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F : (6)04-656 6466/8470
: (6)04-653 2350
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No. Fail : F0415
Tarikh : 2 Disember 2011

Prof. Madya Dr. Zuraini Zakaria
Pusat Pengajian Pendidikan Jarak Jauh
Universiti Sains Malaysia

Puan,

LAPORAN AKHIR SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS)
Tajuk Projek : A Fundamental Study of the Antifungal Activity of Crude Extracts and Extracted Phenols of Common Edible Ferns
No. Akaun : 203/PJJAUH/671126

Dengan hormatnya perkara di atas dirujuk.

2. Terlebih dahulu saya ucapkan ribuan terima kasih di atas satu salinan laporan akhir untuk projek penyelidikan seperti tajuk di atas.

3. Adalah dimaklumkan walaupun projek ini telah selesai, kerjasama Jabatan Bendahari dipohon untuk menguruskan penutupan akaun projek pada selewat-lewatnya **31 Disember 2011**. Tempoh ini bertujuan untuk menyelesaikan semua urusan tuntutan dan bayaran yang telah dibelanjakan di dalam tempoh projek. Walau bagaimanapun, puan dinasihatkan supaya tidak mengeluarkan borang-borang pesanan baru di dalam tempoh ini.

4. Selanjutnya sila ambil perhatian terhadap perkara-perkara berikut sekiranya berkaitan:

(i) Semua penerbitan harus merakamkan penghargaan kepada **Skim Geran Penyelidikan Fundamental (FRGS)** dan puan dipohon mengemukakan satu salinan ke Pejabat ini.

(ii) Bahagian Penyelidikan & Inovasi boleh/akan mengagihkan semula peralatan yang telah dibeli menggunakan peruntukan geran ini seandainya terdapat penyelidik lain yang memerlukan peralatan tersebut.

5. Akhir sekali, tahniah di atas usaha dan kejayaan pihak puan dapat menyelesaikan projek ini dengan jayanya.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"
'Memastikan Kelestarian Hari Esok'

Yang menjalankan tugas,

HAH, HAR, SM

(AMRA OTHMAN)
Penolong Pendaftar
Unit Pengurusan Geran & Kontrak

LAPORAN AKHIR SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS)

Tajuk Projek : A Fundamental Study of the Antifungal Activity of Crude Extracts and Extracted Phenols of Common Edible Ferns

No. Akaun : 203/PJJAUH/671126

s.k. Dekan Penyelidikan
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Perpustakaan Hamzah Sendut
Universiti Sains Malaysia

} Disampaikan satu salinan laporan akhir projek untuk simpanan Perpustakaan

Penolong Bendahari Kanan
Unit Kumpulan Wang Penyelidikan
Jabatan Bendahari
Universiti Sains Malaysia

} Mohon kerjasama pihak puan untuk menguruskan penutupan akaun projek selewat-lewatnya pada **31 Disember 2011** dan mohon kemukakan satu salinan penyata kewangan terakhir ke Pejabat ini untuk tujuan rekod

Pegawai Sains
Pelantar Sains Fundamental
Pejabat Pelantar Penyelidikan
Universiti Sains Malaysia

F0415

BORANG FRGS – P3(R)

FINAL REPORT
FUNDAMENTAL RESEARCH GRANT SCHEME (FRGS)

Laporan Akhir Skim Geran Penyelidikan Asas (FRGS) IPT
Pindaan 1/2010

(JULY 2009 – OCTOBER 2010)

A RESEARCH TITLE : A FUNDAMENTAL STUDY OF THE ANTIFUNGAL ACTIVITY OF CRUDE EXTRACTS AND EXTRACTED PHENOLS OF COMMON EDIBLE FERNS
Tajuk Penyelidikan

PROJECT LEADER : ASSOC. PROF. DR. ZURAINI BT ZAKARIA
Ketua Projek

PROJECT MEMBERS : 1.
(including GRA) 2.
Ahli Projek

PROJECT ACHIEVEMENT *(Prestasi Projek)*

B

ACHIEVEMENT PERCENTAGE			
Project progress according to milestones achieved up to this period	0 - 50%	51 - 75%	76 - 100%
Percentage			√

RESEARCH OUTPUT		
	Indexed Journal	*Non-Indexed Journal*
Number of articles/ manuscripts/ books *(Please attach the First Page of Publication)*		1. **Zuraini Z.**, S. Sasidharan, S. Roopin Kaur & M. Nithiyayini **(2010)** Antimicrobial and antifungal activities of local edible fern *Stenochlaena palustris* (Burm. f.) Bedd. **Pharmacologyonline** No.1 pp 233-237 2. **Zuraini Z.,** Sasidharan S. & Sangetha S. **(2010)** Antifungal activity of the edible ferns : Application for the public health. **International Journal of the Humanities,** Vol.8, Issue 8, pp113-118 3. Sumathy V., Jo Thy Lachumy S., **Zuraini Z.** & Sasidharan S. **(2010)** Effects of *Stenochlaena palustris* leaf extract on growth and morphogenesis of food borne pathogen *Aspergillus niger*, ***Malaysian J of Nutrition*** 16(3) : 439-446
Conference Proceeding *(Please attach the First Page of Publication)*	International	*National*

Intellectual Property *(Please specify)*	

HUMAN CAPITAL DEVELOPMENT

Human Capital	Number				Others (please specify)
	On-going		Graduated		
Citizen	Malaysian	Non Malaysian	Malaysian	Non Malaysian	
PhD Student					
Master Student	1		1		
Undergraduate Student					
Total	1		1		

EXPENDITURE *(Perbelanjaan)*

C

Budget Approved *(Peruntukan diluluskan)* : RM 90,000.00
Amount Spent *(Jumlah Perbelanjaan)* : RM 89,582.28 (Nov 2010)

Balance *(Baki)* : RM 417.72
Percentage of Amount Spent *(Peratusan Belanja)* : 99.54 %

ADDITIONAL RESEARCH ACTIVITIES THAT CONTRIBUTE TOWARDS DEVELOPING SOFT AND HARD SKILLS
(Aktiviti Penyelidikan Sampingan yang menyumbang kepada pembangunan kemahiran insaniah)

D

International		
Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit) **8th International Conference On New Directions In The Humanities**	**29 June – 2 July 2010**	**UCLA, USA**
National		
Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)		

	PROBLEMS / CONSTRAINTS IF ANY *(Masalah/ Kekangan sekiranya ada)*
E	
	RECOMMENDATION (*Cadangan Penambahbaikan*)
F	

RESEARCH ABSTRACT – Not More Than 200 Words (*Abstrak Penyelidikan – Tidak Melebihi 200 patah perkataan*)

G

This study aims to investigate the antifungal activity biological activities of crude extracts and extracted phenols of ferns used in traditional medicine. *Stenochlaena palustris*, *Diplazium esculentum*, *Nephrolepis biserrata* and *Acrostichum aureum* were extracted in methanol and screened against *Aspergillus niger*, *Rhizopus stolonifer* and *Candida albicans*. Results showed that extracts of *D. esculentum* had the most effective results with the widest inhibition zone range of 10-18mm; relative to 7-15mm, 9-12mm and 8-11mm for *S. palustris*, *N. biserrata* and *A. aureum* respectively. This indicated that *D. exculentum* plant extracts had the broadest inhibitory activity to the pathogenic microorganisms and is promising to act as a potential antifungal agent from natural plant source. Thus the extract was further tested for Minimum Inhibitory Concentration (MIC) and Minimum Fungal Concentration (MFC), and compared to commercial antibiotic micanazole nitrate. As a conclusion, all four selected local edible ferns produced considerable antifungal activities, with the *D. esculentum* stem and leave extracts demonstrated a wider antifungal effect. It would be of interest to further investigate, identify, isolate and purify the possible principle components of the fern extracts towards the development of an accountable antifungal property. Thus further tests are to be conducted to enhance these preliminary findings.

Date : 03 MARCH 2011
Tarikh

Project Leader's Signature:
Tandatangan Ketua Projek

COMMENTS, IF ANY/ ENDORSEMENT BY RESEARCH MANAGEMENT CENTER (RMC)
(Komen, sekiranya ada/ Pengesahan oleh Pusat Pengurusan Penyelidikan)

H

....

....

....

Name:
Nama:

Signature:
Tandatangan:

Date:
Tarikh:

Antimicrobial and Antifungal Activities of Local Edible Fern *Stenochlaena Palustris* (Burm. F.) Bedd

Z. Zuraini[1], S. Sasidharan[2], S. Roopin Kaur[1] and M. Nithiyayini[1]

[1]School of Distance Education, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia.

[2]Institute for Research in Molecular Medicine (INFORM), Universiti Sains Malaysia, 11800 USM, Malaysia.

*Corresponding author: Phone +60125323462, email: srisasidharan@yahoo.com

Summary

This current research was conducted to study the uninvestigated (or less investigated) medicinal ferns found in Malaysia in the search for more effective and non-toxic pharmacological natural compounds. Methanol of extracts from winged bean *Stenochlaena palustris* root, stem and leaf extracts were tested for their antimicrobial activity against 15 microbial species, including 10 bacterial pathogens, one yeasts, and four molds using the disk diffusion assay technique. The leaf extract was found to be most effective against all of the tested organisms, followed by the stem and root extracts. The minimum inhibitory concentrations (MICs) of the leaf extracts determined by the broth dilution method ranged from 50 to 12.5 mg/mL. The preliminary results of present investigation appear to indicate that *S. palustris* of Malaysian Edible fern have higher potential antimicrobial properties.

Key words: antimicrobial activity, antifungal activity, fern, *Stenochlaena palustris*

Introduction

Infectious diseases and their control become serious problem in the medical field. Antibiotics usually suggested for the treatment of infectious diseases, have never been pleasing because of their toxic effect and exert a negative impact on the consumer. As an alternative strategy to prevent infectious diseases, natural compound of plants are being tested for their antimicrobial activity and serve as template for new and more effective antimicrobial agents. This research attempted to study uninvestigated (or less investigated) medicinal plants and ferns found in Malaysia in the search for potential, more effective and non-toxic pharmacological natural compounds.

The pteridophytes which constitute ferns and ferns allies, have been known to man for more than 2000 years, and also been mentioned in ancient literature (1, 2). It has been observed that pteridophytes are not infected by microbial pathogens, which may be one of the important factors for the evolutionary success of pteridophytes and the fact that they survived for more than 350 million years. This information's encouraged us to further investigate other tropical fern found in Malaysia for antimicrobial activity.

Stenochlaena palustris (Burm.) Bedd. (Pteridaceae) is a fern trailing over the ground or scrambling high up trees. It is endemic to a large part of tropical areas from southern and northern India through Malaysia to Polynesia and Australia (3). The tender leaves of *S. palustris* are used as a contraceptive by the local people in the central district province of Papua New Guinea (PNG) and in the Nicobar Islands (4, 5). A search for alkaloid-containing plants in New Guinea found the leaves of *S. palustris* to be alkaloid-negative (6). No other chemical studies on this species have been reported. Therefore, the current study was conducted to study the antimicrobial activity of methanolic extract of *S. palustris* against bacterial and fungal strains.

Materials and methods

Materials: The root, stem and leaf of *S. palustris* were collected from Seberang Jaya, Penang, Malaysia, in Mac 2009.

Extraction of plant material: Fern sample was extracted by cold percolation in methanol (consecutively three times) at room temperature for 24 h the resultant extracts were filtered and concentrated to dryness under reduced pressure below 40 ◦C in rotary evaporator.

Antimicrobial activity: Antibacterial and antifungal activities of the fern extracts were investigated by the disk diffusion method (7, 8). The MHA plates, containing an inoculum size of 10^6 colony-forming units (CFU)/mL of bacteria or $2x10^5$ CFU/mL yeast cells or molds spores on SDA and PDA plates, respectively, were spread on the solid plates with an L-shaped glass rod. Then disks (6.0-mm diam.) impregnated with 25 µL of each extract at a concentration of 100.0 mg/mL were placed on the inoculated plates. Similarly, each plate carried a blank disk by adding every solvent control alone in the center, and antibiotic disks (6.0-mm diam.) of 30 µg/mL Chloramphenicol (for bacteria), and 30 µg/mL Miconazole nitrate (for fungi) were also used as positive controls. All of the plates were incubated at 37°C for 18 hours for bacteria and at 28°C for 48 hours for fungi. The zones of growth inhibition around the disks were measured after 18 hours of in incubation at 37°C for bacteria and 48 hours for fungi at 28°C, respectively.

MIC was determined by both broth dilution methods. For broth dilution tests, 0.1mL of standardized suspension of bacteria (10^6CFU/mL) and fungal cell or spores ($5x10^5$CFU/mL) was added to each tube (containing fractions of three extracts at a final concentration of 0 to 20.0mg/mL) and incubated at 37°C for bacteria for 18 hours or at 28°C for fungi for 48 hours. MICs were taken as the average of the lowest concentration showing no growth of the organism and the highest concentration showing visible growth by macroscopic evaluation. Each assay was performed in triplicate.

Results and discussion

The antimicrobial activity data of methanol extract of *S. palustris* are shown in Tables 1 and 2. All the extracts were found to be active in at least one of the microbial strains tested. In general, both bacteria and fungi were found to be susceptible to the test agents than fungi. The preliminary disk diffusion assay (Table 1) of *S. palustris* extracts of root, stem and leaf showed that the leaf extract had the most distinct effect on most of the tested microorganisms, followed by the stem and root extracts. However, two commercial antibiotics were more effective than any of the extract tested. The minimum inhibitory concentration (MIC) values of methanolic extract of *S. palustris* leaf was evaluated and summarized in Table 2. The determination of MIC of root extract was carried out in this study since the extract showed the best activity in the preliminary antimicrobial screening assay. The methanolic extracts of *S. palustris* root gave low MIC values against *Enterobacter aerogenes,Salmonella typhi* and *Azospirilium lipoferum* with concentration of 12.5 mg/ml. However, the *Klebsiella pneumoniae* showed a highest MIC value of 50.0 mg/ml when tested with root extract of *S. palustris*. The MIC values of the extracts seem to be relatively higher. However, being crude extracts, the overall antimicrobial activity screening results are only indicative of the potential of this fern extracts for the medicinal purposes.

The preliminary results of present investigation appear to indicate that *S. palustris* of Malaysian Edible fern especially lower groups (pteridophytes) have higher potential antimicrobial properties. Further studies aimed at the isolation and identification of active substances from the methanol extracts of *S. palustris* may disclose othe rcompounds with better value for antimicrobial agents.

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Table 1: Antimicrobial activity (zone of inhibition)[a] of *Stenochlaena palustris*

Microorganisms	Zone of inhibition (mm)[b]				
	Leaves	Stems	Roots	C	M
BACTERIA					
Gram-positive					
Staphylococcus aureus	14	13	15	24	ND
Bacillus subtilis	10	9	18	22	ND
Micrococcus sp	9	16	8	26	ND
Gram-negative					
Enterobacter aerogenes	16	10	13	25	ND
E. coli	18	14	15	22	ND
Proteus mirabilis	17	24	21	22	ND
Klebsiella pneumoniae	16	17	14	25	ND
Salmonella typhi	–	9	9	21	ND
Azospirilium lipoferum	14	18	20	26	ND
Azobacter	18	12	17	23	ND
FUNGUS					
Penizillium chrysogenum	14	13	9	ND	21
Rhizopus stolonifer	–	–	–	ND	20
Aspergillus niger	11	15	16	ND	24
Fusarium sp.	12	8	10	ND	22
Saccharomyces cerevisiae	15	11	16	ND	21

[a]Disc diffusion technique
[b]The values (average of triplicate) are diameters of zone of inhibition at 100mg/mL of crude extract, 30µg/mL of Chloramphenicol and 30µg/mL of Miconazole nitrate

ND : Not Determined
C : Chloramphenicol
M : Miconazole nitrate

Table 2: Determination of MIC values of root extracts of *Stenochlaena palustris*

Bacteria	Concentration (mg/ml)										
	100.000	50.000	25.000	12.500	6.250	3.125	1.563	0.781	0.391	0.195	0.098
Gram-positive											
Staphylococcus aureus	–	–	–	+	+	+	+	+	+	+	+
Bacillus subtilis	–	–	–	+	+	+	+	+	+	+	+
Micrococcus sp	–	–	–	+	+	+	+	+	+	+	+
Gram-negative											
Enterobacter aerogenes	–	–	–	–	+	+	+	+	+	+	+
E. coli	–	–	–	–	+	+	+	+	+	+	+
Proteus mirabilis	–	–	–	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	–	–	+	+	+	+	+	+	+	+	+
Salmonella typhi	–	–	–	–	+	+	+	+	+	+	+
Azospirilum lipoferum	–	–	–	–	+	+	+	+	+	+	+
Azobacter	–	–	–	+	+	+	+	+	+	+	+

– : Indicates absence of growth

+ : Indicates presence of growth

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Volume 8, Number 8

Antifungal Activity of the Edible Ferns: Application for Public Health

Zuraini Zakaria, Sangetha Sanduran and Sasidharan Sreenivasan

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Antifungal Activity of the Edible Ferns: Application for Public Health

Zuraini Zakaria, Universiti Sains Malaysia, Penang, Malaysia
Sangetha Sanduran, Sunway University College, Jaya, Malaysia
Sasidharan Sreenivasan, Universiti Sains Malaysia, Penang, Malaysia

Abstract: The objective of this study is to specifically evaluate in vitro, the antifungal properties by assaying the crude extracts obtained from some edible Malaysian fern species. Methanolic extracts of leaves, stems and roots of the local ferns Stenochlaena palustris, Diplazium esculentum, Nephrolepis biserrata and Acrostichum aureum were screened against Aspergillus niger, Rhizopus stolonifer and Candida albicans. Tests using disc diffusion method showed a broad spectrum of antifungal activity especially for Diplazium esculentum leaves. The Minimum Inhibitory Concentration (MIC) and Minimum Fungal Concentration (MFC) were also obtained against the three pathogenic fungal strains, and compared to commercial antibiotic micanazole nitrate. Values of MIC of Diplazium leaves for Aspergillus niger, Rhizopus stolonifer and Candida albicans are 100mg/mL, 50mg/mL and 50mg/mL respectively, whereas values of MFC were 200mg/mL, 100mg/mL and 100mg/mL respectively. The study shows that the fern extracts are favourable antifungal agents with potential applications in public heath against diseases. More scientific information available about the antipathogenic properties of these fern extracts is highly demanded and recommended. With a greater popularization of these aspects of properties, there will be hopefully a noticeable change in our traditional knowledge and habits regarding traditional folk medicines and fern consumption.

Keywords: Antifungal Activity, Stenochlaena Palustris, Diplazium Esculentum, Nephrolepis Biserrata, Acrostichum Aureum, Minimum Inhibitory Concentration (MIC), Minimum Fungal Concentration (MFC)

Introduction

PTERIDOPHYTES ARE ONE of the oldest land plant groups on earth and comprise a vast group of vascular cryptograms. The position of the Pteridophytesor ferns, as intermediates between the lower cryptograms and higher vascular plants, has made this plant group fascinating. Since ancient times, ferns have shown interesting nutritional values and many edible species are being consumed (Kumar and Kaushik, 1999). In the tropics, the fern species are widely used in traditional societies as a source of vegetables in the diet of village people. Many edible species have been recorded that make a significant dietary impact. The plant parts like rhizome, stem, fronds, pinnae and spores may also be used in treating many diseases. However their cultivation potential as medicinal plants has received little attention. Various contributions regarding the taxonomy, ecology and distribution of Pteridophyteshave been published from time to time, but enough attention has not been paid towards their medicinal valuable aspects (Dixit, 1975).

At present, efforts have been made to explore the ethno medicinally important Pteridophytes, and their beneficial aspects areproperly documented.Biological screening of these

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edible plants in various studies especially the antifungal activity may eventually lead to drug and health product development for the future.

Approximately 80% of the world's population still depends on traditional medicine as a source of treatment of various diseases. Malaysia is known with its green tropical vegetation and forest whereby its diverse nature are claimed to possess medicinal values. Around 200 species of ferns are regularly eaten in the tropics. Meanwhile the traditional communities in Sarawak, East Malaysia has gathered 16 species of edible ferns from the forest which account for 8-10% of their vegetable dishes (Christensen, 1997). It is therefore of interest to evaluate the effect of our local fern extracts on three fungal strains *Aspergillusniger*, *Rhizopusstolonifer* and *Candida albicans*.

In this study, four types of common edible ferns in Malaysia namely *Stenochlaena palustris, Diplazium esculentum, Nephrolepis biserrata and Acrostichum aureum* are selected and studied for their antifungal activities (Table 1). These popular ferns are normally collected all year round as their rhizomes, stems, young leaves and shoots are harvested for consumption and medicinal treatments.

Table 1: Fern Species used for Antifungal Evaluation

SPECIES	LOCAL NAME	FAMILY	PARTS CONSUMED
Stenochlaenapalustris	pakumiding	Blechnaceae	Young leaves and shoots
Diplaziumesculentum	pakusungai	Athyriaceae	Young leaf buds and shoots
Nephrolepisbiserrata	pakuputih	Oleandraceae	Young leaves and shoots
Acrostichumaureum	pakubesar	Pteridaceae	Young leaves and curled buds

Material and Method

Plant Materials

Fresh plant parts of leaves, stems and rootsof *Stenochlaenapalustris*, *Diplaziumesculentum*, *Nephrolepisbiserrata* and *Acrostichumaureum* were collected from various areas in Penang in March 2009 (Table 1). The fresh plant materials were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles.

Extraction of Plant Materials

Hundred grams of dried powder of each plant parts were extracted with 80% (v/v) methanol (400mL) in flat bottom glass containers at room temperature, through occasional shaking (2000 rpm) for 7 days (Trease and Evans, 1997; Jeffery *et al.*, 2000). The extracts were filtered with muslin cloth and the methanolicsolutions were evaporated to dryness with a rotary evaporator at 40° to 50°C until the extracts became a paste mass (Dongmo*et al.*, 2003). Various amounts of paste were taken and weighed depending on the concentration needed for later tests. Crude extracts were prepared by diluting the paste in dimethyl sulphoxide (DMSO) and stored at 4°C in airtight bottles for further studies.

Antifungal Activity

Microbial Strain

Microbial strains of *Aspergillusniger*, *Rhizopusstolonifer* and *Candida albicans*were obtained from School of Biological Sciences, UniversitiSains Malaysia and maintained on Potato Dextrose Agar medium (PDA) at 28°C.

Screening for the Antifungal Effect

Antifungal activities of the fern methanolicextracts were investigated by the disc diffusion method (Bauer et al., 1966; Alzoreky and Nakahara, 2003). The test microbe was removed aseptically with an inoculating loop and transferred to a test tube containing 5mL of sterile distilled water. Sufficient inoculums were added until the turbidity equaled to 0.5 McFarland which is 2×10^5 CFU/mL fungi cells or spore. The test tube suspension (1mL) was added to 15 to 20mLNutrientAgar and SabouraudDextrose Agarbefore setting aside the seeded agar plate (9 cm in diameter) for 15min to solidify. Three Whatman's filter paper No. 1 discs of 6 mm diameter were used to screen the antimicrobial activity. Each sterile disc was impregnated with 20µL of the extract (corresponding to 100 mg/mL of crude extract); miconazole nitrate (30 µg/mL), as positive control and methanolas negative control. The plates were incubatedat 28°C for 1-5days depending on the tested microbes. Sensitivity of the microorganism species to the plant extracts was determined by measuring the sizes of inhibitory zones (including the diameter of disc) on the agar surface around the disc. Values less than 8mm were considered as not active against microorganisms (Zhu *et al.*, 2005).All of the experiments were performed in triplicate and results were reported as the average of three experiments.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no growth occurs. Only *D. esculentum* extracts were subjected to determine the minimum inhibitory concentration, since it exhibited the most significant activity against the tested fungi. Potato Dextrose Broth medium was prepared and sterilized in universal bottles, each containing 10mL medium. Different amounts of tested extract were added to the broth medium to give the following concentrations: 0.3125 to 100mg/mL. To each flask, 0.5mL of Tween-80 was added as an emulsifying agent. The flasks were inoculated with 0.5mL fungi or spore suspension containing 10^5 fungi or spores per mL and incubated at 28°C for 1-5 days depending on the microbes. The MIC value was determined as the lowest concentration of plant extract in the broth medium that inhibited visible growth of the test microorganisms. Each assay was carried out in triplicate.

Results and Discussion

The screening of antifungal activity was done as an initial procedure to assure whether the extract compounds which were tested have a favourable antimicrobial activity or otherwise. The screening for the antifungal activity of the methanolicextracts was detected using the disc diffusion method as shown in Table 2. The assay results show that in general, plant extracts of *D. escu lentum*had the most effective results where it gave rise to the widest inhibition zone range which was 10-18mm; relativeto 7-15mm, 9-12mm and 8-11mm for *S. palustris*, *N. biserrata* and *A. aureum* respectively. However the commercial antibiotics were more effective than any of the extracts.Generally, in some cases antibiotics do show wider zones if compared with tested plant extracts; though therapeutically, plant extracts have many advantages over antibiotics, such as they are cheaper and readily available in nature where it can be utilized easily (Ezzat, 2001).

From this antifungal assay result, it indicates that *D. exculentum*extracts have a broad inhibitory activity to pathogenic microorganisms and is promising to act as a potential antifungal agent from natural plant source thus it is carried forward for further testing. Values of MIC and MFC (Minimum Fungal Inhibition) of the *D. esculentum*were subsequentlyevaluated and summarized in Table 3. The methanolic extracts of *D. esculentum*gave MIC values of 50mg/mL for *Rhizopusstolonifer* and *Candida albicans*, whereas for *Aspergillusniger* it was 100mg/mL. The MFC values for *Rhizopusstolonifer*, *Candida albicans* and *Aspergillusniger*were 100mg/mL, 100mg/mL and 200mg/mL respectively.

Table 2: Antifungal Activity (Zone of Inhibition) of Various Fern Extracts Compared to Commercial Antibiotic Miconazole(M)

	Leaves	Stems	Roots	M
Fern : *Stenochlaenapalustris*	-	9	7	23
Aspergillus niger	10	-	-	20
Rhizopusstolonifer	11	12	15	24
Candida albicans				
Fern: *Diplaziumesculentum*	18	5	-	25
Aspergillusniger	15	12	-	18
Rhizopusstolonifer	17	13	10	26
Candida albicans				
Fern: *Nephrolepisbiserrata*	-	-	-	22
Aspergillusniger	10	-	12	19
Rhizopusstolonifer	9	11	-	23
Candida albicans				
Fern: *Acrostichumaureum*	9	-	10	23
Aspergillusniger	11	10	-	18
Rhizopusstolonifer	8	-	9	22
Candida albicans				

Table 3: Antifungal Activity of (MIC and MFC) of *Diplaziumesculentum*

Microorganism	MIC (mg/ml) of the extract [b]	MFC (mg/ml) of the extract [b]
Aspergillusniger	100	200
Rhizopusstolonifer	50	100
Candida albicans	50	100

[b] Agar dilution method

Conclusion

This study concludes that all four selected local edible ferns produced considerable antifungal activities. However the *D. esculentum*stem and leaveextracts demonstrated a wider antifungal effect. This particular fernseems to possess a higher potential as an antifungal agent compared to the other three fern species. It would be of interest to further investigate, identify, isolate and purify the possible principle components of the fern extracts towards the development of an accountable antifungal property.Thus further tests are to be conducted to enhance these preliminary findings.

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About the Authors

Dr. Zuraini Zakaria
Zuraini Zakaria is presently serving as a senior Biology lecturer at the School of Distance Education (SDE), Universiti Sains Malaysia (USM). Research projects are centred on Biology and Distance Education programmes. Her research interests lie in extending whatever expertise and knowledge to be disseminated for the benefit of the public. Her research activities on the use of technology in instruction and strategies for teaching will deepen the knowledge, improve and increase student learning and achievement. Whilst the outcomes from her biological research are her tremendous gift to the field of medicinal plants. The new findings from research of our native crops which have been acknowledged internationally and nation wide, will hopefully foster interactions with pharmaceutical and biotechnology industries. Other fields of biology are Plant Taxonomy and Plant Physiology.

Sangetha Sanduran
Sunway University College, Malaysia

Dr. Sasidharan Sreenivasan
Universiti Sains Malaysia, Malaysia

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Effects of *Stenochlaena palustris* Leaf Extract on Growth and Morphogenesis of Food Borne Pathogen, *Aspergillus niger*

Sumathy V[1], Jothy Lachumy S[1], Zuraini Z[1], & Sasidharan S*[2]

[1] *School of Distance Education, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*
[2] *Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*

ABSTRACT

Some synthetic preservatives have become controversial because they have been proven to cause health problems. These increased health concerns have led consumers to prefer food preservatives based on natural products. Hence, *Stenochlaena palustris* leaf extract was used in this study to evaluate the antifungal activity against food borne pathogen, *Aspergillus niger*. The value of minimum inhibitory concentration and minimum fungicidal concentration of leaf extract for this fungus grown on Potato Dextrose Agar medium was 50 mg/ml. IC_{50} value for the hyphal growth of *A. niger* was at a concentration of 17.41 mg/ml. Morphology changes of *A. niger* treated with the fern leaf extract was observed through scanning electron microscope. The thread-like and elongated hyphae cell wall was disrupted, with some appearing flattened and others being broken. Currently, there is growing interest in using natural food preservatives such as medicinal plant extracts for preserving foods to reduce outbreaks of foodborne pathogenic microorganisms. Hence, *S. palustris* appears to have promise as a safe alternative natural product-based food preservative for future generations.

Keywords: Antifungal activity, *Aspergillus niger*, edible fern, food preservative, *Stenochlaena palustris*

INTRODUCTION

Food borne pathogens such as *Aspergillus niger* are widely distributed in nature, causing considerable mortality and morbidity in the population. The black aspergilli has been identified as *A. niger* group by Raper (Yokoyama *et al.*, 2001). *A. niger* is filamentous and belongs to the family of ascomycete. This fungus is ubiquitous in the environment and well used in the fermentation industry (Baker, 2006) but at the same time *A. niger* can also be a very dangerous agent to humans as it can lead to opportunistic infections in humans. Although several synthetic food preservatives are currently used, there is still a necessity to develop new food preservatives based on natural products since synthetic preservatives in food processing has led to the appearance of remarkable side effects such as carcinogenic effects in living organisms, enlarged livers and increased microsomal enzyme activity (Ames, 1983; Ito, Fukushima & Hagiwara, 1983). Due to these limitations, there is increased interest in

* Correspondence author: Dr S Sasidharan; Email: *srisasidharan@yahoo.com*

finding naturally produced food preservatives capable of preserving foods from spoiling while protecting human health (Ebrahimabadi *et al.*, 2010).

Increased consumer demand for organic natural food stuff on one hand and the observation of growing cases of microbial resistance to existing preservatives on the other has encouraged the world food research community towards seeking new natural antimicrobial substances (Ebrahimabadi *et al.*, 2010). The fern kingdom which is known to have a remarkable diversity in producing natural bioactive compounds has attained a special interest. Today, accessing fern materials with antifungal activity is an ideal objective in the field of food preservatives research based on natural products. Ferns are used as food, shelter and as ornamentals (Benjamin & Manickam, 2007). *Stenochlaena palustris* (Burm.) Bedd is a long creeping fern that is commonly found in dry land. *S. palustris* belongs to the family of Blechnaceae and is widely distributed in the tropical areas of Malaysia, Indonesia and Australia. The leaf of this fern is normally eaten as a vegetable. Medically, this fern is used to treat diarrhea and is also used as a contraceptive (Liu *et al.*, 1998). The present research reports the antifungal activity of the leaf extract of *S. palustris* against the food borne pathogen, *A. niger*.

MATERIALS AND METHODS

Plant collection

The fresh *S. palustris* was collected from an oil palm estate, located at Rawang, Selangor, Darul Ehsan, Malaysia, in March 2010 and authenticated by the botanist of the School of Biological Sciences at Universiti Sains Malaysia. The leaves were separated from the stem and roots and washed with clear tap water. After a thorough washing, the leaves were dried in the oven at 40°C for 5 days and grounded to a powder form for extraction. Dried material in general is preferred because there are fewer problems associated with the large-scale extraction of dried material than fresh plant material (Mdee, Masoko & Ellof, 2009).

Preparation of solvent extract

A total of 300 g of the powdered plant material were extracted at room temperature with methanol by the maceration method. After 7 days, the macerated extract was filtered using No 1 Whatman filter paper and the solvent was removed *in vacuo* to yield the crude extract (Pimenta *et al.*, 2003).

Fungal species

The local clinical isolates of *A. niger* from the collection of the School of Biological Sciences, Universiti Sains Malaysia was used. The fungal isolate was subcultured and prepared for the assessment of fern extract activity on Potato Dextrose Agar (PDA) and maintained at 28°C. Spores were harvested when the culture was fully sporulated by flooding the Petri plate with 2 ml of Potato Dextrose Broth (PDB) and rubbing the culture with a sterile bent glass rod. The spore numbers were determined and verified using a haemacytometer to obtain a concentration of 10^6 spores per ml (Uldahh & Knutsen, 2009).

Antifungal assays

The agar diffusion method was used for the antifungal susceptibility test. Agar plates were prepared in triplicates by pouring 20 ml of Potato Dextrose Agar (PDA) into the petri dishes and allowing it to solidify. The inoculum suspension was uniformly spread with an L-shaped glass rod. Sterile 6 mm diameter discs with 20 μl of extract at a concentration of 100 mg/ml, miconazole as positive control at 30 μg/ml and methanol as negative control, respectively were placed in each plate and incubated at 28°C for 72 hours. The zone of inhibition was measured from the edge of the disc to the inner margin of the surrounding fungus (Chandrasekaran & Venkatesalu,2004).

Minimum fungicidal concentration (MFC)

A serial dilution method was used to determine the minimum fungicidal concentration values for the plant extract. Initially the fungal culture was grown in Potato Dextrose Agar (PDA). The crude extract was dissolved in methanol (15% v/v) to prepare the stock solution and serially diluted with PDA at 45°C to obtain the required 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 0.78 mg/ml and 0.39 mg/ml concentration in each agar plate. After the dilution, the solution was mixed well with an L-shaped glass rod and poured onto the petri plates. When the agar solidified, 1 mm plug of the mycelium was cut and placed at the centre of the plates. The plates were sealed and incubated for 48 hours to measure radial growth. Methanol was used as negative control and miconazole as positive control. The growth inhibition percentage of fungal colonies was calculated using the following formula:

$$\text{Growth inhibition (\%)} = \frac{D_c - D_s}{Dc} \times 100$$

where D_c is diameter of colony in control sample and D_s is the diameter of colony in treated sample (Gandomi *et al.*, 2009).

The values were used to plot a graph of percentage inhibition against concentration to determine inhibition concentration at 50% (IC_{50}). The experiment was conducted in triplicates.

Scanning electron microscopy

Scanning electron microscopy (SEM) observation was performed on *A. niger* that was treated with *S. palustris* extract with a concentration of 50 mg/ml. The plate containing 25 ml potato dextrose agar medium was seeded with 1 ml of the *A. niger* conidial spore suspension containing 10^5 spores per ml from a 120 hour-old culture. The extract (1 mL), at a concentration of 50 mg per ml, was then dropped onto the inoculated agar and was further incubated for another 7 days at the 28°C. A methanol-treated culture was used as a control. Segments of 5-10 mm were cut from cultures growing on potato dextrose plates at various time intervals (1, 2, 3, 4, 5, 6, and 7 days) for SEM examination and placed on a planchette and kept covered in a petri plate. A few drops of 1% osmium tetraoxide were dropped in the petri plate outside the planchette and vapour fixed for 1 hour. Subsequent to vapour fixation, the planchette was slushed in nitrogen at -210°C for a few seconds before proceeding with the freeze drying method. The planchette was then kept in a freeze dryer (EMITECH K750) and the sample was freeze-dried for 10 hours. Following drying, the samples were coated with gold-palladium electroplating and the samples were viewed in SEM (FESEM LEO Supra 50 VP, Germany) operating at 15 kv at different magnifications (Kamilla *et al.*, 2009).

RESULTS

The antifungal activity against the extract screened using the disc diffusion method showed inhibition zone diameter values as tabulated in Table 1. The diameter of the inhibition of the leaf extract against *A. niger* was 11 ± 2 mm on the Potato Dextrose Agar medium after 3 days. The values obtained in the minimum inhibitory concentration and minimum fungicidal concentration studies were 50 mg/ml (Table1) against *A. niger* for *S. palustris* leaf extract tested. The IC_{50} value for fungal inhibition was 17.41 mg/ml (Table 2, Figure 1).

The activity of *S. palustris* leaf extract against the morphology of *A. niger* grown on a PDA medium examined by SEM is shown in Figures 2 and 3. All the pictures were taken at the same magnification. The morphology of *A. niger* can be divided into two parts which are the mycelium and head. In *A. niger*, the matured head is composed of many chains of conidia which are arranged compactly. Microscopic observation for the control group featured conidium in globose to somewhat elliptical in shape having

Table 1. Antifungal activity (diameter of inhibition zone, MIC and MFC) of *S. palustris* leaf methanol extract and Miconazole

	Diameter of inhibition (mm)[1]			
Microorganism	Extract (mg/ml)	Miconazole (i g/ml)	MIC(mg/ml) of the extract[2]	MFC(mg/ml) of the extract[2]
	100.00	30.0		
Aspergillus niger	11±2	24±3	50.00	50.00

[1] The values (average of triplicates) are diameter of inhibition ± SD
[2] Agar dilution method

Table 2. Effect of *S. palustris* on hyphal growth of *Aspergillus niger*

	Hyphal growth inhibition at selected concentrations (%)[1]										
	0.195	0.39	0.78	1.562	3.125	6.25	12.5	25	50	100	IC50
Leaf Exract	0	0	5.65+0.9	11.66+2.45	16.25+3.54	49.47+4.21	67.13+3.75	73.5+3.10	100+1.60	100+1.20	17.41
Miconazole	ND[2]	ND	ND	ND	ND	100+1.43	100+1.67	100+1.54	100+1.24	100+1.29	<0.5

[1] Values are mean of three replicates
[2] ND- not determined

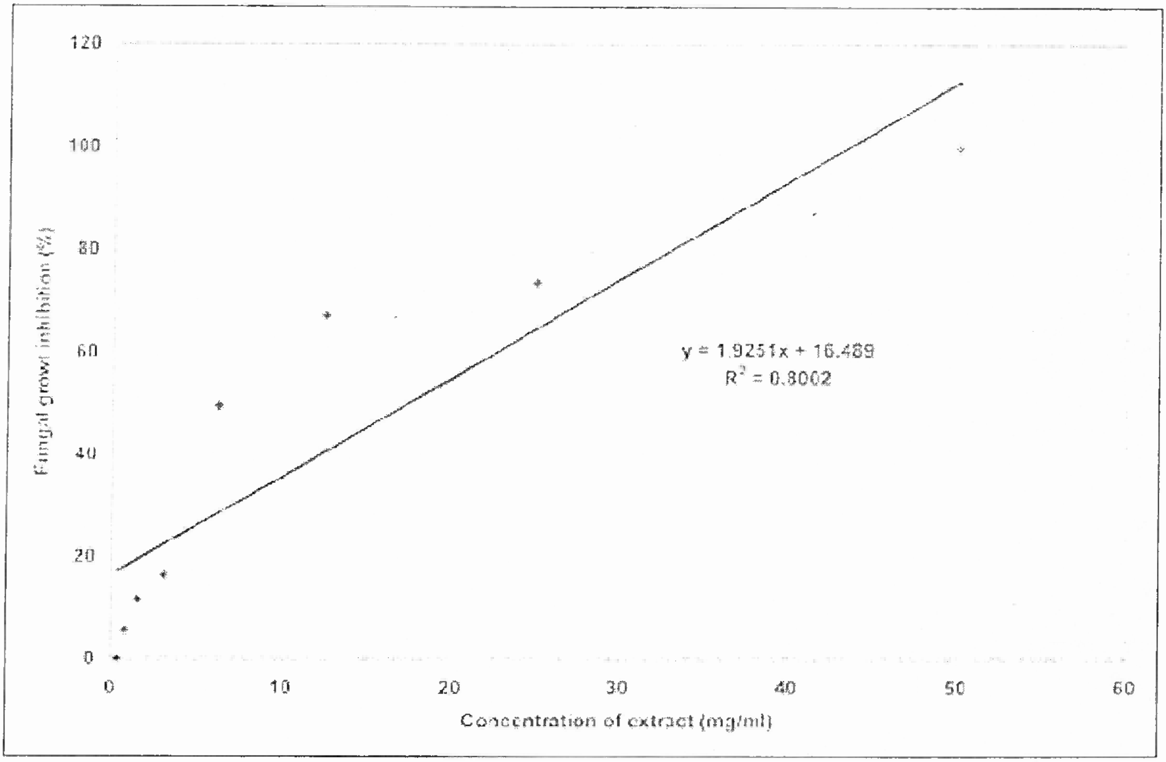

Figure 1. Percentage inhibition against concentration to determine inhibition concentration at 50% (IC_{50})

many conspicuous echinulates (Figure 2a). The conidiophores for the treated group did show minor difference compared to the control group. Spores in the treated group from Day 1 until Day 4 did not show any major disruption on the conidiophores (Figure 2b-e). After Day 5, the spores started shrinking and the round shape was no longer observed. The conidiophores have sharp points with a rough cell wall. In some of the matured heads, breakage of the conidiophores was observed (Figure 2f-h).

The observation for the tread-like and hollow structure of the hyphae is shown in Figure 3. In the control group, *A. niger* was seen to have long, ramified and smooth hyphae . Elongated hyphae looked squashed and damaged in the entire treatment group that received the *S. palustris* leaf extract. Broken hyphae and cell wall disruption was also visible in the microscopic pictures. The disruption in the hyphae structure from Day 1 treatment until Day 7 was very obvious in *A. niger* (Figure 3a-h).

DISCUSSION

Mould infection, especially that caused by *A. niger*, is one of the major problems in patients with AIDS, diabetes, patients undergoing chemotherapy and organ transplant patients (Arif *et al.*, 2009). The drug that is being used currently to control the mould infection leads to some drawbacks that give rise to fungal resistance. There is an urgent need to look for alternative drugs to control mould infection. Focusing on this issue, an experimental study was performed to search for an alternative antifungal drug by testing on a local edible fern, *S. palustris* on *A. niger*. The antifungal activity of *S. palustris* leaf extract against *A.*

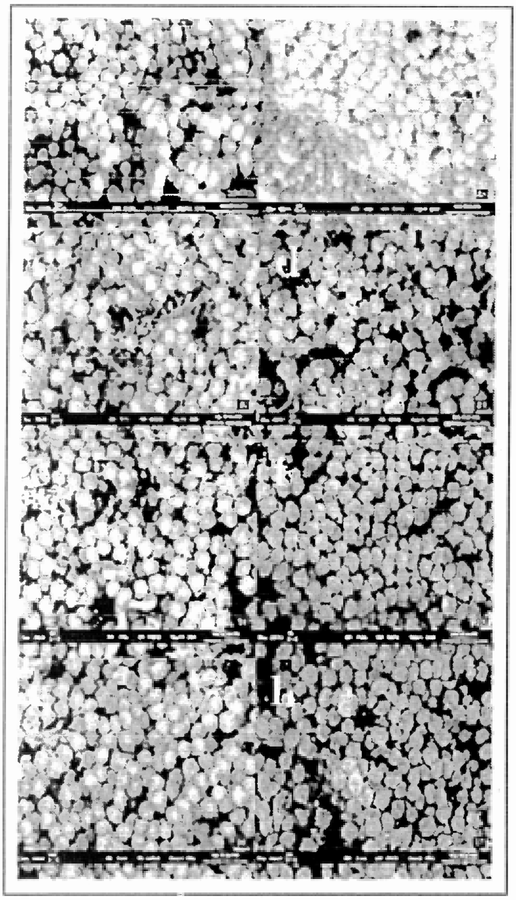

Figure 2. Scanning electron micrographs of *A. niger* conidiophores grown on PDA with and without *S. palustris* leaf extract during 7 days of incubation at 28°C. (a) Control and (b-h) treated conidiophores with 50 mg/ml of extract.

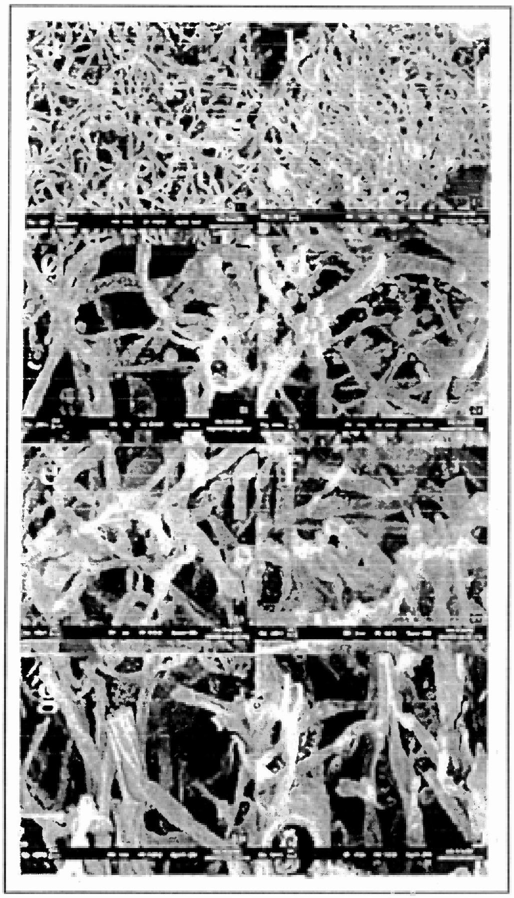

Figure 3. Scanning electron micrographs of *A. niger* mycelium grown on PDA with and without *S. palustris* leaf extract during 7 days of incubation at 28°C. (a) Control and (b-h) flattened and squashed mycelium treated with 50 mg/ml of extract.

niger was evaluated by looking at the zone of inhibition, MIC, MFC, IC_{50} and microscopic study. Fern leaf extract gave rise to low zone of inhibition and the growth of *A. niger* was fully inhibited at 100 mg/ml. Inhibition growth of any microorganism that has been tested with plant extracts is only due to the presence of active compounds in the plant. Generally the extract of any fern consist of mixtures of active and non-active compounds. Alcohol like ethanol, methanol, hexane and many others are commonly used in the extraction process to extract the active compounds from the plant sample. The active compounds are basically known as secondary metabolites and are present naturally in every plant. Examples are flavanoids, alkaloids, terpenoids, tannins and more. It has been reported in previous studies that many potential secondary metabolites possessing antifungal activity have been identified. A study conducted on the screening of antifungal activity of Tanzanian medicinal plants reported that secondary compounds like tannins and saponin were responsible for the antifungal activity (Maregesi *et al.*, 2008).

The antifungal activity of *S. palustris* was effective only beyond 50 mg/ml based on the values obtained from the MIC and MFC in this study. This could be due to many reasons; for instance, the quality and quantity of active compounds that concentrate in the plant parts varies depending on the seasons or the growth cycle. Climate variation and the harvesting of the plants can also lead to certain differences. SEM was performed in this study to support the MIC and MFC values. It is clear from the microscopic observation that the morphology of *A. niger* is altered when it is treated with 50 mg/ml crude extract of *S. palustris* leaf. The crude extract caused more severe injury to the hyphae of *A. niger* than the spores. As the incubation period for the treatment increased, the cell wall of elongated hyphae appeared flattened and broken into halfs. Meanwhile s the spores became more emaciated as the treatment period progressed. Very few studies have reported on the microscopic study of *A. niger* for antifungal activity of a medicinal fern.

CONCLUSION

Based on this study, it was found that *S. palustris* which is one of the commonest ferns found in Asia possesses antifungal activity against *A. niger*. It is supported by the results obtained in the antifungal test. Further studies aimed at the isolation and identification of active substances from the *S. palusris* may disclose other compounds with better value for food preservation as well as natural fern-based medicine.

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