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Antibacterial, cytotoxicity and antiviral activities of *Asplenium nidus*

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ABSTRACT

This study described the chemical and biological activities of leaves and roots of Asplenium nidusfractions. Methanol extracts of leaves and roots were fractionated using hexane, chloroform and ethyl acetate and the phytochemical contents were determined. High alkaloid and terpenoids were detected. Cytotoxicity towards Vero cells determined by MTT assay revealed the concentration that reduces 50% of cell viability (CC_{50}) ranged from 0.12 to 1.87 mg/mL which can be considered assafe. The antibacterial activities of fractions were evaluated against eleven types of bacterial pathogens with theminimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). From the MIC and MBC values leaves chloroform fraction showed bactericidal against several bacteria. The selectivity indices ($SI=CC_{50}$/ MIC) for antibacterial activity of each fractions varied from 0.006 to 0.3 indicating that these fractions have a potential as antibacterial agent. Antiviral activity using Herpes simplex virus Type-1 (HSV-1) showed all of the fractions have antiviral activity with effective concentration (EC_{50}) values between 0.01 to 0.125 mg/mL. The selectivity indices ($SI = CC_{50}/EC_{50}$) for antiviral of each tested fractions ranged from 0.96 to 29.92 indicating the usefulness of the extracts as potential antiviral agents.

Key words: *Asplenium nidus*; phytochemicals; antibacterial; antivirus

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INTRODUCTION

The rapid emergence of resistance microorganisms against available antimicrobial agents are a major concern among scientists and clinicians worldwide. The pathogenic viruses, bacteria, fungi and protozoa are become resistant to the existing drugs due totheir overused [1]. To overcome the drawbacks of the current problems, the demand for new antimicrobial agents keep arising. Medicinal plants contains various active metabolitesfor example tannins, flavonoids and alkaloid that that can be exploited for the treatments of various ailments [2] as they contain antimicrobial properties.

Though economic and medicinal values of higher plants especially the angiosperms have been investigated extensively, pteridophytes have been unfortunately ignored. Pteridophytes are important contributors to the earth's plant diversity as being the second largest group of vascular plants. They form a significant and dominant component of many plant communities [3].

Asplenium nidus Linn is a member of Aspleniceae which is commonly known to the locals as 'langsuyar' or bird's nest fern. It has a rosette- shaped basket consisting of long fron [4]. It has been used widely in traditional preparation. For example the rootstock is used to reduce fever and treat elephantiasis [5]. The leavesare being used as anti-inflammatory agent, to wrap fractured bone or wound due to traumatic injury, for treatment of malaria and jaundice [6].Previous study on crude extracts of roots and leaves against HSV-1 [7] showed that this plant has antiviral activity towards HSV-1. This study is to determine further the antibacterial and antiviral activities of the plant. We also determined the cytotoxicity of the fraction in order to evaluate the safety towards normal cells. The

phytochemical contents will allow us to understand the potential group of plant metabolites that are involved in the biological activity.

EXPERIMENTAL SECTION

Preparation of fractions

The plant material was dried at room temperature and ground to fine powder using Waring mill blender. Methanol extraction was prepared according to [8] with modification. Filtered methanol extracts (ME) was successively partitioned with hexane (HF), chloroform (CF) and ethylacetate (EAF) and resulting fractions were concentrated to dryness using rotary evaporator (Heidolph 2 Laborota 4000, Germany). The weight of extracts yield was determined and the fractions were kept in refrigerator 4°C until used.

Phytochemical screening

The fractions of plant extracts were analyzed for qualitative determination of phytochemicals constituents described by [9].

Bacterial, cells and viruses

Eleven bacterial species were available from the stock culture in the Microbiology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM. Six tested Gram positive bacteria were *Bacillus subtilis* ATCC 11774, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 14506, Methycillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, and MRSA isolated from pharyngitis patient from Serdang Hospital, Serdang, Malaysia, labelled as MRSA Tm. Other five Gram negative bacteria were *Escherichia coli* ATCC 10536, *Enterobactera erogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC BAA1144, *Pseudomonas aeroginosa* ATCC 10145 and *Proteus vulgaris* ATCC 33420. Both Vero cells and Herpes Simplex virus (HSV-1) were available from the stock culture in the Virology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM. Vero cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 5% Fetal Bovine Serum (FBS, JR Scientific), penicillin/streptomycin (NacalaiTesque). Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Antibacterial Evaluation

Antibacterial evaluation was carried out according to [10] and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The fractions were diluted in 10% dimethyl sulfoxide (DMSO, Merck) and 5% Tween 20 (Merck). The stock concentration of fractions of 25 mg/ mL was added in Mueller Hinton broth (MHB; Oxoid UK) and serially diluted two fold (in 96 well microtitre plate) to a final volume of 100 µL until 0.1953 mg/mL. Bacterial suspension adjusted to 0.5 McFarland standards was added to test solutions or antibiotics controls to a final volume of 200 µL/ well. The negative control comprises only MHB. Chloramphenicol (Sigma) was used as positive control. All tests were performed in triplicate. The MIC was recorded as the lowest concentration that produced no visible growth after 24 h incubation at 37 ºC.

Determination of MBC was done by plating 5 µL of an aliquot from the well in the MIC test in well with no bacterial growth and plated onto nutrient agar (NA; Oxoid, UK). Then plates were incubated overnight at 37 ºC. The lowest concentration which showed no growth on the agar was defined as MBC.The selective index (SI) for antimicrobial agents can be calculated by using following formula, SI= CC_{50}/ MIC.

Cytotoxicity Evaluation

The cell viability was evaluated by the method of [10] with minor modifications using the 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyltetrazolium bromide (MTT)reagent. The optical density using a multiwell spectrophotometer (Bio-Rad 680) at wavelength 540 nm. The 50% cytotoxic concentration (CC_{50}) was defined as the sample concentration that reduced cell viability by 50% when compared to untreated controls. The CC_{50} valuewas determined by plotting percentage of viability of cell and concentration of fractions using GraphPad Prism6.

Antiviral Evaluation

Confluent Vero cells (70-80% confluent) were infected with 50 pfu HSV-1 for 2 hours. The inoculum was replaced with overlay medium (FBS 5% and methylcellulose 1% in DMEM) and supplemented with test extracts at different concentration. Infected cultures were incubated in a humidified CO_2 incubator for 48 hours before staining with crystal violet. Viral plaques were calculated under a binocular microscope. Viral inhibition (%) was calculated as follows:

Antiviral activity % = (number of plaque untreated- number of plaques test/ number of plaques untreated virus) ×100 %. Where number of plaques test indicates the plaque counts from virus infection with test extract and number of plaques control indicates the number of plaques derived from virus infected cells only. The concentration that reduced plaque formation by 50% relative to control was estimated from graphic plot and defined as 50% effective concentration (EC_{50}) [10].

RESULTS AND DISCUSSION

The yield of leaves and roots fractionsis stated in Table 1. The fraction yield decreased as the polarity of solvent used for the fractionation increased. The higher yield of fractions in hexane rather than ethyl acetate ascribed to the fact that hexane has lowerpolarity than ethyl acetate [11]. Less polar solvent is more efficient in wall degradation which cause higher yield of fractions [12].

Table 1

Fractions	Yield of fractions (g)	Percentage (w/w)
Hexane Fraction of roots (HFR)	0.9794	0.49
Chloroform Fraction of roots(CFR)	0.7076	0.35
Ethyl Acetate Fractionof roots (EAFR)	0.2326	0.12
Hexane Fraction of leaves (HFL)	0.6435	0.32
Chloroform Fraction ofleaves (CFL)	0.5421	0.27
Ethyl Acetate Fractionof leaves(EAFL)	0.1456	0.07

The phytochemical analysis of*A. nidus* leaves and roots fraction is presented in Table 2. Alkaloid and terpenoids can be found in all of the fractions. Anthraquinone can be found only in ethyl acetate fractions. Results from the present investigation showed that these fractions are very rich in phytochemicalsdifferences in their constituents. The composition of secondary metabolite in extracts are dependent on several factors such as nature, polarity of solvent and solvent concentration [12].

TABLE 2: Phytochemical content of *Asplenium nidus* fractions

Component	ALK	FLA	TER	SAP	TAN	STE	ANT
HFR	√	√	√	×	√	√	×
CFR	√	×	√	×	√	×	×
EAFR	√	×	√	×	√	×	×
HFL	√	×	√	×	√	√	×
CFL	√	×	√	×	√	√	×
EAFL	√	×	√	×	×	×	√

ALK:Alkaloid, FLA:Flavonoid, TER:Terpenoids, SAP:Saponins, TAN:Tannins, STE:Steroids, ANT:Anthraquinon

HFR and HFL showed low antibacterial activitywith MIC and MBC valuesagainst tested bacteria were equal to or more than 25 mg/mL (Table 3). Although, MIC value for HFL against *B. subtilis*is low (3.125 mg/mL) but the MBC is high (25 mg/mL). This indicates that HFL is able to inhibit growth of *B. subtilis* but need higher concentration to kill the bacteria. HFL has a higher antibacterial activity than HFR on the basis of lower MBC value than HFR. However, HFR was able to inhibit and kill for MRSA ATCC 43300 at a lower concentration than HFL.

HFL and HFR are low in polarity and therefore contains phytochemicals that displays lower antibacterial activity. According to [13]polar fraction tends to show higher antibacterial activity than non polar fractions. Result depicted that higher antibacterial activity of hexane fraction against Gram negative bacteria than Gram positive bacteria. This also depend on the presence of lipopolysaccharide at the outerlayer of Gram negative bacteria and thin layer of peptidoglycan. The presence of non polar lipopolysccharide prevent the entry of polar substances while at the same time allowing non polar substances going through. Thus, hydrophobic hexane fractions might the cause of inhibiting Gram negative bacteria.

Chloroform fraction is more polar than hexane. Thus, it had showed antibacterial activity than hexane fraction as shown in Table 3. Chloroform fractions of the leaves have anti-MRSA activity relatively higher in *S. aureus*ATCC 43300 with MIC values of 3.125 mg/mL. As chloroform fraction is more hydrophilic than the hexane fraction, chloroform fraction showed a higher antibacterial activity against Gram-positive bacteria while hexane fraction has a higher antibacterial activity against Gram-negative bacteria. Leaves chloroform fraction is bactericidal activity against *S. epidermidis, E. faecalis, E. coli, P. aeruginosa*, MRSA ATCC 43300, and MRSA Tm and bacteriostatic against *B. subtilis.* Root chloroform fraction was bactericidal against *S. epidermidis, B. subtilis, E. aerogenes, K. pneumoniae, P. vulgaris, and P. aeruginosa.* Root chloroformfraction is bacteriostatic against MRSA Tm.

Table 3: MIC and MBC values of *Asplenium nidus* fractions

	Fractions											
	HFR		CFR		EAFR		HFL		CFL		EAFL	
Test bacteria	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Sa	25	>25	25	>25	25	25	25	25	12.5	>25	6.25	25
Se	25	>25	25	25	6.25	25	25	25	25	25	12.5	12.5
Bs	12.5	25	25	25	25	25	3.125	25	6.25	>25	12.5	>25
Ef	25	>25	12.5	>25	12.5	>25	25	>25	12.5	25	12.5	25
Ea	25	25	12.5	12.5	12.5	12.5	6.25	6.25	12.5	>25	12.5	>25
Ec	25	>25	12.5	>25	6.25	>25	25	>25	12.5	12.5	12.5	25
Kp	>25	>25	12.5	25	12.5	25	>25	>25	12.5	>25	12.5	25
Pv	12.5	25	12.5	25	12.5	12.5	12.5	12.5	25	>25	6.25	6.25
Pa	>25	>25	25	25	12.5	12.5	25	>25	25	25	25	25
Ma	6.25	6.25	12.5	>25	3.125	12.5	12.5	12.5	3.125	3.125	1.563	1.563
MTm	25	25	6.25	>25	6.25	12.5	12.5	25	6.25	6.25	1.563	6.25

Note: Sa= Staphylococcus aureus; Se= Staphylococcus epidermidis; Bs= Bacillus subtilis; Ef= Enterococcus faecalis; Ea= Enterobacter aerogenes; Ec= Escherichia coli; Kp= Klebsiella pneumoniae; Pv= Proteus vulgaris; Pa= Pseudomonas aeruginosa; MA= MRSA ATCC 43300; MTm= MRSA Tm. HFR= hexane fraction Roots; CFR= chloroform fraction roots; EAFR= ethyl acetate fraction roots;HFL=hexane fraction leaves; CFL=chloroform fraction leaves; EAFL= ethylacetate fraction leaves.

Ethyl acetate fraction had the highest hydrophilic properties in all three types of fractions tested. In Table 3, ethyl acetate fraction give MIC and MBC values lower than values showed by the hexane and chloroform fractions. Thus, ethyl acetate fraction displays highest antibacterial activity compared to other fractions. Ethyl acetate fractions also have anti-MRSA activity with MIC values high and low MBC against both strains of MRSA tested. As shown by the hexane fraction and chloroform fraction, EAFL are more active in terms of antibacterial activity compared to EAFR. EAFL was bactericidal against all bacteria tested except against*B. subtilis* and *E. aerogenes*whichcannot be determined. Ethyl acetate fraction root also is bactericidal against all bacteria tested except for*E. faecalis* and *E. aerogenes*.

Study by (14) also showed similar antibacterial activity against *E. coli* and *P. aeruginosa*. However, the study did not show antibacterial activity against *E. aerogenes* and *K. pneumoniae* but almost all fractions used in this study have antibacterial activity against both of these bacteria. This results may caused by the concentration used in this study is higher than the concentration used by [14].

The value of CC_{50} stated in Table 4.CFR showed highest CC_{50} value (1.87 mg/ mL) and also lowest CC_{50}value was found in EAFR (0.12 mg/ mL). All fractions can be considered as non toxic to Vero cells. According to [15], substances have value of CC_{50} more than 0.02 mg/mL are non toxic towards viability of cells. Based on Table 4 all of the fractions are not suitable used as antibacterial agents because SI values lower than 10. The SI values above 10 indicating the usefulness of the fractions as potential antibacterial or antiviral agents [16].

Table4: Selective Indices of *Asplenium nidus* Fractions

Bacteria	**Hexane Fraction**				**Chloroform Fraction**				**Ethyl acetate Fraction**			
	Leaves		**Roots**		**Leaves**		**Roots**		**Leaves**		**Roots**	
	CC_{50} (mg/mL)	**SI**	**CC_{50} (mg/mL)**	**SI**	**CC_{50} (mg/mL)**	**SI**	**CC_{50} (mg/mL)**	**SI**	**CC_{50} (mg/mL)**	**SI**	**CC_{50} (mg/mL)**	**SI**
Sa	0.29	0.012	0.94	0.038	0.14	0.011	1.87	0.075	0.21	0.034	0.12	0.005
Se	0.29	0.012	0.94	0.038	0.14	0.006	1.87	0.075	0.21	0.015	0.12	0.019
Bs	0.29	0.093	0.94	0.075	0.14	0.022	1.87	0.075	0.21	0.015	0.12	0.005
Ef	0.29	0.012	0.94	0.038	0.14	0.011	1.87	0.150	0.21	0.015	0.12	0.01
Ea	0.29	0.046	0.94	0.038	0.14	0.011	1.87	0.150	0.21	0.015	0.12	0.01
Ec	0.29	0.012	0.94	0.038	0.14	0.011	1.87	0.150	0.21	0.015	0.12	0.02
Kp	0.29	-	0.94	-	0.14	0.011	1.87	0.150	0.21	0.015	0.12	0.01
Pv	0.29	0.023	0.94	0.075	0.14	0.006	1.87	0.150	0.21	0.034	0.12	0.01
Pa	0.29	0.012	0.94	-	0.14	0.006	1.87	0.075	0.21	0.009	0.12	0.01
MA	0.29	0.023	0.94	0.150	0.14	0.045	1.87	0.150	0.21	0.134	0.12	0.04
MTm	0.29	0.023	0.94	0.075	0.14	0.022	1.87	0.300	0.21	0.134	0.12	0.02

Note: Sa= Staphylococcus aureus; Se= Staphylococcus epidermidis; Bs= Bacillus subtilis; Ef= Enterococcus faecalis; Ea= Enterobacter aerogenes; Ec= Escherichia coli; Kp= Klebsiella pneumoniae; Pv= Proteus vulgaris; Pa= Pseudomonas aeruginosa; MA= MRSA ATCC 43300; MTm= MRSA Tm; - = unidentifiedHFR= hexane fraction roots; CFR= chloroform fraction roots; EAFR= ethyl acetate fraction roots; HFL=hexane fraction leaves; CFL=chloroform fraction leaves; EAFL= ethylacetate fraction leaves

As shown in Table 5, antiviral activity was observed in all six fractions. The EC_{50} values ranged from 0.01 mg/mL to 0.125 mg/mL with the selectivity indices (SI) of each tested material were between 0.96 and 29.92. The highest SI value (29.92) is shown by CFR. All of the fractions showed promising results as antiviral agents except for EAFR which has low SI value (0.96).The presence of secondary metabolite in these fractions might be the responsible agent for antiherpetic activity. The highest antiviral activity was displayed byCFR and EAF that both contains anthraquinones. It haspreviously been shown that antiviral activity can be contributed by anthraquinones and its derivatives [17].

Table 5: CC50 and EC50 values

Samples	CC_{50} (mg/mL)	EC_{50} (mg/mL)	SI (CC_{50}/EC_{50})
HFL	0.29	0.03125	9.28
CFL	0.14	0.0417	3.35
EAFL	0.21	0.0156	13.46
HFR	0.94	0.125	7.52
CFR	1.87	0.0625	29.92
EAFR	0.12	0.0125	0.96

As conclusion, this study showed that all of the fractions of *A. nidus*are non cytotoxic, has low antibacterial activity and can be used as antiviral agent. Antiviral activity of *A. nidus* can be further explored for antiherpetic properties.

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