Original Article

Phytochemical Screening and Evaluation of Cytotoxic Effect and Antioxidant Activity of Fractions Isolated from *Stenochlaena palustri* (Burm.f.) Bedd. Leaves

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ABSTRACT

Introduction: *Stenochlaena palustris* (Burm.f.) Bedd. is an edible fern from Blechnaceae family, native to India through Southeast Asia to Polynesia and Australia. The study was conducted to evaluate the cytotoxic effect and antioxidant activity of fractions obtained from *Stenochlaena palustris* leaf extracts. **Methods:** *Stenochlaena palustris* (Burm.f.) Bedd. was tested for its antioxidant activity using DPPH assay and *in vitro* cytotoxic effect against HeLa cancer cell line using MTT assay. Major fractions were obtained from ethanol and ethyl acetate leaves extract of *Stenochlaena palustris* (Burm.f.) Bedd. through gravity column chromatography and the secondary metabolites were screened using qualitative phytochemical analysis. **Results:** In DPPH assay, the highest radical scavenging activity was exhibited by fraction 11 of ethanol extract and fraction 4 of ethyl acetate extract at 98.47 ± 0.002% (ED_{50} = 0.120 mg/mL), and 81.38 ± 0.018% (ED_{50} = 0.650 mg/mL), respectively. Meanwhile, ascorbic acid and kaempferol exhibited radical scavenging of 80.95 ± 0.002% (ED_{50} = 0.014 mg/mL) and 98.67 ± 0.006% (ED_{50} = 0.011 mg/mL). As for MTT assay, the percentages of cell viability of both cell lines decreased as the concentration increased. Fraction 7 of ethanol extract and fraction 1 of ethyl acetate extract exhibited the lowest IC_{50} value of 4.58 μg/mL and 8.60 μg/ml, respectively. Doxorubicin hydrochloride showed lowest IC_{50} value of 2.21 μg/mL against HeLa cells. **Conclusion:** The fractions isolated from ethanol and ethyl acetate leaves extract of *Stenochlaena palustris* (Burm.f.) Bedd. exhibited higher cytotoxic effect against HeLa cells, and higher radical scavenging activity.

Key words: *Stenochlaena palustris* (Burm.f.) Bedd, Phytochemical analysis, DPPH and MTT assays.

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INTRODUCTION

Stenochlaena palustris (Burm.f.) Bedd. or commonly known as '*pucuk midin*' or '*kelakai*' among the Malays,[1] is a climbing fern with long-creeping rhizome, 0.5 to 1.0 cm in diameter, green in colour, but turns brownish upon maturity. *S. palustris* is an adaptable species, commonly found at the edges of hot springs, mangroves and fresh water at forest margins. Furthermore, the fern is found growing on a wide range of tress and palms.[2] It is widely distributed throughout Malaysia, India, Myanmar, Yunnan in China, Laos, Thailand, Vietnam, Solomon Islands, south and northern Australia, islands of Fiji, Samoa and Tonga.[3] This species is also present in East Africa as well as on the Indian Ocean islands such as Madagascar.[4] The reddish young fronds are harvested from the wild and eaten as vegetables in countries like Malaysia, Thailand, Philippines, and Indonesia. In Malaysia, the plant is taken raw or cooked with boiling water in treating diarrhoea, while in Sumatra it is used as mild laxative.[1] In Nicobar Islands and the central region of Papua New Guinea, the tender leaves of *S. palustris* are used as a

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contraceptive.[5] The juice of the fern can help treating fever. In Malaysia and Indonesia, the fern is made into paste and applied on the ulcers, wound injuries and bacterial infected skin.[3]

The objectives of the study were to isolate semi-purified fractions from ethanol and ethyl acetate crude extracts of *Stenochlaena palustris* and to determine its antioxidant activity and cytotoxic effect against selected cancer cell lines. The study also qualitatively screened possible secondary metabolites presence, such as flavonoids, alkaloids, phenols, saponins, terpenoids, quinones, glycosides and tannins.

EXPERIMENTAL

Plant materials

Stenochlaena palustris leaves (4.0 kg) were collected from a swampy land at Cherang Ruku, Pasir Puteh, Kelantan, Malaysia (Coordinate: 5.8710843,102.4832757) on 3rd of June 2014. The plant was authenticated by Dr. Goh Teik Khiang, Department of Agriculture and Food Science, UTAR, Perak. A voucher specimen (140615) was kept at the Biomedical Science Laboratory, University Tunku Abdul Rahman, and Malaysia. The matured leaves were air dried and were crushed into powdered form. The powdered leaves were soaked ethyl acetate and ethanol (95%), respectively and the filtrates were concentrated using rotary evaporator at 40 to 60°C (R-200, Buchi). This step was repeated twice with soaking period of three days. The weights of ethanol extract were 53.07 g, meanwhile ethyl acetate was 9.20 g and these extracts were kept at 4°C.

Phytochemical screening

Test for the presence of flavonoids, alkaloids, phenols, saponins, terpenoids, quinones, glycosides and tannins in the crude extracts of *Stenochlaena palustris* leaves were carried out using modified methods.[6,7]

Gravity column chromatography

The ethyl acetate and ethanol extracts, respectively were added to column (4 cm x 50 cm) containing packed silica (silica was added approximately 2/3 height of the column for better separation). Hexane (100%) was first flowed through the column and the polarity of the solvent was slowly increased using various combinations of solvents such as hexane: ethyl acetate, methanol, and ethanol. Examples of gradients system used are hexane: ethyl acetate (10:1; 10:2, 10:3, and increasing), ethyl acetate alone (100%), followed by ethyl acetate: methanol (10:1; 10:1.5 and increasing), finally were flushed out with only methanol (100%). Approximately 200 mL of eluate was collected and concentrated using rotary evaporator. The 50 fractions obtained were subjected to thin layer chromatography (TLC) for combination of similar compounds. These fractions were dried in the oven at 37°C and the dry weights of the fractions were tabulated as Table 1A.

Cell culture and subculture

Human epithelial carcinoma cell line, HeLa (ATCC®CCL-2™) was cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (JR Scientific, Inc, USA) and incubated in 5% CO_2 incubator (BINDER, Germany) at 37°C. The cells were sub cultured upon reaching 80% confluency. HeLa cells were trypsinised using trypsin-EDTA (0.25%) (Sigma, USA) for 10 min. Once cells detached, complete medium was added to the cells to deactivate the trypsin activity. The mixture was then centrifuged at 1000 rpm for 10 min. The supernatants were removed, and the pellets were re-suspended with complete medium. The cells were then aliquoted to few culture flasks containing complete medium and incubated in 5% CO_2 incubator.

DPPH assay

Stock samples of fractions obtained from both extracts were prepared at 10 mg/mL using absolute methanol (acetone-free). DPPH assay was conducted using 96-well microliter plate. Approximately 150 μL of methanol (analytical grade) were added into the 96-well plate. Later, 50 μL ascorbic acid, kaempferol and fractions, respectively were added into the well and were further diluted to obtain concentrations ranging from 0.16 to 5.00 mg/mL. Methanol was used as negative control. Fifty microliter of 0.2 mM DPPH reagent (Sigma-Aldrich, USA) was added into each well and incubated at room temperature in dark for 30 min. After incubation, the absorbance was read at 517 nm using microliter plate reader (Tecan, USA). These steps were repeated for thrice and the average absorbance values were calculated. The percentage of radical scavenging activity was calculated based on the following formula and graph of percentage of radical scavenging against concentration were plotted.[8]

Percentage of radical scavenging = $[(A_{NC} - A_{sample}) / A_{NC}] \times 100\%$: where, A_{NC} = average absorbance of negative control; A_{sample} = average absorbance of sample.

MTT Assay

The fractions were prepared at 10 mg/mL using 100% DMSO (Merck, Germany) and were further diluted with basic medium at various concentrations ranging from 6.25 to 100.00 μg/mL. The DMSO content in MTT

Table 1A: Fractions collected from ethanol and ethyl acetate extracts, respectively via gravity column chromatography.

Fractions collected from ethanol crude extract	Weight(g)	Fractions collected from ethyl acetate crude extract	Weight(g)
1	0.10	1	0.10
2	0.07	2	0.07
3	0.10	3	0.07
4	0.09	4	0.46
5	0.24	5	0.36
6	0.06	6	0.08
7	0.08	7	0.77
8	**0.03**	8	0.14
9	0.07	9	0.34
10	0.06	10	0.07
11	0.10	11	0.10
12	0.07	12	0.11
13	0.08	13	0.15
14	0.13	14	0.22
15	0.22	15	0.20
16	**0.41**	16	0.51
17	0.17	17	0.04
18	0.17	18	0.32

Table 1B: Phytochemical results of the ethanol and ethyl acetate leaves extracts of *Stenochlaena palustris*.

Metabolites	Observation
Alkaloids	+++
Flavonoids	+++
Phenols	+++
Saponins	+++
Terpenoids	++
Quinones	-
Glycosides	+
Tannins	+

+++ Higher amount; ++ moderate amount; + lesser amount; - not detected

assay was <1%. The concentration of cells used was 1.0×10^5 cells/mL. Approximately, 100 μL of respective cells were seeded into the 96-well plate and incubated in 5% CO_2 incubator for 24h. About 50 μL of fractions, doxorubicin (Fisher Scientific, USA) and 1% DMSO were added into respective wells. The cells were incubated for 72h. MTT reagent (5 mg/mL) (20 μL) was added into the wells and incubated further for 4h. After incubation, 200 μL of 100% DMSO was added into the wells. The colour changes from yellow to purple were observed and the absorbance was read at 570 nm using microliter plate reader (Tecan, USA). The graphs of percentage of cell viability against concentration were plotted and the IC_{50} values were determined from the graph. Percentage of cell viability was calculated using the formula:

Percentage of cell viability = $(A_{sample} / A_{NC}) \times 100\%$; Where, A_{sample} = average absorbance of the sample, A_{NC} = average absorbance of the negative control.[9] MTT assay was repeated thrice.

Data analysis

The data obtained from both assays were tabulated and analysed using Microsoft Office Excel 2013. The results are expressed as mean values and standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS

Phytochemical Screening

The presence of several secondary metabolites such as alkaloids, flavonoids, phenols, saponins, terpenoids, glycosides and tannins were detected in fractions obtained from both extracts Table 1B. These major metabolites were detected based on colour changes,

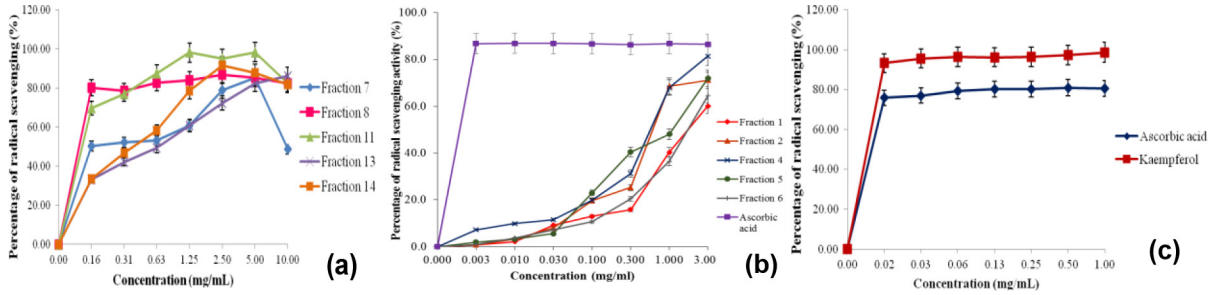

Figure 1: The percentage radical scavenging activity of samples at various concentrations (a) Fractions 7, 8, 11, 13 and 14 isolated from ethanol extract; (b) Fractions 1, 2, 4, 5 and 6 from ethyl acetate extract; (c) Ascorbic acid and kaempferol.

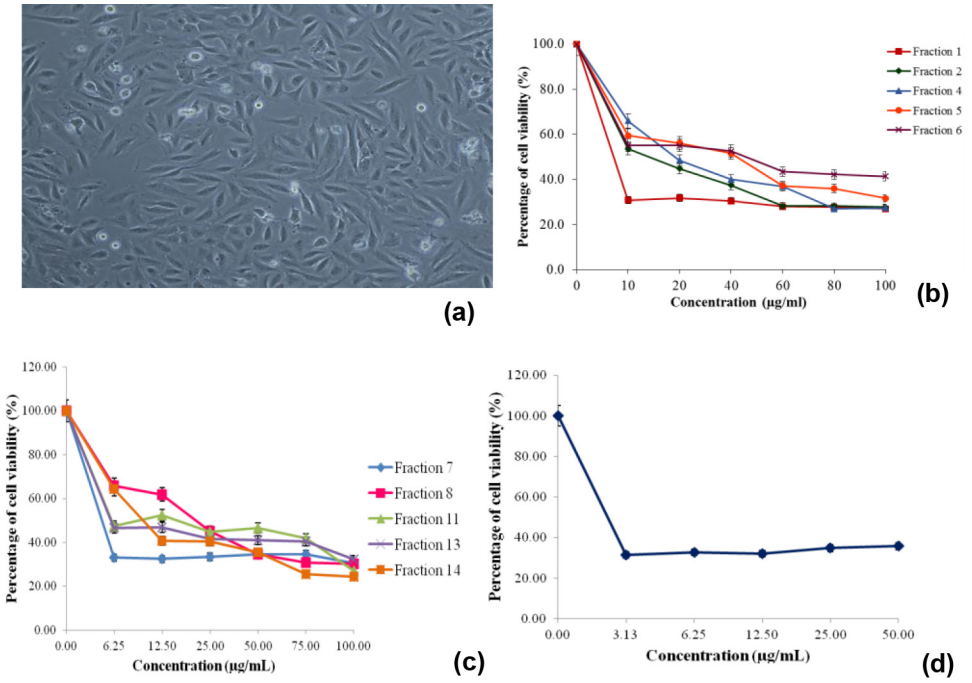

Figure 2: (a) Morphology of HeLa cancer cell lines cultured in DMEM at 80 to 90% confluency under magnification of 100×; The percentage viability of HeLa cells at 72h incubation using different concentration of (b) fractions 1, 2, 4, 5 and 6 of ethyl acetate extract, (c) Fractions 7, 8, 11, 13 and 14 of ethanol extract, (d) Doxorubicin hydrochloride.

formation of precipitation or formation of persistent forms qualitatively. A reddish-brown precipitate was formed to confirm the presence of alkaloids in all the fractions. Besides, higher amount of saponins, flavonoids and phenols were also detected. On the other hand, lower number of glycosides and tannins were identified. No quinones were detected in the qualitative phytochemical screening.

DPPH assay

Few selected fractions from both extracts and positive controls showed higher radical scavenging activity as the concentration increases. Fractions 7, 8, 11, 13 and 14 from ethanol extract exhibited higher radical scavenging activity, and among them fraction 11 showed 98.47% at 5.00 mg/mL. The ED_{50} value of fraction 11 was 0.120 mg/mL.

Meanwhile, fractions 1, 2, 4, 5 and 6 from ethyl acetate extract showed potential radical scavenging activity. Fraction 4 exhibited the highest radical scavenging activity of 81.38% at 3.000 mg/ml, followed by fraction 5 with 71.90%. The Ed_{50} value of fraction 4 is 0.650 mg/mL. Positive controls, kaempferol and ascorbic acid showed 98.67% and 80.95%, respectively Figure 1.

MTT assay

Confluent HeLa cells as shown in Figure 2(A) were used in MTT assay. The percentage viability of HeLa cells decreased as the concentration of fractions increased and there was a slight fluctuation in between the concentrations Figure 2 (B) and (C). Fractions 1 and

4 of ethyl acetate extract exhibited the lowest cell viability against HeLa cells upon treatment with 26.90% (IC_{50}: 8.60 μg/mL) and 26.92% (IC_{50}: 19.40 μg/mL), respectively. Meanwhile, Fractions 13 and 14 of ethanol extract showed cell viability of 32.39% (IC_{50}: 5.83 μg/mL) and 24.43% (IC_{50}: 10.16 μg/mL), respectively. The IC_{50} values obtained for all the fractions were less than 50.00 μg/mL. On the other hand, the IC_{50} value of doxorubicin hydrochloride was 2.21 μg/mL and killed almost all the HeLa cells Figure 2 (D).

DISCUSSION

In the presence of antioxidant molecules, the DPPH free radicals will be reduced leading to the changes in colour of DPPH reagent from violet to yellow or colourless.[10] Fractions obtained from both extracts showed potential antioxidant activity may due to the presence of higher amount of alkaloids, flavonoids and phenols based on qualitative phytochemical screening results. Medini *et al.* (2014) reported that antioxidant capacity is highly associated with phenolic content and most of the radical scavenging activity of plants is derived from phenols.[11] The presence of functional groups such as hydroxyl groups, keto groups, conjugated double bonds, β-ionone ring may contribute to its higher antioxidant activity.[12] The hydroxyl groups help to reduce the free radicals by donating a hydrogen atom, while hydrophobic side chains allows the metabolites to penetrate into biological membranes.[13] to exert free radical scavenging activity. Phenolic compounds isolated from plants were known to trigger redox reactions, reduce and stabilise highly oxidising free radicals by donating hydrogen atoms and electrons.[14]

Human cervical cancer cell line (HeLa) is the oldest and most widely used cancer cell line in human structural and molecular studies. HeLa cells exhibited epithelial-like morphology and was inhibited by the fractions isolated from both extracts. The inhibition was evaluated using MTT assay, a colorimetric assay by quantifying mitochondrial succinate dehydrogenase activity of proliferating cells.[15] Succinate dehydrogenase is a mitochondrial reductase enzyme that cleaves the tetrazolium ring and catalyses the conversion of water soluble tetrazolium salt to an insoluble purple formazan. Cancer cells are highly proliferating cells and possess high succinate dehydrogenase activities.[9] Due to the presence of active secondary metabolites in the fractions as reported, HeLa cells possibly may undergo destruction effect either alter its genetic constituents, reducing micro hypoxic environments in tumours or targeting cell cycle that enhance the killing effect on the cancer cells.[16] Flavonoids were proven as therapeutic agents for cancer prevention as it reduces cancer cells proliferation and significantly decreases the expression angiogenesis marker, vascular endothelial growth factor (VEGF) in ovarian cancer cells. Furthermore, it has been shown to inhibit cell proliferation in a dose dependent manner by regulating cyclin-dependent kinase 1 (CDK1) and cyclin B, a marker for transition of G2 to M phase, and by regulating a tumour suppressor gene which plays a key role in cell cycle arrest, p53 in MCF-7 breast cancer and HeLa cervical cancer cells.[17] Thus, inhibition of Hela cancer cells upon the treatments of various fractions could possibly followed the cell cycle arrest signalling pathway and thus stops the cells from proliferating further.

The bioactive constituents from the fractions isolated from both extracts could be the root factor for a potential cytotoxic agents and antioxidant activity. However, further isolation and purification, followed by structural elucidation may reveal the potential pure compounds that exert its bioactivity.

CONCLUSION

Fractions obtained from *Stenochlaena palustris* leaves are potential cytotoxic and antioxidant agents. Therefore, further studies should be carried out to evaluate its bioactivity using pure compounds in developing potential pharmaceutical drugs.

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CONFLTCIT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATIONS USED

HeLa: Human Cervical Cancer Cell Line; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DPPH:** 1,1-diphenyl-2-picrylhydrazyl; **IC_{50}:** Half-maximal inhibitory concentration; **ED_{50}:** Half-maximal effective concentration.

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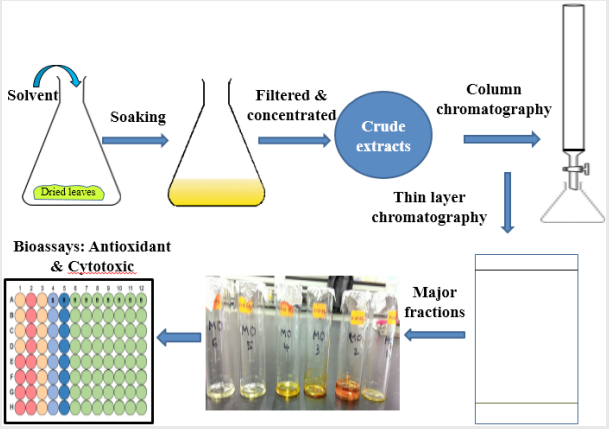
PICTORIAL ABSTRACT



SUMMARY

- The leaves of *Stenochlaena palustris* were soaked in various organic solvents at room temperature with occasional shaking.
- The leaves were extracted using cold solvent technique. The crude extracts obtained were further isolated using gravity column chromatography.
- The major fractions were visualized using thin layer chromatography.
- These fractions were further evaluated for antioxidant activity and cytotoxic effect.

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