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Antifungal Activity of *In-vitro* Grown *Earliella Scabrosa*, a Malaysian Fungus on Selected Wood-degrading Fungi of Rubberwood

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Abstract: *Rubberwood is prone to attack by saptain mold, wood decaying fungi and wood borers. Rubberwood preservation treatment is usually done through conventional chemical methods, which could cause environment and public health concern. A promising biocontrol agent from natural resources with a broader spectrum of disease control and high yield is needed. In this study, the growth of Malaysian white-rot fungus, Earliella scabrosa (E. scabrosa) isolated from the Malaysian forest was carried out in a shake flask culture. The antifungal activities of E. scabrosa extract against selected wood-degrading fungi of rubberwood were elucidated via broth dilution method using a 96-well microtitre plate for minimum inhibitory concentration assay. The composition of the major compounds extracted from the mycelium was determined by gas chromatography mass spectrometer (GC-MS). It was observed that the maximum biomass production was obtained when E. scabrosa was incubated in media with initial pH 6.5 or when incubation temperature was at 30°C or when agitation speed was 150 rpm. This fungal strain also showed higher antifungal activity against seven wood-degrading fungi of rubberwood with Minimum Inhibitory Concentration (MIC) values ranging between 0.61 and 5.00 $\mu g\ \mu l^{-1}$. The GC-MS spectrum indicated that this fungus produced several compounds, such as 2(3H)-furanone, 5-heptyldihydro-, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- and triacetin. This shows that E. scabrosa, which belongs to the group basidiomycete, is capable of producing active ingredients to inhibit the growth of fungi on the rubberwood.*

Keywords: *Earliella scabrosa*, biomass production, antifungal activity, wood degrading fungi, minimum inhibitory concentration

1. INTRODUCTION

Rubber trees (*Hevea brasiliensis*), which belong to the family Euphorbiaceae, were indigenous to the Amazon Valley of South America and were introduced in the Peninsula Malaysia more than a century ago. The trees were replanted every 25–30 years when they were uneconomical for latex production. It had been reported that the earlier rubberwood was utilised for timber and timber-based products, while the felled trees were used as fuelwood.[1,2]

However, in the late 1970s, Malaysia started to commercialise the rubberwood, especially for industries that used fuel wood (e.g., drying and smoking of sheet-rubber, tobacco curing and brick making), the charcoal industry and the block board industry.[1] Nowadays, it can be used to make a wide range of products, such as rubberwood-based panels (e.g., particle board, plywood and medium-density fibreboard), furniture and joinery-products, floor tiles and parquet, and mouldings.[2] Yet the fungal decay and deterioration problem has reduced the utilisation of rubberwood in wood processing industries.

Generally, chemical preservatives such as boron compounds are commonly impregnated into rubberwood in order to preserve it from fungal attack. Still today, there is a great concern on the impact of wood preservation formulations on the environment and human health. Due to that, this chemical preservation had narrowed down only to the utilisation of rubberwood, particularly in the furniture industry.[3] Interest in using natural antifungal compounds in the development of wood protection strategies for eco-products is absolutely needed. Currently, biological control agent is a promising alternative to chemical control in wood preservative sector.

Antifungal secondary metabolites isolated from the heartwood of plants had been considered to contribute to the tree resistance against the wood-degrading fungi.[4] As an example, biological control of the wooden distribution poles using *Trichoderma spp*. has been studied by Score and Palfreyman,[5] in which *Trichoderma spp.* displayed a killing action against the dry soft fungus, *Serpula lacrymans* in *in-vitro* test. This phenomenon provided the ecology chemical interactions, and hence established the existence of biochemical pathways with the sole purpose of producing mycotoxins and other natural products in fungi.[6,7]

White rot fungi are categorised under physiological grouping, which belongs to the basidiomycetes group. These fungi produce active ingredients of bioherbicide, bioinsecticide and biofungicides products.[7] *Earliella scabrosa* (*E. scabrosa*) is a monotypic genus of fungi in the family Polyporaceae. It produces enzymes for dyes biotransformation in solid-state fermentation as reported by Guerra et al.[8] The results showed that this white rot fungus has a higher decolourisation percentage of Navy FNB and Red FN-3G dyes as compared to *Trametes maxima* and *Ganoderma zonatum*. Rationally, the use of fungal crude enzyme for dyes transformation could be considered a friendly proposal with the environment for biological treatment of polluted effluents.[8]

Though the effectiveness of *E. scabrosa* in its antifungal activity has not yet been reported, it has a potential use as a biocontrol agent in the rubberwood protection sector. This article examines the growth condition of the white rot

fungus *E. scabrosa* in shake flask cultivation, and its ability to exhibit antifungal activity in order to inhibit the growth of selected wood-degrading fungi of rubberwood.

2. EXPERIMENTAL

2.1 Fungus Strain

The fungus strain, *E. scabrosa* was obtained from Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. The stock culture was grown on malt extract agar (MEA) at 30°C and maintained on agar slant prior for subsequent studies.

2.2 Wood-degrading Fungi Used

Selected wood-degrading fungi strains of *Pycnoporus sanguineus*, *Schizophyllum commune*, *Lentinus sp.*, *Lentinus strigosus*, *Microporus affinis* and *Microporus xanthopus* were collected from Biocomposite and Protection of Timber Forest Products Laboratory, FRIM, Kepong, Malaysia. The stock cultures were grown at 30°C and maintained on malt extract agar slant for subsequent studies.

2.3 Mycelia Suspension Preparation

Mycelia suspension was prepared by suspending mycelia discs from seven-day-old culture plate in a sampling bottle containing sterilised distilled water and 0.1% (v/v) Tween 80. A disc of 5 mm diameter was punched on the mycelia mats of the agar plate using sterilised cork borer. A total of 10 discs for every 100 ml of sterilised water were vortexed for 5 min in order to make the mycelia suspensions became homogenous.

2.4 Crude Extract Preparation

10 ml (10% v/v) of the mycelia suspension was added to 90 ml medium in 250 ml Erlenmeyer flask. The medium composition used in this study consists of 26.9 g l^{-1} yeast extract, 10.0 g l^{-1} malt extract, 49.2 g l^{-1} glucose, 1.0 g l^{-1} KH_2PO_4, 1.0 g l^{-1} K_2HPO_4, 0.93 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 2.0 g l^{-1} $(NH_4)_2SO_4$. Before transferring the mycelia suspension into culture media, the media was sterilised at 121°C for 15 min. The media initial pH was 6.5. The culture was incubated in an incubator shaker at 30 ± 1°C, 150 rpm for 5 days. The culture broth was then harvested and centrifuged at 4000 rpm (Eppendorf Centrifuge

5702, Germany) for 15 min. The mycelia produced were filtered prior placing it in an oven at 60°C for 24 h before weighing. The biomass concentration was determined as shown in Equation 1.

$$\text{Mycelia dry weight (g/l)} = \frac{\text{g(mycelia + filter paper)} - \text{g(filter paper)}}{\text{Volume measure (l)}} \quad (1)$$

2.5 Effect of Culture Condition on Mycelia Growth

Selection of the culture conditions on *E. scabrosa* growth was done by employing one-factor-at-a-time (OFAT) techniques carried out in a 250-ml Erlenmeyer flask. In this study, the main three parameters chosen were initial pH of the media (4.5–8.5), incubation temperature (25°C–45°C) and agitation rate (0–450 rpm). In each respective experiment, one parameter would be changed while the other parameters remained to identify the profile trend. All experiments were performed in triplicate.

2.6 Extraction Procedure

Dry mycelia (100 g) were boiled either in water or in methanol (ratio 1 g: 20 ml) for 48 h. The percentage yield for water and methanol extract was about 0.68% and 1.01%, respectively. The crude extract obtained was then dried and kept at 4°C for further analysis.

2.7 Minimum Inhibitory Concentration (MIC) Assay

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of fungal mycelia extract at which no growth of wood-degrading fungi was observed after the incubation period. Dimethyl sulfoxide (DMSO) at the concentrations used did not interfere with the reaction conditions.[9]

Crude extract from *E. scabrosa* were prepared and tested for antifungal agent against selected wood-degrading fungi such as *Pycnoporus sanguineus*, *Schizophyllum commune*, *Lentinus sp.*, *L. sajor-caju*, *L. strigosus*, *Microporus affinis* and *M. xanthopus*. The antifungal activity of *G. trabeum* was evaluated by broth dilution method. All experiments were performed using a 96-well microtitre plate and carried out in triplicates. Briefly, the mycelia of each strain were prepared from 48-h broth cultures, and each suspension was standardised to 0.5 McFarland standard turbidity. Then, the crude extract obtained was dissolved into 5% DMSO. Each well contained malt extract broth with 90 µl of serially diluted crude extracts and was inoculated with the mycelial suspension of wood-degrading fungi to a final volume of 100 µl. The control contained only malt

extract broth. The growth of microorganisms was determined by adding 20 µl of yellow tetrazolium MTT reagent dissolved in distilled water (5 µg μl^{-1}). The results were obtained after an incubation period of 1 h. The clear colour wells indicated the presence of growth inhibition whereas the dark bluish colour wells indicated the absence of growth inhibition.

2.8 Analytical Method

The crude extract was dissolved with 70% (v/v) ethanol prior to the gas chromatography mass spectrometry (GC-MS) analysis. In this study, a GC-MS was used to analyse the sample quantitatively by referring to the molecular weight of the compounds in a library (Model: NIST) that was incorporated into it. The gas chromatography analyses were performed using a Perkin Elmer Clarus 600 gas chromatograph equipped with an ELITE-5 column. The gas chromatography was coupled to the Perkin Elmer 600T mass spectrometer. The oven temperature was programmed at 65°C for 4 min and then increased to 280°C at a rate of 8°C min^{-1}.

3. RESULTS AND DISCUSSION

3.1 Effect of Initial Medium pH on Mycelia Growth

The pH of the growth medium played an important role by inducing morphological changes in the fungal mycelia. It had been reported that various species of mushrooms, such as basidiomycetes and ascomycetes provided good mycelia growth under moderately or slightly acidic pH during submerged cultivation.[10,11] Figure 1(a) shows that *E. scabrosa* grew fairly well in acidic, neutral and alkaline environments (pH 5.5–7.5).

It was observed that the best growth (20.74 ± 0.85 g l^{-1}) was obtained in acidic medium of pH 6.5. Then, the vegetative growth of 15.34 ± 0.63 g l^{-1} biomass, which was the second best, was also recorded in slightly alkaline medium (pH 7.5), followed by growth at acidic medium of pH 5.5. It could be deduced that *E. scabrosa* preferred a lower acidic and alkaline medium. This result was quite similar to the study by Fasidi and Akwakwa,[12] who reported that acidic pH values of 5.5–6.5 could provide a good mycelia growth of *Pleurotus tuber-regium*. However in this study, only 1.74 ± 0.39 g l^{-1} biomass was observed for pH 4.5, suggesting that very strong acidic or alkaline environments were inhibitory for the fungus growth. Therefore, the pH value of 6.5 was suggested for use in further study.

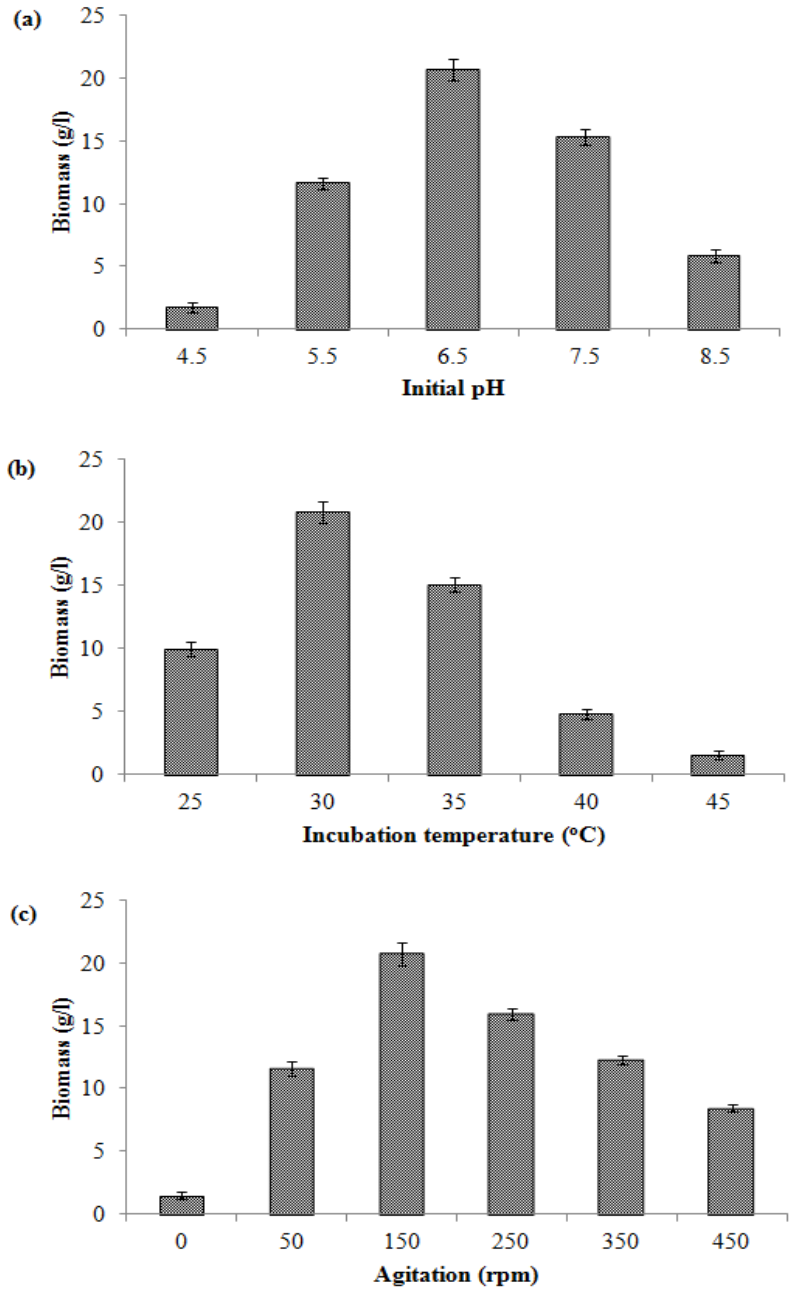

Figure1: Biomass production of *E. scabrosa* under different physical conditions after 5 days incubation, i.e., (a) initial pH, (b) incubation temperature and (c) agitation speed.

Besides pH, temperature was also another important environmental factor that influenced the growth of filamentous fungi. Figure 1(b) revealed that the best biomass production (20.82 ± 0.88 g l^{-1}) by *E. scabrosa* was attained at 30°C. This phenomenon might be due to the fact that this range of temperature (30°C–35°C) was suitable for mycelia growth, as suggested by Yang and Liau.[13] There is a significant decrease in biomass production at higher temperature [Figure 1(b)]. This observation agrees with the study by Gbolagade et al.,[14] who reported that growth of *L. subnudus* was inhibited at extremely low and high temperatures. This could be the result of the metabolic activities of fungus, which was usually reduced at extremely lower temperature, and denaturation of fungal enzymes occurred at higher temperature. Taking into consideration of the above factors indicated that a moderate temperature of 30°C was chosen for subsequent studies.

Agitation speed was also a notable factor in the fermentation process since it would increase the amount of dissolved oxygen in the cultivation medium. As in this study, the effect of agitation speed (0–250 rpm) on biomass production by *E. scabrosa* in a shake flask culture was evaluated using single factor experiments, as shown in Figure 1(c). Results showed that the biomass increased sharply with increase in agitation speed from 0 to 50 rpm. In contrast to an optimum rotating speed of 100 rpm for mycelia growth of *Aspergillus niger* hyphae during the production of tannase,[15] the best yield of this study as in Figure 1(c) was achieved at agitation speed of 150 rpm (20.69 ± 0.90 g l^{-1}). This result was similar to that reported by Yang and Liau,[13] who found that the best yield of *Ganoderma lucidum* growth was achieved at rotation speed of 150 rpm. Beyond this point, a reverse trend was observed [Figure 1(c)]. This condition could be due to the fungal cell disturbances as a result of agitation speed, which increased the shear stress on the mycelia.[13,15] Therefore, the agitation speed of 150 rpm was used for further study.

3.2 Anti-fungal Activity

Antifungal activity from the crude extract of *E. scabrosa* was assayed against seven types of wood-degrading fungi isolated locally. The antifungal activity was evaluated using MIC assay as shown in Table 1.

Table 1: Antifungal activities from the crude extract of *E. scabrosa* against selected wood-degrading fungi.

Wood-degrading fungi	Minimum inhibitory concentration, MIC (μg μl^{-1})	
	Water extract	Methanol extract
Pycnoporus sanguineus	>5	>5
Schizophyllum commune	>5	>5
Lentinus sp.	2.50	1.25
Lentinus sajor-caju	5.00	1.25
Lentinus strigosus	5.00	2.50
Microporus affinis	1.25	0.61
Microporus xanthopus	1.25	0.61

**Tested concentration was at 5.0 μg μl^{-1}. Activity concentration: weak activity: MIC > 5.0 μg μl^{-1}; moderate activity: 1.0 μg μl^{-1} < MIC ≤ 5.0 μg μl^{-1}; strong activity: MIC ≤ 1.0 μg μl^{-1}.*

The aqueous and methanol extracts from *E. scabrosa* showed antifungal activity against the five wood-degrading fungi tested, except in the study on *P. sanguineus* and *S. commune*. Among the wood-degrading fungi tested, *M. affinis* and *M. xanthopus* showed susceptible growth inhibition to the water extract of *E. scabrosa*, in which the MIC value was 1.25 μg μl^{-1}. Meanwhile *Lentinus* sp., *L. sajor-caju* and *L. strigosus* showed MIC values of 2.50 μg μl^{-1}, 5.00 μg μl^{-1} and 5.00 μg μl^{-1} (Table 1). On the other hand, the methanol extracts from *E. scabrosa* also effectively inhibited growth of four wood-degrading fungi tested. The MIC values obtained from the methanol extract of *E. scabrosa* against *Lentinus sp., L. sajor-caju, L. strigosus, M. affinis* and *M. xanthopus* were 1.25 μg μl^{-1}, 1.25 μg μl^{-1}, 2.50 μg μl^{-1}, 0.61 μg μl^{-1} and 0.61 μg μl^{-1} (Table 1), respectively. This phenomenon might be due to the fact that different proteins from different fungi exert different antimicrobial activities with different mechanisms such as inhibiting alternate microorganism-secreted enzymes, or they could interfere directly with the carrier proteins.[9]

Likewise, the MIC results listed in Table 1 also summarised that the methanol extracts gave better antifungal activity compared to aqueous extract. This could be due to the fact that the alcohol extract provided a more complete extraction, in which less polar compounds possessed antifungal properties.[17] In addition, the methanol extracts could provide relatively wider spectrum of antimicrobial activity as compared to the aqueous extract.[18]

3.3 GC-MS Analysis

The interpretation of the mass spectrum GC-MS was conducted using the database of the National Institute Standard and Technology (NIST). The spectrum of the unknown component from a sample was compared with the spectrum of the known components stored in the NIST library (version 2.0). Then, the name, molecular weight and structure of the components of the tested materials were ascertained. Figure 2 and Table 2 show the GC-MS spectrum obtained from the *E. scabrosa* methanol extract. The most abundant compound identified were 2R,3S-9-[1,3,4-Trihydroxy-2-butoxymethyl]guanine (24.02%), followed by L-Glucose (11.51%).

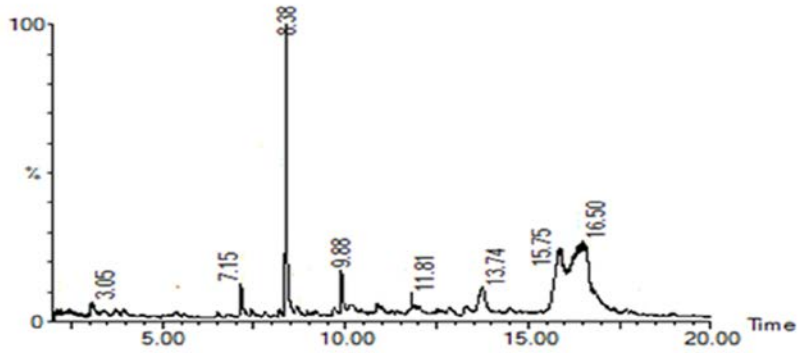

Figure 2: GC-MS chromatogram for the *E. scabrosa* methanol extract.

The results in Table 2 are just a preliminary phytochemical screening of *E. scabrosa* methanol extract that showed the presence of carbohydrate, triglyceride, phenolic compounds, tannis, flavonoids and proteins. Among all the components, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP) is one of the anti-microbial present in the *E. scabrosa* methanol extract with retention time (RT) and peak area of 8.38 min and 7.23%, respectively. Figure 3 shows that the mass spectrum of the unknown compound was similar to the mass spectrum of DDMP based in the NIST library database. This active compound has been found in the crude extract of *P. sanguineus* and *S. commune*, respectively. Also, it could be used to inhibit the growth of various type of wood-degrading fungi within the range of 0.10–5.00 μg μl^{-1}.[7,9] Besides, its ability on the biological functions has also been studied, including anti-alpha-glucosidase activity in patients with diabetes mellitus, reactive oxygen-scavenging activity, arylamine and 2-acetoxyacetylaminofluorene (2AAAF)-induced DNA damage in Chinese hamster ovary cells, and anti-tumour, anti-oxidant, anti-microbial and anti-inflammatory activities.[7,9,19,20]

Table 2: Chemical components identified in the *Earliella scabrosa* methanol extract via GC-MS analysis.

No.	RT (min)	Name of the compound	Molecular formula	MW	Confidence interval	Peak area (%)
1	3.06	Oxiranemethanol, (R)-	$C_3H_6O_2$	74	Diverse functional group	1.56
2	7.15	4,5-Diamino-2-hydroxypyrimidine	$C_4H_6N_4O$	126	Diverse functional group	1.33
3	8.38	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	Diverse functional group	7.23
4	9.88	2(3H)-Furanone, 5-heptyldihydro-	$C_{11}H_{20}O_2$	184	Ester	0.70
5	11.81	Triacetin	$C_9H_{14}O_6$	218	Ester	0.50
6	13.74	Sucrose	$C_{12}H_{22}O_{11}$	342	Diverse functional group	3.38
7	15.75	L-Glucose	$C_6H_{12}O_6$	180	Diverse functional group	11.51
8	16.50	2R,3S-9-[1,3,4-Trihydroxy-2-butoxymethyl]guanine	$C_{10}H_{15}N_5O_5$	285	Diverse functional group	24.02

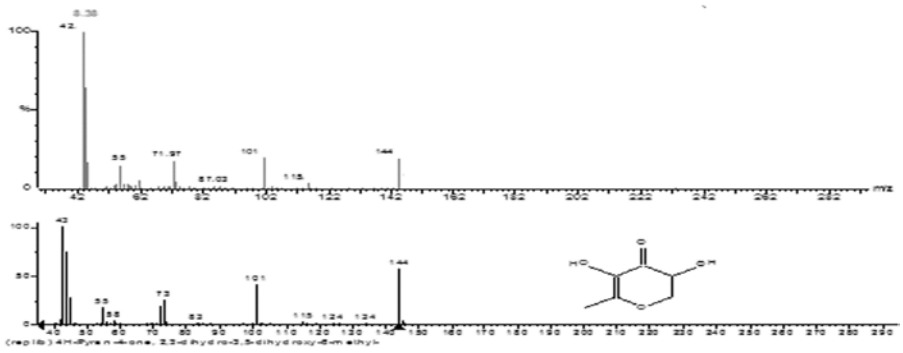

Figure 3: Mass spectrum for the compound at 8.38 min and 4H-pyran-4-one,2, 3-dihydro-3,5-dihydroxy-6-methyl- (DDMP) with its structure.

In addition, other compounds found in the extracts (Table 2), such as Triacetin (RT: 11.81, 0.50%) and 2(3H)-Furanone, 5-heptyldihydro- (RT: 9.88, 0.70%), could be amongst the important agents that inhibited the growth of the wood-degrading fungi.[21] Overall, a number of compounds with known toxicity were found to be produced by *E. scabrosa* under the conditions that were determined to maximise biomass production. Further optimisation studies need to be carried out using the optimum conditions described in this study so as to increase the yield of the active compounds.

4. CONCLUSION

In this study, the maximum *E. scabrosa* biomass production in a shake flask culture was obtained when initial culture medium was at pH 6.5 or incubation temperature 30°C or when agitation speed was 150 rpm. The water and methanol extracts provided antifungal activities against the selected wood-degrading fungi of rubberwood within MIC values range from 0.61–5.00 μg μl^{-1}. Several chemical components, such as 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP), Triacetin and 2(3H)-Furanone, and 5-heptyldihydro- were present in the methanol extract, which could play an important role in inhibiting the growth of selected wood-degrading fungi. Hence, it is intended that the present work will contribute to an understanding and determining the physical conditions (e.g., initial medium pH, incubation temperature and agitation speed) for *E. scabrosa* mycelia growth and also its antifungal activities.

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