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# Phytochemical Screening and Antibacterial Activity of *Lignosus rhinocerotis* (Cooke) Ryvarden Grown in Open Field and Indoor

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**Highlights**

- Chemical screening and antibacterial activities of *Lignosus rhinocerotis* (Cooke) Ryvarden.
- FEA extract did not contain flavonoid in phytochemical screening test.
- At 30 mg/mL, ethyl acetate extracts inhibited *Staphylococcus aureus* moderately.

**Article Info**

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**Keywords**

*Lignosus rhinocerotis (Cooke) Ryvarden*
*Phytochemical screening*
*Antibacterial activity*
*Disk diffusion test*

**Abstract**

*Lignosus rhinocerotis* (Cooke) Ryvarden, known as tiger milk mushroom is a rare and valuable medicinal mushroom that is widely used throughout Southeast Asia and South China for treating several ailments. This study was conducted to screen the phytochemicals present in *L. rhinocerotis* (Cooke) Ryvarden sclerotium grown from two different environments, open field and indoor, and evaluate the antibacterial activity. In this study, phytochemical screening of ethyl acetate and methanolic extract of *L. rhinocerotis* (Cooke) Ryvarden sclerotium were done using various chemical tests to identify the compounds present in the extracts. All the extracts were then tested for antibacterial activity against three different bacteria including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* at the concentrations of 20 and 30 mg/mL using disc diffusion method. Based on the phytochemical screening result, extracts of *L. rhinocerotis* (Cooke) Ryvarden demonstrated the presence of steroids, terpenoids, alkaloids, and flavonoids. The antibacterial assays revealed that the ethyl acetate extracts from open field and indoor cultivations exhibited moderate activities against *S. aureus* at 30 mg/mL with the values of the inhibition zone being 13.3 ± 0.67 mm and 11.0 ± 0.58 mm, respectively.

## 1. INTRODUCTION

Mushrooms have been consumed by humans around the world for centuries either as foods or medicines. The delicate aroma and texture of edible mushrooms have boosted their popularity as a nutritious food [1,2]. Numerous findings reported that mushrooms contain high levels of functional proteins and polyunsaturated fatty acids and low levels of fats and cholesterols [3–6]. In Malaysia, some of the edible mushrooms, such as oyster mushrooms (*Pleurotus ostreatus*), shiitake (*Lentinus edodes*), and paddy straw mushroom (*Volvariella volvacea),* have been cultivated commercially to meet the market demand. Nevertheless, wild edible mushrooms such as *Grifola frondosa*, *Lignosus rhinocerotis* (Cooke) Ryvarden, *Lentinus squarrosulus*, and *Schizophyllum commune* have drawn attention mainly because these mushrooms contain highly nutritional and medicinal values [7].

*Lignosus* is a genus of wild medicinal mushrooms belonging to the Polyporaceae family. To date, eight species of *Lignosus* were identified [7] and three species found in Malaysia are *L. rhinocerotis* (Cooke) Ryvarden [8,9], *L. tigris,* and *L. cameronensis* [10]. *L. rhinocerotis* (Cooke) Ryvarden is known as the 'tiger milk mushroom' or 'cendawan susu harimau' by local people in Malaysia. This mushroom can only be found in specific geographic regions including South China, Thailand, Malaysia, Indonesia, the Philippines,

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Papua New Guinea, New Zealand, and Australia [11]. Local and indigenous people in Malaysia have used this mushroom as a traditional remedy to treat various medical conditions such as asthma, cough, fever, food poisoning, and as a general tonic [12,13]. Previous biological studies show the mycelium and sclerotium extracts of *L. rhinocerotis* (Cooke) Ryvarden possess numerous bioactivities such as anti-asthmatic [14–16], anti-inflammatory [17,18], antimicrobial [19], antioxidant [20,21], anti-proliferative [17,22], antiviral [23], anti-diabetic [24,25], immunomodulatory [26,27], and neuritogenic properties [28–30] .

Due to its rarity and high value of medicinal properties, the demand for *L. rhinocerotis* (Cooke) Ryvarden is booming. Previously, *L. rhinocerotis* (Cooke) Ryvarden was only accessible in the wild forest and the collection process was time and energy consuming [31]. Realizing the necessity for domestication, since the 2000s, many efforts on commercial cultivation of *L. rhinocerotis* (Cooke) Ryvarden in controlled environment were done and finally it was made successful in Malaysia [32,33]. Along with cultivation in a controlled environment, *L. rhinorecotis* (Cooke) Ryvarden was also grown successfully in open field where the environment mimics the wild forest [34]. Both successful domestication methods for *L. rhinocerotis* (Cooke) Ryvarden are positively solving the supply issue and accelerating studies on its potential as functional food, dietary supplement, and cosmetic active ingredient.

Throughout many years, various studies have been done on the pharmacological effect between cultivated and wild-growing *L. rhinocerotis* (Cooke) Ryvarden, and most of the studies were focused on its primary metabolites, such as β-glucan and protein [18]. However, a paucity of phytochemical screening of secondary metabolites was performed between cultivated and wild-growing *L. rhinocerotis* (Cooke) Ryvarden. Nallathamby et al. (2016) reviewed numerous studies on *L. rhinocerotis* (Cooke) Ryvarden, revealing the mycelium and sclerotium extracts exert various bioactivities. Of that, many studies were focused on its antioxidant, anticancer, immunomodulatory, and neuritogenic properties; however, only one study was done in 2012 on its antimicrobial activities against human pathogenic microorganisms using the disc diffusion method. Studies have shown that different cultivation methods will produce different types and/or concentration levels of secondary metabolites, which will exhibit different biological activities [35]. There is lack of knowledge in terms of secondary metabolite identification, with only one scientific report on the *in vitro* antimicrobial activity of *L. rhinocerotis* (Cooke) Ryvarden. The present study was conducted to identify the secondary metabolites present in the mushroom and evaluate their antibacterial activities, as the extracts of *L. rhinocerotis* (Cooke) Ryvarden could be a potential source of highly effective antibacterial agent.

## 2. MATERIAL METHOD

### 2.1. Mushroom Materials

The sclerotium powder of *L. rhinocerotis* (Cooke) Ryvarden grown in the open field and indoor cultivation site were provided by Nas Agro Farm (Sepang, Selangor) and Ligno Biotech (Balakong Jaya, Selangor), respectively.

### 2.2. Preparation of Extracts

The powdered mushroom (200g) was extracted using the maceration method at room temperature sequentially with 500 ml of ethyl acetate and methanol for six hours each and filtered using Whatman filter paper No. 1. The filtered solutions were evaporated and concentrated using rotary vacuum evaporator at a temperature of 40 ℃ under reduced pressure until all solvent have been evaporated and the crude extracts were concentrated. All dried extracts were kept in the 4 ℃ refrigerator until further use.

### 2.3. Phytochemical Screening

Preliminary phytochemical screening of extracts of *L. rhinocerotis* (Cooke) Ryvarden was carried out and performed to investigate the presence of steroids, terpenoids, alkaloids, and flavonoids in the mushroom extracts using the previous procedures by [36] and [21] with slight modifications.

**Screening for Steroids (Salkowski Test)**

Approximately 15 mg of dried extract was dissolved in 5 mL of chloroform then a few drops of concentrated sulfuric acid were carefully added. Formation of a brown ring indicated the presence of steroids.

**Screening for Alkaloids**

Extract (10 mg) was added into a test tube and stirred with 4 mL of 1% hydrochloric acid then 1 mL of Mayer's reagent (Potassium mercuric iodide solution) was added. Formation of a white/cream colour indicates the presence of alkaloids.

**Screening for Terpenoids (Salkowski Test)**

Extract (15 mg) was added to a test tube containing 5 mL of chloroform then 3 mL of concentrated sulfuric acid was added which forms a layer. Reddish brown colouration of the interface indicates terpenoids.

**Screening for Flavonoids**

The powdered extract (5 mg) was dissolved in 3 mL water and filtered. Two millilitres of diluted sodium hydroxide (10%) were added to the filtered extract solution. Appearance of yellowish colouration was produced and later was changed from yellow to colourless by adding dilute hydrochloric acid, indicating the presence of flavonoids.

**2.4. Screening of Antimicrobial Activity**

**Test Microorganisms, Growth Medium, and Controls**

Two different concentrations of *L. rhinocerotis* (Cooke) Ryvarden extracts, 20 and 30 mg/mL, were tested against three types of bacteria: one gram-positive (*Staphylococcus aureus* [ATCC BAA-997]) along with two gram-negative bacteria (*Escherichia coli* [ATCC 25922] and *Pseudomonas aeruginosa* [ATCC 2785]). Mueller Hinton (MH) agar was used to culture all of the test microorganisms. Gentamicin (10µg/disc) was used as a reference antibiotic (positive control) while ethyl acetate and methanol were used as negative control.

**Antibacterial Activity**

The antibacterial assay for this study was screened and evaluated using the Kirby-Bauer disc diffusion method described by [37]. The four extracts were diluted separately with ethyl acetate and methanol to get two different concentrations of 20 mg/mL and 30 mg/mL. Mueller Hinton agar was used to culture *S. aureus*, *E. coli*, and *P. aeruginosa.* The agar was firstly autoclaved at 121 ℃ for 20 minutes and was then allowed to cool to about 50 ℃ prior to use. All of the tested bacteria were cultured onto sterile MH agar and were incubated at 37 ℃ for 24 hours. Suspension of inoculum was prepared by mixing the single colony of each microorganism in sterilised distilled water. The suspension was then compared with McFarland standards. The inoculum suspension was swabbed evenly onto the respective agar plates using a sterilised swab stick. The plates were then left to dry for about ten minutes before the filter paper discs that had been impregnated with different concentrations of different types *L. rhinocerotis* (Cooke) Ryvarden extracts were placed gently onto the inoculated agar surface. Standard antibacterial agent, gentamicin (10µg/disc), was also placed firmly on inoculated agar plates. All the plates were incubated at 37 ℃ for 24 hours. The diameter of the zone of inhibition (mm) was measured, including the disc (6mm), to assess the antibacterial activity. Zero inhibition is denoted by the diameter of the disc itself which is 6 mm. All experiments were performed in triplicate.

### 2.5. Statistical Analysis

Statistical analysis was done using SPSS 20.0 software. Statistical comparison between the means was done using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test for multiple comparisons. The values were expressed as mean ± standard error mean (SEM) and the data was statistically significant with $p < 0.05$.

## 3.RESULTS AND DISCUSSION

### 3.1. Extraction Yield of *L. rhinocerotis* (Cooke) Ryvarden

The yield percentages of ethyl acetate (FEA) and methanol (FM) extracts grown in open field and yield of ethyl acetate (IEA) and methanol (IM) extracts grown indoor were recorded (Table 1). The yield percentages of FEA, FM, IEA, and IM were 0.29%, 0.66%, 0.88% and 2.82%, respectively. Indoor extracts of *L. rhinocerotis* (Cooke) Ryvarden had consistently higher percentage yields than open air extracts of *L. rhinocerotis* (Cooke) Ryvarden.

***Table 1.*** *Extraction yield (mg), yield percentage, and colour of extracts from L. rhinocerotis*

| Sample | Extraction Yield (mg) | Yield Percentage (%) | Paste colour |
|---|---|---|---|
| **FEA** | 145.6 | 0.29 | Yellowish |
| **FM** | 328.9 | 0.66 | Dark yellowish |
| **IEA** | 441.7 | 0.88 | Brownish |
| **IM** | 1412 | 2.82 | Dark brownish |

### 3.2. Phytochemical Screening

Phytochemical screening was used to determine the presence of phytochemical constituents such as phytosterol, alkaloids, terpenoids, and flavonoids in all extracts (Table 2). Results from the phytochemical screening showed the presence of steroids and terpenoids in all extracts derived from both open field and indoor samples, regardless of the different environment of growing. Our findings suggest the presence of terpenoids in the extracts of *L. rhinocerotis.* This is in agreement with previous studies, in which genomic sequence results of *L. rhinocerotis* encoded 12 sesquiterpene synthases (STSs), one non-ribosomal peptide synthetase (NRPS), and a polyketide synthase (PKS) [38,39]. Three sesquiterpene synthases, which were highly expressed on sclerotium of *L. rhinocerosus,* were successfully cloned and heterologously expressed in yeast and (+)-torreyol and α-cadinol were isolated. The presence of other sesquiterpenes, such as germacrene D-4-ol, selina-6-en-4-ol, β-elemene, β-cubebene, and cedrene, were also detected using gas chromatography mass spectrometry (GCMS). Our study showed that alkaloids were only present in methanolic extracts from both open field and indoor samples, suggesting the alkaloids extracted are polar derivatives since they were absent from the ethyl acetate extracts. The presence of flavonoids was observed in all extracts except the FEA sample. Semi-polar flavonoid derivatives were absent in FEA, suggesting that these compounds are not present in the *L. rhinocerotis* (Cooke) Ryvarden grown in the open field due to environmental factors. Studies have shown that different cultivation methods will produce different types and/or concentration levels of phytochemical constituents [35]. The presence of various phytochemical constituents in the extracts revealed that *L. rhinocerotis* (Cooke) Ryvarden contained a variety of bioactive compounds which may produce various biological activities in humans, including antibacterial activities.

***Table 2.*** *Phytochemical screening of extracts from L. rhinocerotis (Cooke) Ryvarden*

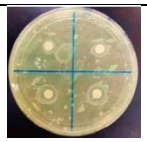
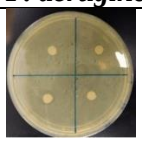
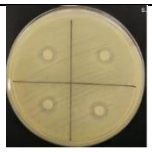
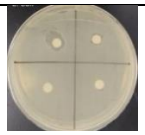
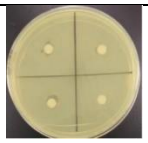
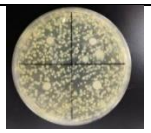
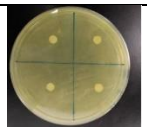
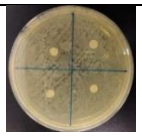
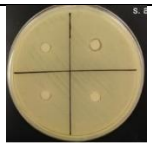
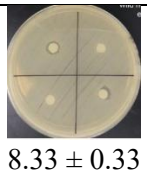
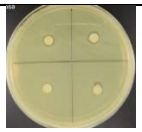
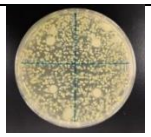
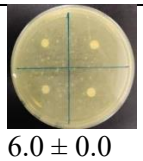
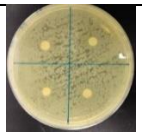
| Phytochemicals | FEA | FM | IEA | IM |
|---|---|---|---|---|
| **Steroids** | + | + | + | + |
| **Alkaloids** | - | + | - | + |
| **Terpenoids** | + | + | + | + |

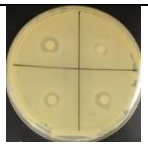
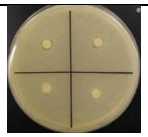
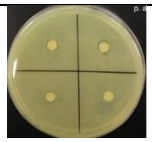
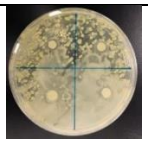
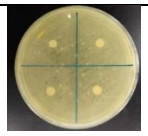
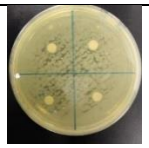
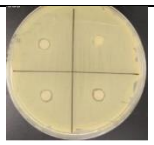
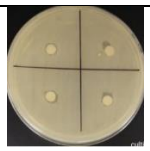
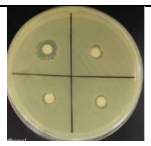
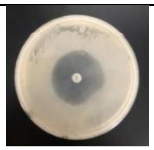
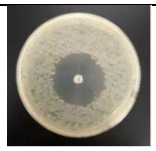
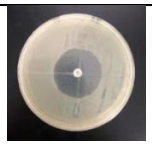
| | | | | |
|---|---|---|---|---|
| **Flavonoids** | - | + | + | + |
| **+ = present; - = absent** | | | | |

### 3.3. Antimicrobial Activity

The inhibitory effect of extracts from *L. rhinocerotis* (Cooke) Ryvarden was determined against *S. aureus*, *E. coli*, and *P. aeruginosa* strains. The activity was quantified using the inhibition zone method, and the results were compared to those obtained with the standard drug, Gentamicin (35–36 mm), as shown in Table 3. The inhibitory effect is dose-dependent, where all of the tested microorganisms were more sensitive to the extracts with higher concentration (30 mg/mL compared to 20 mg/mL). All extracts prepared at 30 mg/mL and 20 mg/mL show weak inhibition activities against all three bacterial strains, with the exception of FEA and IEA extracts at a concentration of 30 mg/mL, which display moderate inhibitory effects against *S. aureus*, with 13.3 ± 0.67 mm and 11.0 ± 0.58 mm, respectively. A previous study also reported similar findings that extracts of *L. rhinocerotis* (Cooke) Ryvarden exhibited mild to moderate antimicrobial activity at 30 mg/mL and suggested that the antimicrobial activity of the extracts may be attributed to the presence of various phytochemical constituents [19]. Our study found that all of the extracts show no inhibition effect against Gram-negative bacteria, *E. coli* and *P. aeruginosa* strains, which might be due to the complexity of Gram-negative bacterias' cell wall, which contains a double membrane in comparison to Gram-positive bacterias' unique glycoprotein/teichoic acid membrane [40]. Another previous study shows the essential oil containing sesquiterpenes, including α-cadinol, exhibited potential antimicrobial properties against four pathogenic strains (*S. aureus*, *Bacillus cereus*, *E. coli*, and *Salmonella typhi*) [41].

***Table 3.*** *Antimicrobial activity of wild ethyl acetate extracts against selected microorganisms (mean ± SEM)*

| | | **Diameter of zone of inhibition (mm)** | | |
|---|---|---|---|---|
| **Extract** | **Concentration** | **Microorganisms** | | |
| | **mg/mL** | ***S. aureus*** | ***E. coli*** | ***P. aeruginosa*** |
| **FEA** | 20 |  6.0 ± 0.0 |  6.4 ± 0.7 |  6.37 ± 0.33 |
| | 30 |  13.3 ± 0.67 |  8.0 ± 0.6 |  6.67 ± 1.67 |
| **FM** | 20 |  6.0 ± 0.0 |  6.0 ± 0.0 |  6.4 ± 0.0 |
| | 30 |  7.33 ± 0.33 |  8.33 ± 0.33 |  7.67 ± 0.33 |
| **IEA** | 20 |  6.0 ± 0.0 |  6.0 ± 0.0 |  6.0 ± 0.0 |

| | | | | |
|---|---|---|---|---|
| | 30 |  11.0 ± 0.58 |  8.33 ± 0.33 |  7.17 ± 0.44 |
| **IM** | 20 |  6.37 ± 0.03 |  6.0 ± 0.0 |  6.0 ± 0.0 |
| | 30 |  7.67 ± 0.33 |  7.3 ± 0.3 |  8.3 ± 0.3 |
| **Gentamicin** | |  36.0 ± 0.0 |  35.0 ± 0.0 |  35.0 ± 0.0 |

16 mm above: highly inhibited; 11–15 mm: moderately inhibited; 6–10 mm: weakly inhibited.

## 4. CONCLUSION

The maceration method at room temperature using methanol as the extraction solvent successfully yielded a higher percentage of extract yields for indoor samples of *L. rhinocerotis* compared to open field samples. Preliminary phytochemical screening of both ethyl acetate and methanolic extracts of open field and indoor samples of *L. rhinocerotis* (Cooke) Ryvarden revealed the presence of steroids, terpenes, alkaloids, and flavonoids. For the antimicrobial study, the results demonstrated that the FEA and IEA extracts exhibited a moderate inhibitory effect against *S. aureus*. As *L. rhinocerotis* (Cooke) Ryvarden could be a potential source in developing new antimicrobial agents, further studies will be required to isolate the secondary metabolites and specify their possible mechanism for antibacterial activity.

## CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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