as the MIC_{50} of the extract.

DPPH assay

Four hundred micrograms (400 µg) of the dried extracts was weighed using an analytical balance and was dissolved in 1mL of 95% ethanol in a microcentrifuge tube. Two hundred microliters (200µL) of the extract was added to the first lane of the 96-wells microplate with 100µL of 95% ethanol in the succeeding lanes. Two-fold dilution was done using a multichannel pipette discarding the last 100µL from the last lane. Addition of 100 µL of 0.2 mM DPPH radical was done to each well producing a range of concentration from 200 µg/mL to 0.1 µg/mL of the extract. Blank controls were also done. The mixture was shaken using a vortex mixer for 30 minutes before subjecting to the spectrophotometer set at 515 nm for absorbance readings. The scavenging activity (SA) (%) of the extracts on the DPPH radical was computed using the formula:

$$\text{SA} = [(Ac - As)/Ac] \times 100$$

Where *Ac* is the absorbance of the control and *As* is the absorbance with the extract. Tests were done in triplicates and a calibration curve was constructed with ascorbic acid as the standard.

Data analysis

Data were analyzed using the SPSS program and GraphPad Prism. Half maximal effective concentration (EC_{50}) was also computed from the SA values of each ethanolic extract. Shapiro–Wilk test was performed to determine if the data were in normal distribution. Mann-Whitney’s test was done to determine which sample extract has significantly different antimicrobial activity when paired with ciprofloxacin. The test was also done to assess differences between the EC_{50} values of each extract against the ascorbic acid standard. Differences with p-value of less than 0.05 ($P < 0.05$) were considered as statistically different.

RESULTS

Diameters of zone of inhibitions from the activities of the mushroom extracts are shown in Table 1. *G. applanatum*, *G. lucidum* and *P. pinisitus* exhibited zones of inhibition ranging from 6.55 ± 0.23 mm to 7.43 ± 0.29 mm against *S. aureus*. The rest of the macrofungi did not exhibit anti-staphylococcal activity.

D. confragosa, *F. rosea*, *G. lucidum*, *M. xanthopus* and *P. pinisitus* showed antimicrobial activities against *S. aureus* with an MIC_{50} ranging from 1250 µg/mL to 10000 µg/mL (Table 2). Only *G. applanatum* and the *L. elegans* showed no significant MIC_{50} values against *S. aureus*.

The radical-scavenging effects of the samples were observed by the capacity to decrease the absorbance of the DPPH solution at 515nm. EC_{50} values of the crude ethanolic macrofungal extracts are shown in Figure 2. In general, extracts exhibited DPPH radical scavenging as most have an activity of above 50% at 200 µg/mL ($\log[\text{extract}]$: 2.3). Notable effective

Table 1 : Mean ± SEM diameters of zone of inhibition (mm) of crude ethanolic macrofungal extracts against *S. aureus*.

Mushroom Extract	<i>S. aureus</i>
<i>Daedaleopsis confragosa</i>	-
<i>Fomitopsis rosea</i>	-
<i>Ganoderma applanatum</i>	7.12±0.88
<i>Ganoderma lucidum</i>	7.43±0.29
<i>Lenzites elegans</i>	-
<i>Microporus xanthopus</i>	-
<i>Polyporus pinistus</i>	6.55±0.23
95% Ethanol	-
1% DMSO	-
Ciprofloxacin	24.28±0.15 (S)

Table 2 : MIC_{50} (µ g/mL) of ethanolic extracts from eight mushrooms against *S. aureus*

Mushroom Extracts	<i>S. aureus</i>
<i>Daedaleopsis confragosa</i>	1250
<i>Fomitopsis rosea</i>	2500
<i>Ganoderma applanatum</i>	-
<i>Ganoderma lucidum</i>	10000
<i>Lenzites elegans</i>	-
<i>Microporus xanthopus</i>	2500
<i>Polyporus pinistus</i>	2500

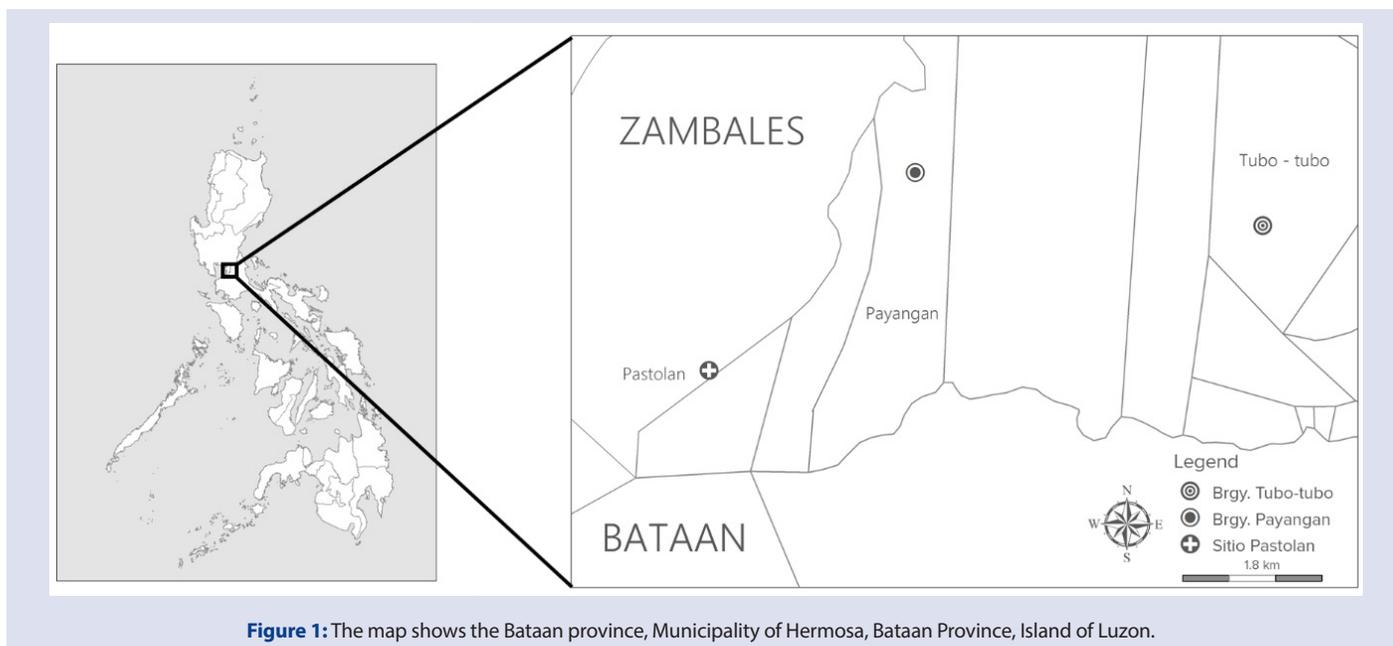


Figure 1: The map shows the Bataan province, Municipality of Hermosa, Bataan Province, Island of Luzon.

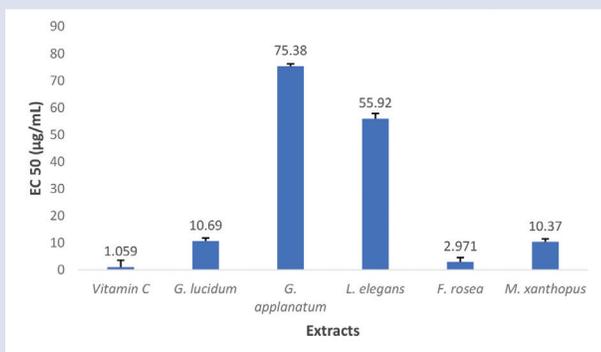


Figure 2: DPPH radical scavenging activities of macrofungal extracts from Bataan, Philippines. Each value was expressed as mean \pm SEM (n=3).

antioxidant activities due to low to moderate EC₅₀ values include *F. rosea*, *G. applanatum*, *G. lucidum*, *M. xanthopus*, with *F. rosea* having the highest antioxidant activity among all the extracts tested (3.0 µg/mL).

DISCUSSION

There were few published literatures on the mushrooms species present in the Philippines. There were also few researches on the antimicrobial activity of mushrooms species in the country.⁸ Majority of the antimicrobial activity screening studies in the Philippines used terrestrial plants. Providing baseline information on the bioactivities of these mushroom species will contribute knowledge on macrofungi in the Philippines as it is not well documented at present.⁷

Mushrooms are advantageous as source of bioactive compounds over plants because their fruiting body can be produced in shorter period of time. Different wild mushrooms have been previously reported to have high antimicrobial and antioxidant activity. Phytochemical analysis from previous studies have shown that antioxidants found in mushrooms

are mainly from phenolic compounds (phenolic acids and flavonoids), tocopherols, ascorbic acid and carotenoids.⁹

The minimal to no zone of inhibition against *S. aureus* did not necessarily mean the absence of antimicrobial activity of the macrofungal extracts. Diffusion assay is not suited to crude extracts with less polar compounds because they are hardly soluble in water and their hydrophobic nature inhibits the diffusion of the extract through the agar media.¹⁰

This limitation of diffusion was addressed by broth microdilution method showing better antibacterial activity. The results of broth microdilution assay revealed higher antimicrobial activity compared to Kirby Bauer disc diffusion assay. *D. confragosa* showed the highest MIC₅₀ against *S. aureus* based on this study. This was supported by previous literature which showed that *D. confragosa* had MIC₅₀ value of 5 mg/mL against *S. aureus*. Previous studies also showed that *D. confragosa* contains tannins and flavonoids which are both major classes of antimicrobial compounds present in macrofungi.^{2,11,12}

F. rosea, *G. lucidum*, *G. applanatum* and *M. xanthopus* have statistically comparable DPPH scavenging effects ($p < 0.05$) to the standard vitamin C. No correlation was observed between the antioxidant and antibacterial properties of the macrofungi tested. Based on previous studies, *F. rosea* contains triterpenes but it has no antiradical activity against DPPH radicals.¹³ It is possible that other phytochemicals present in *F. rosea* is responsible for the excellent antioxidant activity obtained in this study. Exopolysaccharides have been previously isolated from *G. lucidum* which exhibited antioxidant properties.¹⁴ Previous study has shown that polysaccharides from *G. applanatum* has antioxidant activity.¹⁵ Previous reports on *M. xanthopus* showed it contains high amount of ascorbic acid and antioxidant activity.¹⁶

CONCLUSION

D. confragosa, *F. rosea*, *G. lucidum*, *M. xanthopus* and *P. pinisitus* showed antimicrobial activities against *S. aureus* with MIC₅₀ ranging from 1250 µg/mL to 10000 µg/mL. *F. rosea*, *G. lucidum*, *G. applanatum* and *M. xanthopus* have statistically comparable DPPH scavenging effects ($p < 0.05$) to the standard vitamin C. These Philippine macrofungi can be further explored as potential sources of antibacterial and antioxidant compounds. Isolation of these compounds in further studies can lead to

the eventual clinical application of mushrooms as agents against bacteria and oxidative stress.

ACKNOWLEDGEMENT

This work was financed by the UP Manila National Institutes of Health Faculty Research Grant (Project Code: NIH 2015-007).

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

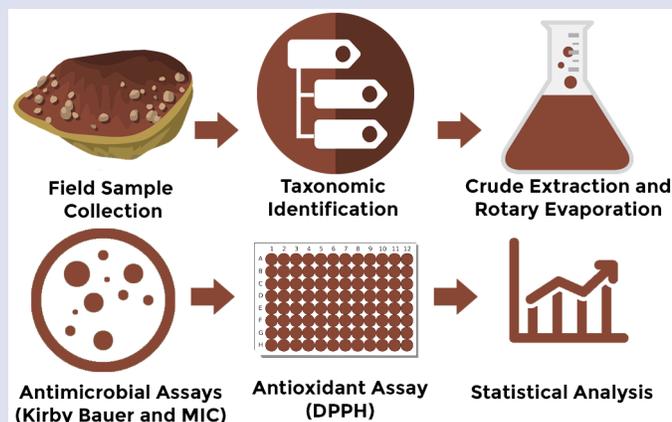
ABBREVIATION USED

DMSO: Dimethyl sulfoxide; **DPPH**: 2,2-diphenyl-1-picrylhydrazyl; **EC₅₀**: Effective concentration 50.

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GRAPHICAL ABSTRACT



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SUMMARY

- Selected Philippine macrofungi collected from the Municipality of Hermosa, province of Bataan, island of Luzon showed anti-staphylococcal activity independent of the anti-oxidant activity. These macrofungi are *Daedaleopsis confragosa*, *Fomitopsis rosea*, *Ganoderma applanatum*, *Ganoderma lucidum*, *Lenzites elegans*, *Microporu xanthopus*, *Polyporus pinistus*. The isolation of biologically active compounds as antibacterial agents can be further studied.

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