Mal J Nutr 16(3): 439 - 446, 2010

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 45ºC 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 -210ºC 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 (ì 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

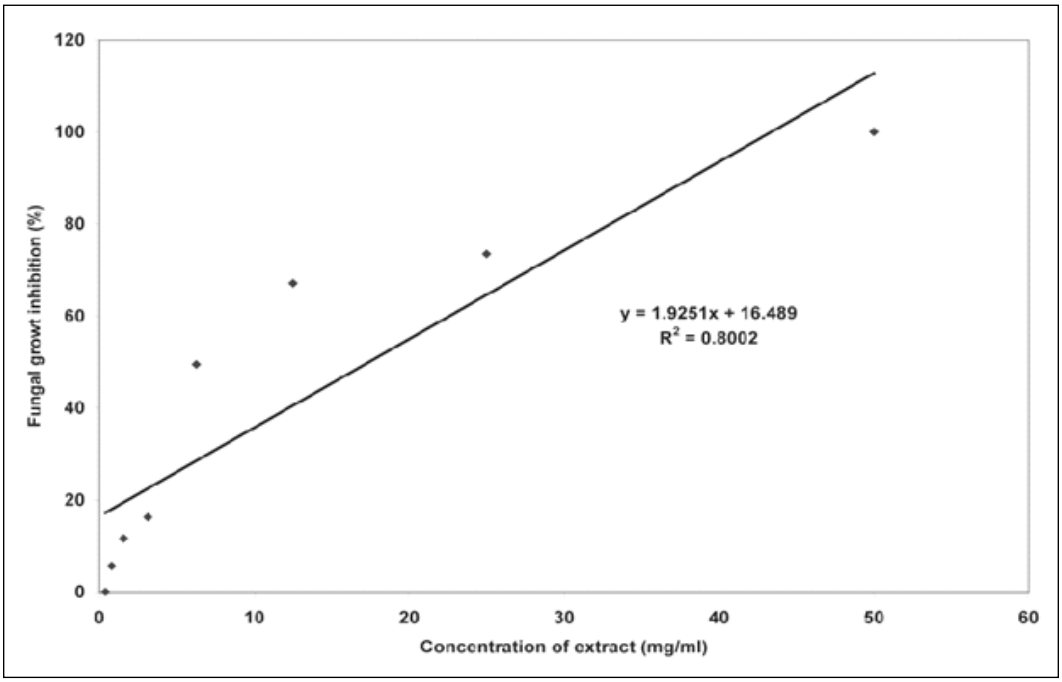

Figure 1. Percentage inhibition against concentration to determine inhibition concentration at 50% (IC_{50})

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.*

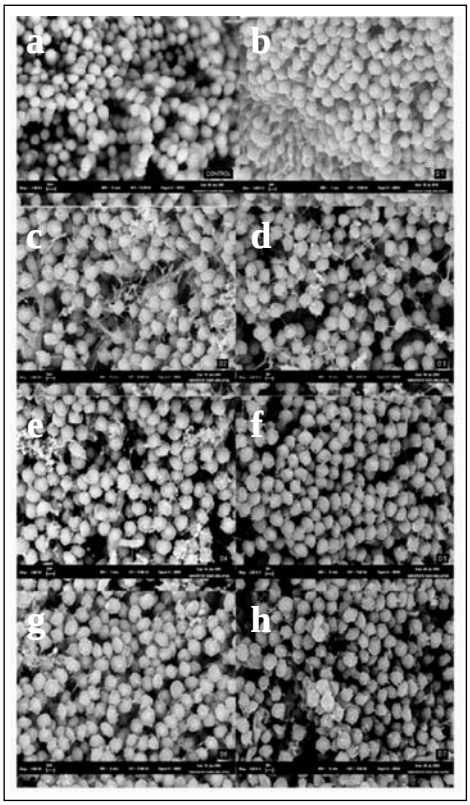

Figure 2. Scanning electron micrographs of *A. niger* conidiophores grown on PDA with and without *S. palustris* leaf extract during 7 days of incubation at 28ºC. (a) Control and (b-h) treated conidiophores with 50 mg / ml of extract.

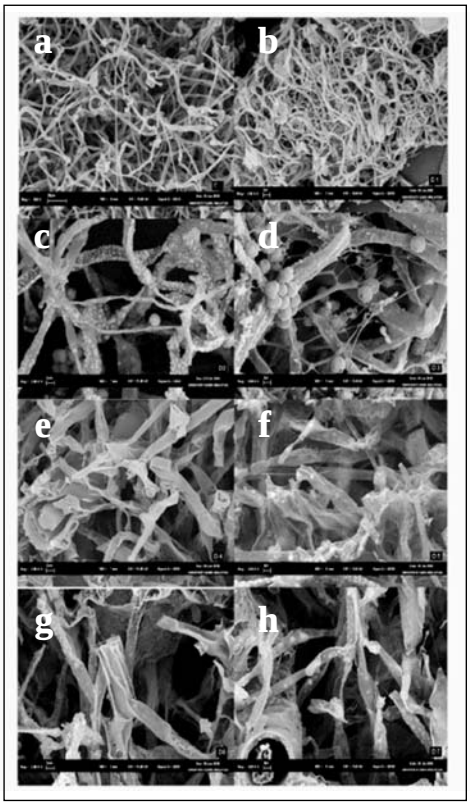

Figure 3. Scanning electron micrographs of *A. niger* mycelium grown on PDA with and without *S. palustris* leaf extract during 7 days of incubation at 28ºC. (a) Control and (b-h) flattened and squashed mycelium treated with 50 mg / ml of extract.

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.

ACKNOWLEDGEMENT

Veloo Sumathi and Subramanion Jothy Lachumy are supported by the USM Fellowship Scheme from the Institute for Postgraduate Studies (IPS) of Universiti Sains Malaysia.

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