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PHYTOCHEMISTRY, ANTIBACTERIAL AND ANTIVIRAL EFFECTS OF THE FRACTIONS OF *Asplenium nidus* LEAVES AQUEOUS EXTRACT

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ABSTRACT

In this study the phytochemical content, antibacterial and antiviral potentials of *Asplenium nidus* leaves aqueous extract fractions was described. Leaves aqueous extract was fractionated using chloroform, hexane and ethyl acetate. Phytochemical screening revealed the presence of alkaloid, flavonoids and terpenoids in all fractions with anthraquinones available only in the ethyl acetate fraction. Safety of the fractions on Vero cells was determined from CC_{50} value i.e. the concentration that reduces 50% of cell viability. The fractions are not cytotoxic with CC_{50} value ranged from 0.78 to 32 mg/mL. The antibacterial activities of the fractions were evaluated against fifteen pathogenic bacteria by determining the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). The MIC and MBC values for the ethyl acetate fraction showed highest bactericidal activity against fourteen bacteria. The antibacterial selectivity indices (SI = CC_{50}/MIC) for the fractions ranged between none to 40.94. The fractions have antiviral potential against Herpes Simplex Virus Type I (HSV-1) with effective concentration that reduces 50% of plaque formation (EC_{50}) were between 0.056 to 0.54 mg/mL and selective index (SI = CC_{50}/EC_{50}) of the fractions ranged between 14 to 59. As a conclusion, fractions from the aqueous extract of *A. nidus* have potential as antibacterial and antiviral agents that may be attributed by the anthraquinones content.

Key words: *Asplenium nidus*, phytochemical content, cytotoxicity, bactericidal activity, anti HSV-1 activity

INTRODUCTION

The genus *Asplenium* (family Aspleniceae) consists of more than 700 species of ferns. *Asplenium nidus* Linn locally known as *langsuyar* or bird's nest fern is an epiphytic fern. Traditionally, the leaves were used as antipyretic agent to treat elephantiasis, emollient in cough and chest diseases (Benjamin & Manickam, 2007). The leaves also hosts for a diversity of fungal endophytes that contributes to secondary metabolites with low antibacterial activity (Ibrahim & Japri, 2015). Previous study had shown antibacterial activity of the *A. nidus* leaves crude methanol extract (Lai *et al.,* 2009) and the fractions of methanol leaf and root extracts (Tahir *et al.,* 2015). Methanol and aqueous crude extracts of the leaves and roots also showed antiviral activity against herpes simplex virus type-1 (HSV-1) (Tahir *et al.,* 2014).

Hence, this study is done to determine the phytochemical content, cytotoxicity, antibacterial and antiviral activities of the different fractions from the leaves aqueous extract. Determining the fractions cytotoxicity are important to evaluate the safety of fractions towards normal cells before further evaluation on the effects on the microbes. Screening for the phytochemical contents will allow the understanding of plant group of metabolites involved in the antimicrobial activities.

MATERIALS AND METHODS

Preparation of fractions

The leaves were dried at room temperature and were ground to fine powder using Waring mill blender. Aqueous extraction (AE) was prepared by

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hot technique with 100 g of dry *A. nidus* leaves were heated (up to 100°C) in 1 L of distilled water for 20 minutes. The AE were then filtered by Whatman filter paper No. 1 and centrifuged at 4000 rpm for 2 minutes. Fractionation was performed by successive partitioning of the AE with chloroform, hexane and ethyl acetate to produce chloroform fraction (CF), hexane fraction (HF) and ethyl acetate fraction (EAF) respectively. The rotary evaporator (Heidolph 2, Laborota 4000, Germany) were used to concentrate the fractions. The weight of the three fractions were determined and kept in refrigerator at 4°C until used.

Phytochemical screening

Qualitative determination of phytochemicals constituents of the fractions were analysed including alkaloid, flavonoids, terpenoids, saponin, tannin, steroid and anthraquinones using methods previously described by Harborne (1973).

Cells, bacteria and virus

Fifteen bacterial species were obtained from the stock culture in the Microbiology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). Gram positive bacteria include; *Bacillus subtilis* ATCC 11774, *Streptococcus pyogenes* ATCC 122344, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 11632 and methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300. Gram negative bacteria were *Escherichia coli* ATCC 10536, *Enterobacter aerogenes* ATCC 13048, *Pseudomonas aeroginosa* ATCC 10145, *Proteus mirabilis* ATCC 12453, *Proteus vulgaris* ATCC 33420, *Salmonella typhimurium* ATCC 51812, *Serratia marcescens* ATCC 13880, *Shigella sonnei* ATCC 29930, *Vibrio cholerae* and *Vibrio fluvialis*. Vero cells as host cell and HSV-1 clinical strain as test virus were available from the Virology Laboratory stock collection, 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), 100U/L non-essential amino acid (Sigma, Life-science) and 100U/L penicillin/streptomycin (Nacalai Tesque). Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cytotoxicity evaluation

The cytotoxicity of fractions was evaluated by the method of Mossman (1983) using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5 diphenyltetrazolium bromide (MTT) reagent. The optical densities were measured at wavelength 540 nm using a multiwell spectrophotometer (Bio-Rad 680). The 50% cytotoxic concentration (CC_{50}) was determined by plotting the percentage of viable cells against the test fractions concentration using GraphPad Prism 6. The sample concentration that reduced cell viability by 50% when compared to untreated controls is considered as the CC_{50} value.

Antibacterial evaluation

Antibacterial activity was evaluated by determining the Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the procedure described in BSAC (1991). Fractions stocks at 25 mg/ mL were prepared in 5% Tween 20 (Merck) and 10% dimethyl sulfoxide (DMSO, Merck). Stocks were serially diluted two fold in Mueller Hinton broth (MHB; Oxoid UK) in 96 well microtiter plate to a final volume of 100 µL to the concentration of 0.1953 mg/mL. Test bacterial suspensions were prepared to have similar density to 0.5 McFarland standards before test solutions or antibiotics controls were added to a final volume of 200 µL/ well. Negative control wells were added with MHB only. Chloramphenicol (Sigma) serves as positive control. All tests were done in triplicate. The lowest concentration with no visible growth after 24 h incubation at 37ºC was recorded as the MIC. To determine the minimum bacteriocidal concentration (MBC), an aliquot of 5 µL from the well in the MIC test with no bacterial growth was plated onto the nutrient agar (NA; Oxoid, UK). The plates were incubated at 37ºC overnight. MBC was defined as the lowest concentration which showed no growth on the agar. Selective indices (SI) for antibacterial capabilities were determined by dividing CC_{50} value with MIC value.

Antiviral Evaluation

HSV-1 at 50 pfu were infected for 2 hours on 70-80% confluent Vero cells. The inoculum was aspirated and overlay medium containing FBS 5%, methylcellulose 1% in DMEM and supplemented with test extracts at different concentration (0, 5, 2.5, 1.25, 0.625, 0.325, 0.1625, 0.0625 mg/mL). Infected cultures were incubated in humid incubator with 5% CO_2 for 48 hours. Plaques are made visible by staining with crystal violet for 45 minutes. After incubation, viral plaques were enumerated using inverted microscope. Antiviral activity was determined as viral inhibition percentage (%) that can be calculated as follows:

$$\text{Viral inhibition percentage (\%)} = \frac{\text{number of plaque (untreated)} - \text{number of plaques (test)}}{\text{number of plaque (untreated)}} \times 100$$

with number of plaques (test) indicates the number of plaques visible after virus infection and treated with test extract. Number of plaque (untreated) indicates the number of plaques derived from virus infected cells with no treatment. The concentration that reduced viral plaque formation by 50% relative to no treatment control was estimated from plotted graph and defined as 50% effective concentration (EC_{50}) (Fayyad *et al.,* 2013).

RESULTS AND DISCUSSION

The initial weight of *A. nidus* aqueous extracts of leaves is 3.87 g. The yields after fractionation of the extract are stated in Table 1. According to Sultana *et al* (2009) increment of yield of extractions is due to several factors such as polarity of solvent, concentration of solvent and technique used in the extraction process. The fractions yield in this study showed the increment in yield with the increasing polarity of solvents. The solvent used in this study have different degree of polarities with hexane is non- polar, chloroform (semi-polar) and ethyl acetate (polar). This can be also related to the ability of the compounds to dissolve in the polar solvent (Jones & Fleming, 2010).

The presence of active compounds from different fractions of *A. nidus* is shown in Table 2. All of the fractions in this study have shown the presence of alkaloids, flavonoids and terpenoids. Flavonoids from plant such as quercetin, naringin, hesperetin and catechin affect in reduction of infectivity and reduced intracellular reaction replication of HSV-1, polio-virus type 1 and parainfluenza virus type 3 (Kaul *et al.*, 1985). Terpenoids also have been reported to exhibit potent inhibitory activity against herpes simplex virus (Niedermeyer *et al.,* 2002). While, anthraquinones is an aromatic organic compound which can be soluble in the organic solvent. The previous phytochemical screening from methanol leaves extract done by Tahir *et al* (2015) showed composition of metabolite such as alkaloid and terpenoids with anthraquinones only in ethyl acetate fraction. Anthraquinones is only present in ethyl acetate fraction compared to the other solvents. Ethyl acetate is polar, organic solvent that has ability to extract anthraquinones, an organic compound (Xu *et al.,* 2010).

The MIC and MBC values of the fractions, positive and negative controls for selected bacteria are shown in Table 3. MIC and MBC is the technique used to determine bactericidal activity of any antimicrobial agent of against selected microorganism. Bactericidal activity is considered if the MBC value is not more than four times the value of MIC (French, 2006). Chloroform fraction (CF) and ethyl acetate fraction (EAF) are bactericidal towards all of tested bacteria except *S. sonnei*. The fractions showed different antibacterial activity towards Gram positive and Gram negative bacteria. The difference in sensitivity of bacteria against fractions may be explained by the morphological differences between these organisms. Gram negative bacteria have an outer phospholipid membrane carrying the lipopolysaccharide which makes the cell wall more impermeable to the fractions. While Gram positive bacteria are more susceptible since they have only an outer peptidoglycan layer which is not an effective permeability barrier (Arias *et al.,* 2004).

EAF has the highest antibacterial activity than other fractions with lower MIC value and its SI value (Table 4) is highest for most of the tested bacteria. Anthraquinones which is only present in EAF might be the contributing factor affecting the antibacterial activity which has also been reported in the earlier study (Tahir *et al.,* 2015). Anthraquinones display antimicrobial effect through redox reactions. Its complex irreversibly binding with nucleophilic amino acids in microbial proteins often lead to inactivation and loss of function (Sher, 2009). The antimicrobial activity was proven when anthraquinones isolated from different part of *Cassia nodosa* are effective against pathogenic microbial strain such as *E. coli, S. aureus* and *P. aeruginosa* (Yadav *et al.,* 2013).

The antiviral activity of fractions against HSV-1 was summarized in Table 5. The CC_{50} ranged between 0.78 to 32 mg/mL. While EC_{50} of each tested fraction were 0.056 to 0.54 mg/mL with the SI values is more than 10. According to Gad (2000) these fractions are not toxic to Vero cells as

Table 1. Yield of fractionation of *A. nidus* leaves

Fractions	Yield of fractions (g)	Percentage (w/w)
Hexane fraction (HF)	1.1293	29.18
Chloroform fraction (CF)	1.9240	49.72
Ethyl acetate fraction (EAF)	3.0359	78.45

Table 2. Phytochemical content of *A. nidus* fractions

Component Fractions	ALK	FLA	TER	SAP	TAN	STE	ANT
HF	√	√	√	–	–	–	×
CF	√	√	√	–	–	–	×
EAF	√	√	√	–	–	–	“

Note: ALK: alkaloid, FLA: flavonoid, TER: terpenoid, SAP: saponins, TAN: tannins, STE: steroid, ANT: anthraquinones. √ = Positive, – = negative.

Table 3. Value of MIC and MBC of hexane, chloroform and ethyl acetate fraction of *A. nidus* of aqueous extract

	Bacteria	Hexane fraction		Chloroform fraction		Ethyl acetate fraction		Chloromphenicol	MHB
		MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/L)	
1	MRSA	6.25	6.25*	3.125	12.5*	3.125	6.25*	16	–
2	*S. aureus*	6.25	25*	3.125	12.5*	0.782	3.125*	>16	–
3	*S. epidermidis*	12.5	>25	6.25	25*	6.25	25*	>16	–
4	*S. pyogenes*	>25	>25	6.25	6.25*	6.25	6.25*	16	–
5	*B.subtilis*	12.5	>25	6.25	25	6.25	25	2	–
6	*E. coli*	>25	>25	6.25	25*	3.125	12.5*	8	–
7	*S. sonnei*	25	>25	12.5	>25	6.25	>25	>128	–
8	*S. typhimurium*	6.25	25*	3.125	12.5*	1.563	6.25*	32	–
9	*P. vulgaris*	>25	>25	3.125	12.5*	3.125	12.5*	64	–
10	*P. mirabilis*	6.25	12.5*	3.125	6.25*	3.125	3.125*	>128	–
11	*P. aeruginosa*	>25	>25	12.5	25*	6.25	12.5*	200	–
12	*S. marcescens*	>25	>25	6.25	25*	3.125	12.5*	128	–
13	*E. aerogenes*	25	>25	3.125	12.5*	1.563	6.25*	>128	–
14	*V. cholera*	6.25	>25	3.125	12.5*	3.125	12.5*	>100	–
15	*V. fluvialis*	>25	>25	12.5	12.5*	1.563	1.563*	>100	–

Notes = * bactericidal activity, 1–5 = Gram positive bacteria, 6–15 = Gram negative bacteria, – = no activity.

Table 4. Value of CC_{50}, MIC and SI of hexane, chloroform and ethyl acetate fraction of *A. nidus* of aqueous extract

Bacteria	Hexane fraction		SI	Chloroform fraction		SI	Ethyl acetate fraction		SI
	CC_{50} (mg/mL)	MIC (mg/mL)		CC_{50} (mg/mL)	MBC (mg/mL)		CC_{50} (mg/mL)	MIC (mg/mL)	
MRSA	6.25	6.25	1	0.7813	3.125	0.25	32	3.125	10.24*
S. aureus	6.25	6.25	1	0.7813	3.125	0.25	32	0.782	40.94*
S. epidermidis	6.25	12.5	0.5	0.7813	6.25	0.125	32	6.25	5.12
S. pyogenes	6.25	>25	–	0.7813	6.25	0.125	32	6.25	5.12
B. subtilis	6.25	12.5	0.5	0.7813	6.25	0.125	32	6.25	5.12
E. coli	6.25	>25	0.25	0.7813	6.25	0.125	32	3.125	10.24*
S. sonnei	6.25	25	1	0.7813	12.5	0.063	32	6.25	5.12
S. typhimurium	6.25	6.25	–	0.7813	3.125	0.25	32	1.563	20.47*
P. vulgaris	6.25	>25	1	0.7813	3.125	0.25	32	3.125	10.24*
P. mirabilis	6.25	6.25	–	0.7813	3.125	0.25	32	3.125	10.24*
P. aeruginosa	6.25	>25	–	0.7813	12.5	0.063	32	6.25	5.12
S. marcescens	6.25	>25	–	0.7813	6.25	0.125	32	3.125	10.24*
E. aerogenes	6.25	25	0.25	0.7813	3.125	0.25	32	1.563	20.47*
V. cholera	6.25	6.25	1	0.7813	3.125	0.25	32	3.125	10.24*
V. fluvialis	6.25	>25	–	0.7813	12.5	0.063	32	1.563	20.47*

Notes = * SI more than 10.

Table 5. Value of CC_{50}, EC_{50}, and SI of different fractions against HSV-1

Fractions	CC_{50}	EC_{50}	SI
Hexane	6.25 mg/mL	0.32 mg/mL	20
Chloroform	0.78 mg/mL	0.056 mg/mL	14
Ethyl acetate	32 mg/mL	0.54 mg/mL	59
Acyclovir	> 200 µg/mL	12.9 µg/mL	–

– = undefined due to maximum concentration used in this study at 200 µg/mL.

the CC_{50} value is more than 0.02. The SI value above 10 indicates the usefulness of the any substances as potential antiviral agents (Dargan, 1998). The highest of SI value shown by the ethyl acetate fraction. The SI values of the aqueous fractions in this study were higher than methanol fractions that previously done by Tahir *et al* (2015). Although, the similarities between this study and previous study is the presence of anthraquinones causes the fractions have higher antiviral activity. But, the

antiviral activities of the fractions were still lower than the crude extracts which have been shown by Tahir *et al* (2014). One possible explanation is that contents presents are differ between crude extract and fractions. The crude extract of leaves have more phytochemical composition than fractions including tannins that might be increasing effectiveness of antiviral effects against HSV-1. Tannins have been reported to be inhibiting HSV-2 penetration to cells and cell nucleus (Cheng *et al.,* 2002). Thus it is not surprising to express such effects against the tested microbes in this study.

CONCLUSION

All of the fractions of *A. nidus* showed anti-bacterial activities against tested bacteria as well as anti HSV-1. Fractions can be further explored of its potential due to the non-toxicity nature of the fraction from leaves aqueous extract are safe. From the results of this study, it is pertinent to say that organic solvents play a vital role in revealing bioactive compound. Alkaloid, flavonoids, terpenoids are important compound available in the *A. nidus* aqueous extract for antibacterial and antiviral activities which increases the SI value.

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