



## Antibacterial activity of Endolichenic Fungi Isolated from *Dirinaria applanata* against Nosocomial Pathogens

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### ABSTRACT

The conduct of this study generally aimed to investigate the antibacterial activity of endolichenic fungi isolated from *Dirinaria applanata* against nosocomial pathogens. Specifically, it sought to determine the zone of inhibition of the endolichenic fungal crude extracts at various concentrations against nosocomial pathogens (*Staphylococcus aureus*; *Klebsiella pneumoniae*; *Escherichia coli*; *Enterobacter agglomerans*; and *Pseudomonas aeruginosa*) and compare the zone of inhibition of the various concentrations of endolichenic fungal crude extracts against nosocomial pathogens. In this study, the host lichen was characterized using published identification keys and online keys and online catalogues. The isolated endolichenic fungi were morphologically characterized. Mass production of endolichenic fungi was carried out to increase the amount of fungal crude extracts. Presence of secondary metabolites in the fungal crude extracts was determined through Thin-Layer Chromatography while its antibacterial activity against the nosocomial pathogens was tested through paper disk diffusion method. This study utilized Two-way analysis of variance (Two-Way ANOVA) to determine zone of inhibition of the various concentrations of endolichenic fungal crude extracts against nosocomial pathogens. In the light of the findings of this study, it can be concluded that the endolichenic fungi isolated from *Dirinaria applanata* has potential antibacterial activity against nosocomial pathogens specifically for gram-positive *Staphylococcus aureus* and the widely known multidrug resistant nosocomial strain *Pseudomonas aeruginosa*. It was also found out that the antibacterial activity of the endolichenic fungal crude extracts is seemingly evident at 400 mg/mL concentration. It is recommended to increase the concentration of fungal crude extracts and determine the minimum inhibitory concentration.

**Keywords:** *Dirinaria applanata*, endolichenic fungi, nosocomial pathogens, Paper Disk Diffusion Assay, Thin Layer Chromatography

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### INTRODUCTION

Nosocomial infections are hospital acquired infections (HAIs). Pathologically, these are contaminations in a health care facility that are distinct to patient's original condition, and dauntingly remains a significant challenge to health care facilities. In a global sight, hospital-acquired infections have distressed a huge number of patients including its financial losses, and high mortality rate. According to estimate reported by World Health Organization approximately 15% of all hospitalized patients suffer from these infections [23]. In point of fact, these infections are responsible for 4% to 56% of mortality rate among neonates, with incidence rate of 75% in South-East Asia and Sub-Saharan Africa [25]. In this sense, no health care facility in the world is exempted from nosocomial infections.

In the Philippines, Universal Health Care (Kalusugan Pangkalahatan) remains the core concern in public health. That is to say, the indicators available in describing quality health care include net death rate, and net infection rate. Net infection rate caters hospital-acquired infection that occurs beyond 72 hours during hospitalization. Ideally, hospitals should strive for 0.0% net infection rate. However, based on the data gathered, 36.67% had 0.0% net infection rate while 63.33% had an average net infection rate below 1% (DOH, 2011). In an urban setting, 28% of patients with hospital-acquired infections were reported by tertiary level hospital [24]. Alarmingly, pneumonia was found to be the most common HAI (35%). Poor infection control practice is a reflection of non-compliance to nosocomial infection monitoring. Philippines is classified as low- and middle-income country, and its pecuniary heavy load could weaken its hold on standard hospital measures [2].

In Region II, the Department of Health takes delivery on Quarterly Physical Report of Operation to provide essential updates relative to nosocomial infections. To this end, it develops appropriate measures

to enhance the quality of hospital patient care by ensuring proper operation on infection control practices. In December 2013, the Cagayan Valley Medical Center- a tertiary medical center, envisioned 0% rate of hospital-acquired infections. Unfortunately, 0.37% was reported. Net death rate means death that has occurred more than 48 hours after admission. Net death rate was given a physical target of 0% rate. Conversely, 1.81% was reported.

In 2017, the Department of Health issued a report with reference to the leading causes of morbidity, and mortality on nosocomial infections. For morbidity details, upper respiratory tract infection was the topmost fear while urinary tract infection appeared fourth on the list. For the record, pneumonia was the leading cause of mortality with a rate of 58.18% out of 2, 092 cases.

Bacteria, viruses, and fungal parasites are disease-causing microorganisms. Surprisingly, the most common pathogenic microorganism triggering nosocomial infections are bacteria [6]. Some thrive in the human body, and produce an antagonistic effect when the patient's immune system is compromised. There are several drugs available in the market to stop the chain of infection brought by hospital-acquired infections. However, the large spill of bacterial infection is behaving negatively to the public, and even worldwide. At present, there is a vast issue on research that pharmacologically active compounds need to be introduced for drug innovation [4].

One identified potential source of this bioactive compounds is Lichen. Lichens are organisms made up of two separate species (namely lichen and algae) that are mutually symbiotic with one another. They contain endolichenic fungi, having diverse synergistic effect. They hold bioactive secondary metabolites vital for drug modernization [14]. Studies have shown that these metabolites have antibacterial properties. Among the lichens studied, *Dirinaria applanata* has a promising bioactive molecule as evidenced by its high phenolic content, and scavenging capacity of DPPH radicals. The macrolichens are capable of manufacturing bioactive compounds [13].

Several studies on antimicrobial activities of endophytic fungi have been reported. To date, few studies on the medicinal usefulness of fungi have been conducted in the Philippines. Given its rich potential source of novel compounds, this study aimed to assess the antibacterial activities of the crude culture extracts of endolichenic fungi isolated from *Dirinaria applanata* against nosocomial infections.

## MATERIAL AND METHODS

### Research Design

A 5 by 4 Factorial Design was used for this experiment. Considerable attention was given to this experiment which aimed to determine the antibacterial activity of Endolichenic Fungi Isolated from *Dirinaria applanata* against nosocomial pathogens like *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter agglomerans*, and *Pseudomonas aeruginosa*. Four levels of concentration of extracts with triplicate samples were prepared namely: 100 mg/mL, 200 mg/mL, 300 mg/mL, and 400 mg/mL.

### Research Locale

The host lichen was gathered from Luquilu, Cabagan, Isabela. The identification of the said host lichen, isolation of endolichenic fungi, and the testing of antibacterial activity were conducted at the Central Laboratories in the University of Santo Tomas.

### Collection, Identification of Host Lichens, and Isolation of Endolichenic Fungi

The host lichen was collected at Luquilu, Cabagan, Isabela and was identified to be *Dirinaria applanata* through Thalline Spot Test. The endolichenic fungi were isolated from the host lichen.

### Antibacterial Assay Using Paper Disk Diffusion Method

Test Bacteria. The following test bacteria were used in this study: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter agglomerans*, and *Pseudomonas aeruginosa*. All of the test bacteria were initially grown on Mueller Hilton Agar (MHA) at 35°C for 24 hours. Following culture, the test bacteria were suspended in 10 mL MHB tubes, and standardized to 0.5 McFarland [7].

Paper disk diffusion assay. The standardized test bacteria were swabbed on MHA plates. Sterile discs were impregnated with 30 µL of endolichenic fungal crude extracts with concentrations of 100, 200, 300, and 400 mg/mL, and were placed on MHA plates [23]. Ethyl acetate was used as the negative control while tetracycline, and chloramphenicol were used as the positive control. All of the culture plates were incubated at 35°C for 24 hours. Following incubation, using the Vernier caliper, the diameters of the zone of inhibition were measured in triplicates, and the mean of these measurements were used. The zone of inhibition (ZOI) was interpreted in accordance with CLSI Standard for zone of inhibition. For Tetracycline < 14- Resistant, 15-18- Intermediate and, > 19- Susceptible; for Chloramphenicol < 12- Resistant, 13-17- Intermediate and > 18- Susceptible.

## Data Analysis

This study utilized Two-way analysis of variance (Two-Way ANOVA) to determine zone of inhibition of the various concentrations of endolichenic fungal crude extracts against nosocomial pathogens.

## RESULTS AND DISCUSSION

Burgeoning antibacterial resistance among species of nosocomial pathogens requires alternative options to fight the multidrug resistance of these organisms and therefore, several researchers have focused on the discovery of new antimicrobial agents. In this sense, numerous studies have been made on antimicrobial activity of natural products [3]. One of the potential sources of natural products are endolichenic fungi isolated from host lichen.

### Antibacterial Activity of Endolichenic Fungi Isolated from *Dirinaria appplanata*

Table 1 shows the summary of the mean zone of inhibition of Endolichenic Fungi (ELF) Isolated from *Dirinaria appplanata* at various concentrations against nosocomial pathogens *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter agglomerans*, and *Pseudomonas aeruginosa*. It can be gleaned that both *Staphylococcus aureus*, and *Pseudomonas aeruginosa* have shown appreciable extent of susceptibility to the isolated endolichenic fungi at 200mg/mL concentration while the the gram-negative species *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter agglomerans* have been leveled to be resistant to the isolated fungi given the same concentration. Subsequently, at 400mg/mL concentration, with *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, *Enterobacter agglomerans* have jointly demonstrated to be susceptible with lichen-associated fungi under study. Conversely, *Klebsiella pneumoniae* consistently manifested to be resistant even at higher concentrations while *Escherichia coli* was found to have intermediate susceptibility.

This clearly implies that when both of the investigated doses of isolated endolichenic fungi are used against with *Staphylococcus aureus*, and the multidrug resistant strain *Pseudomonas aeruginosa*, the growth of these nosocomial pathogens at the infection site will be inhibited by this potential antibacterial agent. In contrast, *Enterobacter agglomerans* have been proven to be sensitive at 400 mg/mL, whereas *Escherichia coli* have no known therapeutic effect at this concentration. Strangely, *Klebsiella pneumoniae* can still build its colonies at the locus of nosocomial infection despite the presence of the ELF prepared at two studied concentrations.

Table 1. Mean comparison on the antibacterial activity of different concentrations of endolichenic fungi isolated from *Dirinaria appplanata* against nosocomial pathogens.

Concentration Levels	Mean Zone Inhibition per Nosocomial Pathogen				
	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>E. agglomerans</i>	<i>P. aeruginosa</i>
200mg/mL	20.58	7.31	10.32	7.43	27.58
400mg/mL	35.04	10.16	16.96	29.81	31.69
Tetracycline (+ control)	30.37	23.62	19.74	24.65	28.68
Chloramphenicol (+ control)	33.74	32.06	24.75	25.32	41.54

\*CLSI Standard for zone of inhibition

For Tetracycline < 14- Resistant 15-18- Intermediate > 19- Susceptible

For Chloramphenicol < 12- Resistant 13-17- Intermediate > 18- Susceptible

Table 2 shows the antibacterial activity of Endolichenic Fungi Isolated from *Dirinaria appplanata* at various concentrations compared with the positive control tetracycline, and chloramphenicol. Results of the Analysis of Variance on zone of inhibition when grouped according to type of nosocomial pathogen reveals F-ratio of 31.228 whose associated p-value is 0.000. Further, it can be deduced that there is significant difference on the antibacterial activity depending on the level of concentration. Also, it is evident that the strain of nosocomial pathogens, and dosage of isolated endolichenic fungi simultaneously influenced antibacterial activity.

Table 2. Tests of effects on the antibacterial activity of different concentrations of endolichenic fungi isolated from *Dirinaria appplanata* against nosocomial pathogens.

Source	Df	Mean Square	F	p-value	Decision
Concentration	3	1112.414	48.395**	.000	Reject Ho
Nosocomial Pathogen	4	717.814	31.228**	.000	Reject Ho
Nosocomial Pathogen * Concentration	12	126.664	5.510**	.000	Reject Ho
Error	70	22.986			
Total	90				
Corrected Total	89				

These findings initially connote that, the zone of inhibition is significantly influenced by the type of treatment. There are at least two types of treatment that significantly differed in the zone of inhibition as shown in table 2a. Drug of last resort for *P. aeruginosa* – chloramphenicol has the highest zone of inhibition at 31.48 mm, seconded by tetracycline. This finding has high significant difference among the means of varying treatment types except for tetracycline, and 400 mg/mL level concentration which apparently show to be comparable with one another. This suggests, that the varying concentrations are less reactive to the test organisms as compared to the positive control. However, the effect of the positive control - tetracycline is comparable with the endolichenic fungi isolated from *Dirinaria applanata* prepared at 400mg/mL concentration.

It has been shown that the antibacterial activity of the isolates is concentration dependent. This is consistent with the findings of several studies which explicated that antimicrobial agent activity is concentration dependent [18, 5].

Table 2a. Comparison among means (LSD) on the antibacterial activity of different concentrations of endolichenic fungi isolated from *Dirinaria applanata*.

Level of Concentration	Means	Mean Differences			
		200 mg/mL	400 mg/mL	Tetra-cycline	Chloram-phenicol
200mg/mL	14.64	-			
400mg/mL	24.73	10.77*	-		
Tetracycline	25.41	10.77*	0.68	-	
Chloramphenicol	31.48	16.84*	6.75*	6.07*	-

\*Mean differences are significant at 0.05

The test organisms *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter agglomerans*, and *Pseudomonas aeruginosa* reacted differently with the treatment as shown in table 2b, where the multidrug resistant *Pseudomonas aeruginosa* has the widest zone of inhibition at 29.63 mm. Table 2.b shows the result of post-hoc analysis on the zone of inhibition of the endolichenic fungal crude extract when grouped according to strains of nosocomial pathogen. The antibacterial endolichenic fungi isolates were more effective in reducing the zones of inhibition in *Staphylococcus aureus*, and *Pseudomonas aeruginosa* which did not show significant variation to the susceptibility testing. The zones of inhibition for these bacteria were significantly higher than the rest of the bacteria. Surprisingly, both *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were found to be susceptible to the endolichenic fungi extracts among the five strains of nosocomial pathogen under investigation.

Such result is due to the presence of secondary metabolites in endolichenic fungal crude extract that exhibit antibacterial activity; namely, alkaloids [11,3], triterpenes [20], sterols, phenols, tannins, flavonoids [1], glycosides, and indoles [21].

The nosocomial pathogens used are all gram negative except *Staphylococcus aureus*. In general, the most susceptible to antibiotics are gram positive organisms like *Staphylococcus aureus* [9]. Meanwhile, gram negative bacteria are inherently more resistant to antibiotics because of their glycolyx, a structure that impedes the flow of antibiotics to the inside of the bacteria. Moreover, among all these gram-negative organisms, the opportunistic pathogen *Pseudomonas aeruginosa*, a strict aerobe, is the most resistant to antibiotics, and is considered to be the most prevalent nosocomial strain [8]. This highly resistant bugs have various virulence factors such as pili, exo-enzyme A, exotoxins, etc. [18].

This finding can be probably attributed to the fact that the extract contained phenol, alkaloid, triterpene, and glycosides. Phenol, and natural phenolic compounds significantly reduced the biofilm formation by *P. aeruginosa* [12]. Biofilm are communities of microorganisms attached to a surface. It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents [17]. On the other hand, alkaloids are plant secondary metabolites which have been shown to have potent pharmacological activities [10]. One study revealed that the effects of alkaloid on *P. aeruginosa* were bacteriostatic which means that this secondary metabolite has the ability to inhibit bacterial colony formation [16].

Alternatively, triterpenes significantly attenuate production of QS-regulated virulence factors, and biofilm formation in *P. aeruginosa*, at the sub-lethal concentration. This compound remarkably interferes in initial stages of biofilm development by decreasing the exopolysaccharide production, and cell surface hydrophobicity. Research findings suggest that some classes of triterpenes can serve as potential anti-infective in controlling chronic infection of *P. aeruginosa* [19].

Furthermore, the isolated endolichenic fungal crude extract contained glycosides. Aminoglycosides, which belong to the broad class of glycosides, are the worldwide approved treatment of choice for nosocomial *Pseudomonas aeruginosa* infection[15].

This may suggest that the synergistic effect of these secondary metabolites have contributed to the bactericidal effect of the extracts against the opportunistic gram-negative *Pseudomonas aeruginosa* bacilli, as well as the gram-positive *Staphylococcus aureus*.

Table 2b. Comparison among means (LSD) on the antibacterial activity of endolichenic fungi isolated from *Dirinaria applanata* on nosomial pathogens.

Nosocomial Pathogens	Means	Mean Differences				
		<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>E. agglomerans</i>	<i>P. aeruginosa</i>
<i>S. aureus</i>	27.81	-				
<i>K. pneumoniae</i>	8.73	-19.08*	-			
<i>E. coli</i>	13.64	-14.17*	4.91*	-		
<i>E. agglomerans</i>	18.62	-9.19*	9.89*	4.98*	-	
<i>P. aeruginosa</i>	29.63	1.82	20.9*	15.99*	11.01*	-

\*Mean differences are significant at 0.05

Figure 1 illustrates the interaction between type of treatment and species of nosocomial pathogens. It can be seen from this figure that among all the test bacteria, *P. aeruginosa* showed the highest zone of inhibition. Obviously, between the two levels of concentration, the wider diameter for Zone of Inhibition is at 400 mg/L. Further, it showed that there is interaction between treatment and type of nosocomial pathogen.

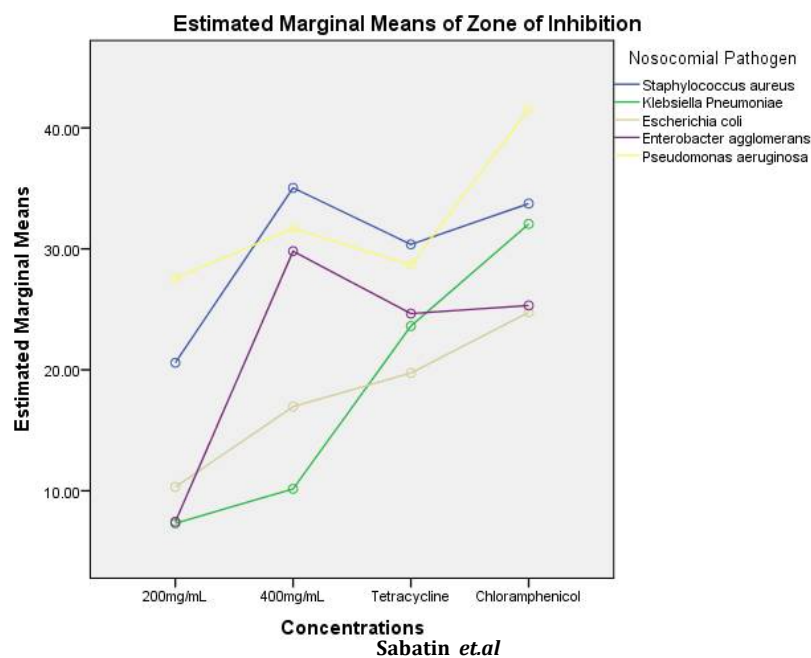


Figure 1: Interaction between type of treatment and species of nosocomial pathogens.

## CONCLUSION

In the light of the findings of this study, it can be concluded that the endolichenic fungi isolated from *Dirinaria applanata* have potential antibacterial activity against nosocomial pathogens, specifically for gram-positive *Staphylococcus aureus*, and the widely known multi drug-resistant nosocomial strain *Pseudomonas aeruginosa*. In view of great impression, it was found out that the antibacterial activity of the endolichenic fungal crude extracts is seemingly evident at 400 mg/mL concentration.

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