Proteomic Analysis of Anti-Cancer Effects of *Streblus Asper* Root Extract on HeLa Cancer Cells

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Cervical cancer is the third most common cancer affecting women worldwide. This occurs despite having precancerous screening and HPV vaccination implemented vigorously as a definitive intervention. Natural plant like *Streblus asper* has been discovered to offer great hope in treating and preventing cancers. In this study, we explored the potential of *S.asper* to inhibit the growth of cervical cancer cell line by using liquid chromatography mass spectrometry (LCMS). Upon analysis, seventy-six proteins that are common to both untreated and treated groups were identified. Of this, 14 proteins are found differentially expressed more than 2-fold changes. Based on past literature, we selected 7 proteins that are closely associated with treatment effects. These include Dermcidin, Keratin, type I cytoskeletal 9, Tropomyosin alpha-4 chain, Myristoylated alanine-rich C-kinase (MARCKS), Tumour protein D52, Folate receptor alpha, and Parathymosin. Pathway enrichment analysis by Reactome revealed 9 related pathways which include metabolism of protein, post-translational protein modification, signalling by Rho GTPases, signalling by NOTCH, cell cycle, cellular senescence, signalling by WNT, transcriptional regulation by TP53, and cellular responses to stress. These findings may improve our understanding on the related significant mechanism involving anti-cancer effects of *S.asper* on the cervical cancer cell line.

Keywords: Anticancer, Apoptosis, Cervical cancer, Natural product, Proteomics, *Streblus asper*. 

Cervical cancer has been ranked third most common cancer among females in the world.1 Cervical cancer arises from uncontrolled proliferation of cells at the cervix. In cervical malignancy, squamous cell carcinoma constitutes approximately 90% of the cases while another 10% is referred to as adenocarcinoma.2 Cervical cancer typically develops in a cervical transformational zone via *Human Papillomavirus* (HPV) transmission, viral persistence, progression of a clone of persistently infected cells to pre-cancer and invasion.3 As of today, numerous studies reveal various risk factors pertaining to cervical cancer development. These include *Human Papillomavirus* (HPV) infection, tobacco use, and long use of hormonal contraceptive pills.4,5,6,7 Among that, HPV infection has been identified to be the major risk factor that is responsible for up to 95% of cervical
malignancies.8 The HPV's can be divided into two groups. There are low-risk group that are mostly associated with benign genital warts and the high-risk group that causes cervical cancer.9 HPV type 16 and 18 are the most commonly identified in invasive cervical cancer.10 In accordance with this, the HPV vaccination programme was introduced as a protective tool against this threat. This method has been widely accepted across the globe with approximately 70 countries establishing HPV vaccination as part of their national immunization programme.11 This trend has continued to be the practice for almost two decades. However, throughout the implementation, many weaknesses have been identified. HPV vaccination is considered expensive with the averaging cost of getting fully vaccinated from $1.49 to $18.94 per woman.12 This has been found to hamper the efforts to widen the coverage of HPV vaccination especially in low- and middle-income countries.13 In addition, studies have found that parents with low knowledge on HPV vaccination are less likely to have their daughters vaccinated.14 This was driven by the fear of its possible adverse effects.15 Cervical cancer is generally controllably provided if the intervention is initiated at an early stage. Precancerous screening strategy is seen to offer great hope for the cervical cancer patient. A pre-cancerous screening which also known as Pap smear is a screening tool used to detect pre-cancerous changes like Cervical Intraepithelial Neoplasia(CIN) 1, CIN 2, and CIN 3. Women aged 30- to 49-year-old are recommended to undergo a screening process from time to time.16 This screening strategy has proven to provide effective protection for women above the age of 30 from cervical cancer mortality.17,18 In another study, the Pap smear examination was observed to contribute to a 4% reduction in mortality.19 Despite its effectiveness, the Pap smear implementation has been observed to struggle against several challenges. These include lack of knowledge, failure in identification of eligible population, having difficulties in access that lead to demotivation of participation, having weaknesses in screening programme operation, insufficiency in monitoring and follow-up upon non-responders, and inadequacy of systematic monitoring of treatment.20 As for treatment, women who undergo radiotherapy and chemotherapy are unavoidably experienced adverse effects. Radiotherapy has been evidenced to cause urologic complications which include radiation cystitis, lower urinary tract dysfunction, stricture disease, fistula formation, and the development of second primary cancer.21 Radiotherapy is also often associated with acute side effects like erythema, desquamation, hair loss, mucositis, diarrhoea, pneumonitis, marrow ablation, nausea, and vomiting. While late or chronic side effects often result in fibrosis, necrosis, nerve damage, myelitis, telangiectasia, and stricture.22 Chemotherapy, on the other hand, is primarily subjected to toxicities. It is observed to increase the risk of ovarian dysfunction in older age at the time of treatment.23 The anti-angiogenic agent which is commonly used in treating gynecologic malignancy is frequently found to produce various adverse events. These include hypertension, left ventricular dysfunction and congestive heart failure, acute vascular event, and bleeding tendencies.24 Based on these setbacks, an alternative approach to control its incidence and development is deemed to be imperative. In accordance with this, we suggested S.asper to be used as potential anticancer agent in this study. S.asper Lou is a family of Moraceae. It is a tree that normally grows indigenously in tropical countries like Sri Lanka, Malaysia, Thailand, the Philippines, and India.25 From root to leaf, S.asper extract and its constituents traditionally exploited to treat a diversity of maladies.26 Studies found that its extract can be used as anti-filarial, anti-fungal, anti-inflammatory, anti-microbial, anti-viral, anti-oxidant and anti-hyperglycemic, anti-diabetic, and anti-cancer.27,28,29,30,31,32,33,34,35,36,37 In addition, the role of S.asper extract as anti-cancer had been proved in cancers like osteosarcoma (HOS cells), tongue carcinoma (SCC-15 cells), mouse lymphocytic leukaemia (P388 cells), and human nasopharyngeal epidermoid carcinoma (KB cells).26,38,25 However, its possible effects against certain other types of cancer like cervical cancer remain uncertain. In this study, we unravelled the potential of S.asper to suppress cervical cancer cell line (HeLa cells) by studying the treatment effects at the proteomic level.

MATERIALS AND METHOD

Plant extract
The Streblus asper plant was obtained
from a nursery in TasekGelugor, Penang, Malaysia. The authenticity was later confirmed by Associate Professor Dr Md. Azman bin PkmSeeni (Malaysian Institute of Pharmaceuticals and Neutraceuticals). *S.asper* roots were washed with distilled water and air-dried in an air-conditioned room for 2 weeks until it was completely dehydrated. The roots were ground into powders using Retsch SM 100 grinder. The ground powder was boiled with distilled water for 30 minutes and the outcome solution was filtered using 0.75 mm filter size. The filtrate was then freeze-dried. Upon usage, the freeze-dried powder’s weight was measured using analytical balance and diluted with double deionized distilled water according to the requirement.

**Cell culture and treatment**

All American Tissue Culture Collection cells used in this study were sub-cultured from ATCC with Catalog No. CCL-2™. This cervix adenocarcinoma cell line, HeLa cells (ATCC Catalog No.CCL-2™) was bought from ATCC, Manasus, VA, USA. HeLa cells were maintained in DMEM supplemented with 10% FBS, 1% sodium pyruvate and 1% penicillin-streptomycin bought from Life Technologies, USA. Cells were incubated in 37°C humidified CO₂ incubator, with 5% CO₂ and 95% of air. The medium was replaced every 72 hours until it reached 80%-90% of cell confluency before sub-culturing was done. Three to ten cell passages were used in the experiment. As for protein analysis, *S.asper* treatment was performed on HeLa cells using the half maximal inhibitory concentration (IC₅₀) dose (0.25 mg/ml) obtained from our previous study.

**Protein digestion**

The cell lysates prepared were undergone protein digestion. Prior to that, acetone precipitation of protein was done to eliminate substances that could interfere with LCMS application. The supernatant of prepared cell lysates was removed, and 80% cold acetone was added 6 times of the sample volume to the sample tube. The tube was inverted 3 times and incubated at “20±%C in a freezer overnight. The next day, the sample tube was spun at 6000x g for 10 minutes. Then acetone was decanted, and the pellet was dried in a speed vacuum. Pellet was resuspended in ammonium bicarbonate 50mM, pH 8.0. Then 100 µg of total protein samples were re-suspended in 100 µl of ammonium bicarbonate (NH₄HCO₃). 100 µl of 0.05% Rapigest™SF was added to each sample. Samples were shaken using Vortex. Then the samples were concentrated to a volume of 100 µl using Vivaspin column MWCO 3000. After that, samples were centrifuged at 14000 rpm (20800 x g), for 10 – 15 minutes. The sample was then heated on a thermomixer at 80°C for 15 minutes. 5 µl of 100 mM DTT was added to each mixture and incubated at 37°C for 30 minutes in thermomixer. Then samples were added with 5 µl of 200 mM Iodoacetamide and incubated at room temperature for 45 minutes. 5 µl (0.2 µg/µl) of trypsin was then added to the reaction and each mixture was incubated overnight at 37°C. The trypsin digestion reaction was stopped by adding 1µl of concentrated trifluoroacetic acid (TFA) and incubated at 37°C for 20 minutes. The mixtures were then centrifuged at 14000 rpm (20800 x g), for 10 minutes. Supernatants were collected and stored at -80°C prior to use.

**LCMS analysis**

Peptide samples were evaporated down to 10 µl per sample. Each sample was mixed with 200 µl of formic acid and filtered using 0.45µm regenerated cellulose membrane syringe filter. The LC-MS analysis was conducted using Orbitrap Fusion mass spectrometer coupled with Dionex 3000 Ultimate RSLCnano (Thermo Fisher Scientific) liquid chromatography system. EASY-Spray Column Acclaim PepMap™ C18 (100 A0, 2 µm particle size, 50 µm id x 15 cm) was used as the analytical column whereas Easy column C18 (2 cm, 0.1 mm i.d., 5 µm) was used as the pre-column. The MS2 spectra were analysed by ion trap MS (ITMS) using the following parameters: rapid scan rate with a resolving power of 60000, AGC target of 1.0e2 (100), 1.6 m/z isolation window, and a maximum injection time of 250 ms. Precursors were fragmented by collision-induced dissociation (CID) and high-energy collision dissociation (HCD) at normalised collision energy of 30% and 28%. Each sample was analysed thrice. Raw data obtained was analysed using Thermo Scientific™ Proteome Discoverer™.

**Bioinformatic analysis**

Thermo Scientific™ Proteome Discoverer™ was used to analyse the peptide identified from raw data. There are another three types of bioinformatics tools employed to further analyse the data obtained. These include Perseus,
Panther and Reactome. Based on the list of protein and its expression obtained from an analysis by Perseus, another analysis was done using Panther (Protein Analysis Through Evolutionary Relationships) software Version 13.0 (http://pantherdb.org/). This is a classification system with a large curated biological database of biomolecules (genes, proteins or transcripts) families and their functionally related subfamilies. This tool is part of the Gene Ontology Reference Genome Project and is effective for high-throughput analysis. An analysis was done using this tool to explain the biological function, cellular localisation, molecular function, and protein class of identified protein obtained previously. Other than Perseus and Panther, another tool known as Reactome version 64 (http://reactome.org) was used in the analysis. Reactome is a curated pathway database that provides comprehensive analysis and interpretation of pathway knowledge. Significant pathways were

Fig. 1. Total ion chromatograms (TICs) plot for the (A) untreated cells and (B) treated cells.
Fig. 2. GO analysis illustrates classes of proteins differing between untreated and treated cells. Protein with significant differences between the two groups was subjected to GO classification in terms of (A) biological process, (B) molecular function, and (C) cellular localisation.
chosen from the analysis according to the number of proteins involved in it and the nature of the pathways.

RESULTS

Protein identification

After the raw mass spectrometry data were loaded into Thermo Scientific™ Proteome Discoverer™ Software Version 2.1, de novo sequencing and database search was performed by accessing the Uniprot_homo_sapiens, a public sequence database for protein identification purpose. There were 4392 peptide-spectrum matches (PSM), 539 peptide group, and 122 protein groups identified in the untreated samples. On the other hand, there were 3266 PSM, 412 peptide group and 102 protein groups identified in the S.asper-treated samples.

The total 224 proteins that were found in both cells (untreated and treated) were further analysed using bioinformatic data interpretation tool that is available online, Panther. This was done to obtain the information on proteins classification based on its biological process, molecular function, and cellular localisation. As shown in Figure 2.A, most of the proteins identified from untreated cells are involved in the cellular process (32.9%), followed by the metabolic process (25.3%), and cellular component organization or biogenesis (12.0%). While most of the proteins identified from treated cells are involved in the cellular process (32.6%). This is followed by a metabolic process (27.9%), and cellular component organization or biogenesis (12.4%). In Figure 2.B, most of the proteins identified from the untreated group are involved in binding (57%), followed by catalytic activity (18.6%), structural molecule activity (15.1%), translation regulator activity (3.5%), transporter activity (2.3%), antioxidant activity (1.2%), signal transducer activity (1.2%), and receptor activity (1.2%). On the other hand, for treated cells, most of the proteins that are involved in binding (56.6%). This is followed by catalytic activity (21.1%), structural molecule activity (13.2%), translation regulator activity (6.6%), transporter activity (1.3%), and antioxidant activity (1.3%). The cellular localisation for untreated cells is classified as shown in Figure 2.C. These include cell part (40.6%), organelle (32.3%), macromolecular complex (16.7%), membrane (6.3%) and extracellular region (3.1%), and synapase (1.0%). As for treated, most of the protein is involved in the cell part (43.3%). This is followed by organelle (32.8%), macromolecular complex (17.9%). Few of the protein is involved in the membrane (3.0%) and extracellular region (3.0%).

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**Fig. 3.** A Venn diagram comparing the unique and common proteins found in the untreated and treated group.
Protein quantification

Of 122 proteins identified in untreated groups, and 102 proteins identified in *S.asper*-treated group, only 76 proteins are common to both groups (Figure 3). These identified proteins were further analysed using a software namely Perseus. This software was used to identify the differentially expressed proteins in both groups. For this purpose, quantitative variations in protein abundance per injection between the protein group of the untreated and treated samples were represented by log2 ratios of normalised volume obtained by the Thermo Scientific™ Proteome Discoverer™ Software Version 2.1. The values were subjected to T-test (P<0.05).

Upon analysis, 54 of them were found differentially expressed. Of that, 14 proteins were up-regulated, 5 proteins appeared with no changes, and 35 proteins were down-regulated. The list of 14 differentially expressed proteins with more than 2-fold changes was listed as in Table 1.

Pathways analysis

Pathway enrichment analysis was carried out on common proteins with at least 2 peptides. The analysis was done using Reactome, an online bioinformatics tool. From that, 9 pathways that play a meaningful role in cancer were selected as listed in Table 2.

**DISCUSSION**

The MS analysis revealed 76 common proteins found in both groups. Of this, several proteins were selected according to its relationship with cancers as discussed in the past literature. *S.asper* mediated the upregulation of galectin-1

### Table 1. Differentially expressed proteins with at least 2-fold changes

<table>
<thead>
<tr>
<th>No</th>
<th>Protein Description</th>
<th>MW[kDa]</th>
<th>Fold Change</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Histone H4</td>
<td>11.36</td>
<td>3.03202</td>
<td>Up</td>
</tr>
<tr>
<td>2</td>
<td>Plasminogen activator inhibitor 1 RNA-binding protein</td>
<td>44.938</td>
<td>2.60309</td>
<td>Up</td>
</tr>
<tr>
<td>3</td>
<td>Galectin-1</td>
<td>14.706</td>
<td>2.19671</td>
<td>Up</td>
</tr>
<tr>
<td>4</td>
<td>10 kDa heat shock protein, mitochondrial</td>
<td>10.925</td>
<td>2.16321</td>
<td>Up</td>
</tr>
<tr>
<td>5</td>
<td>Isoform 2 of Dermcidin</td>
<td>12.406</td>
<td>2.01046</td>
<td>Down</td>
</tr>
<tr>
<td>6</td>
<td>Keratinocyte proline-rich protein</td>
<td>64.093</td>
<td>2.02556</td>
<td>Down</td>
</tr>
<tr>
<td>7</td>
<td>Nascent polypeptide-associated complex subunit alpha, muscle-specific form</td>
<td>205.295</td>
<td>2.03476</td>
<td>Down</td>
</tr>
<tr>
<td>8</td>
<td>Serum albumin</td>
<td>69.321</td>
<td>2.11567</td>
<td>Down</td>
</tr>
<tr>
<td>9</td>
<td>Keratin, type I cytoskeletal 9</td>
<td>62.027</td>
<td>2.14074</td>
<td>Down</td>
</tr>
<tr>
<td>10</td>
<td>Tropomyosin alpha-4 chain</td>
<td>28.504</td>
<td>2.15584</td>
<td>Down</td>
</tr>
<tr>
<td>11</td>
<td>Myristoylated alanine-rich C-kinase</td>
<td>31.536</td>
<td>2.83294</td>
<td>Down</td>
</tr>
<tr>
<td>12</td>
<td>Isoform 3 of Tumour protein D52</td>
<td>26.367</td>
<td>2.86387</td>
<td>Down</td>
</tr>
<tr>
<td>13</td>
<td>Folate receptor alpha</td>
<td>29.799</td>
<td>2.89709</td>
<td>Down</td>
</tr>
<tr>
<td>14</td>
<td>Parathymosin</td>
<td>11.523</td>
<td>3.58596</td>
<td>Down</td>
</tr>
</tbody>
</table>

### Table 2. Pathways to which the common proteins found were annotated

<table>
<thead>
<tr>
<th>No</th>
<th>Pathway Name</th>
<th>Number of Proteins</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Metabolism of proteins</td>
<td>13</td>
<td>0.167477704</td>
</tr>
<tr>
<td>2</td>
<td>Post-translational protein modification</td>
<td>11</td>
<td>0.07638734</td>
</tr>
<tr>
<td>3</td>
<td>Signalling by Rho GTPases</td>
<td>7</td>
<td>0.00443948</td>
</tr>
<tr>
<td>4</td>
<td>Signalling by NOTCH</td>
<td>5</td>
<td>0.003193056</td>
</tr>
<tr>
<td>5</td>
<td>Cell Cycle</td>
<td>5</td>
<td>0.183772998</td>
</tr>
<tr>
<td>6</td>
<td>Cellular Senescence</td>
<td>4</td>
<td>0.008353885</td>
</tr>
<tr>
<td>7</td>
<td>Signalling by WNT</td>
<td>4</td>
<td>0.001169484</td>
</tr>
<tr>
<td>8</td>
<td>Transcriptional Regulation by TP53</td>
<td>4</td>
<td>0.102837272</td>
</tr>
<tr>
<td>9</td>
<td>Cellular responses to stress</td>
<td>4</td>
<td>0.136380287</td>
</tr>
</tbody>
</table>
and 10 kDa heat shock protein. It is also found to downregulate the expression of dermcidin, keratin, type I, tropomyosin alpha-4 chain, myristoylated alanine-rich C-kinase, tumour protein D52, folate receptor alpha, and parathymosin.

Galectins, in general, are α-galactoside specific endogenous lectins with low molecular weight. It plays a role in cell growth, cell activation, and cell-cell, cell-matrix adhesion which include binding to carcinoembryonic antigen, laminin and metalloproteinase.\textsuperscript{39} Prototype galectin which has single carbohydrate recognition domain (CRD), comes in different forms and these include galectin-1, galectin-2, galectin-5, galectin-7, galectin-10, galectin-11, galectin-13, galectin-14, galectin-15.\textsuperscript{40} Galectin-1 is found overexpressed in many forms of human tumour. Louka, et al., (2017) discovered that galectin-1 is upregulated in breast cancer compared to benign breast lesion.\textsuperscript{41} The study observed the elevation of galectin-1 expression corresponds to the increased activity of MMP-2 and MMP-9. Matrix metalloproteinases (MMP) is typically responsible for the degradation of most extracellular matrix proteins during organogenesis, growth and normal tissue turnover.\textsuperscript{42} This means that the increased activity of galectin-1 may serve as a way for cancer metastasis and invasion. Galectin-1 was also found increased in colon cancer, liver cancer, pancreatic cancer, and cervical cancer.\textsuperscript{43,44,45,46} In cervical cancer, overexpression of galectin-1 often associated with invasion and metastasis.\textsuperscript{47} In our recent study, galectin-1 was found increased in abundance after treatment with \textit{S. asper}. This indicated that galectin-1 was initially low in cervical cancer which seems consistent with studies that found galectin-1 to be underexpressed head and neck squamous cell cancer and uterine cancer.\textsuperscript{48,49}

Heat shock proteins (HSPs) are chaperone that play significant role in protecting its client protein from being degraded, hypoxic, thermally and oxidatively stressed.\textsuperscript{50} HSPs are categorized into six families depending on their relative molecular sizes. These include HSP27, HSP40, HSP60, HSP70, HSP90, and family of large HSPs (HSP110 and HSP170).\textsuperscript{51,52,53} The chaperonage function is mediated by facilitating correction of the misfolded proteins, maintaining the innate structure and function of their client protein.\textsuperscript{54,55} In the case of cancer, HSPs are considered as the regulators as they protect oncoprotein associated with cancer proliferation, differentiation, and progression. HSP10 which was identified in this study is a chaperone located in the mitochondria. Past literature reported that overexpression of HSP10 is found in several tumours like exocervical carcinoma, large bowel and uterine exocervix, and serous ovarian carcinoma. The studies discovered that elevated HSP10 level is associated with carcinogenesis and this chaperone protein employ different mechanisms to influence tumour initiation and progression.\textsuperscript{56,57,58} Contrary to expectation, we found HSP10 to be initially low in HeLa cells compared to its level after \textit{S. asper} treatment. In other words, \textit{S. asper} seems to cause HSP10 to be up-regulated in HeLa cells. The detailed mechanism that results in this pattern of expression remains elusive.

Dermcidin has been found to act as a growth and survival factor in breast cancer.\textsuperscript{59} This is concluded when overexpression of dermcidin increases cell proliferation and cell resistance to oxidative stress. This finding substantiates Brauer et al. (2014)’s work, where a significantly high level of dermicidin was found corresponded with an early progression of N-methyl nitrosourea-induced breast cancer.\textsuperscript{60} In a different study, the oncogenic effect of dermcidin in breast cancer was found exerted via ERBB signalling.\textsuperscript{61} The result demonstrated a reduction of dermcidin level upon \textit{S. asper} treatment. \textit{S. asper} seems to cause suppression of dermcidin oncogenic effects which include cancer growth and progression. However, the evidence regarding the expression of dermcidin in cervical cancer is still limited.

Based on past reports, there are 12 keratins identified in cervical carcinoma. These include keratin 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, 18, and 19.\textsuperscript{62} Expression of keratin was also found in breast cancer. According to the study, ninety per cent of all their breast cancer samples have had the expression of keratin 7, 8, 18, and 19.\textsuperscript{63} Keratins have been found contributing to cell size determination, translation control, proliferation, cell type-specific, organelle transport, malignant transformation and various stress responses.\textsuperscript{64} In cancer cells, studies confirm that keratins actively involved in cancer cell progression, metastasis, and response to treatments.\textsuperscript{65,66} This support a finding of which overexpression of keratin particularly
keratin-18 presence in breast cancer correlates with poor prognosis of the disease. Oppositely, down-regulation of keratins provide a sign of improvement and better prognosis for cancer. This pattern of expression can be seen in our study whereby S. asper exhibit the ability to suppress the expression of keratins in cervical cancer.

Another protein known as tropomyosin alpha-4 chain was differentially expressed in our study. Tropomyosin plays a crucial role in regulating the contraction of the muscle. The expression of tropomyosin has been considered as varies depending on its subtype and the type of tumor. Pieces of evidence explain that tropomyosin-4 is up-regulated in esophageal carcinoma and colon cancer. In a different study, Pawlak and co-investigators (2004) found that the level of tropomyosin-1 and -2 were markedly reduced in urinary bladder carcinoma. Bae et al. in 2005 identified the presence of tropomyosin in squamous cervical cancer but no difference in expression pattern was observed between cancerous tissue and normal cervical tissue. In a more recent study, tropomyosin-4 was proved lowly expressed in invasive squamous cervical carcinoma compared to Cervical Intraepithelial Neoplasia (CIN) and normal epithelium. Hence more molecular explanation is required to acquire better insight on the association of tropomyosin-4 and cervical cancer.

Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) protein has been found involved crucially in cell development and regeneration. Upregulation of MARCKS protein increases the activity of cell motility. Besides, it was found associated with the development of intimal hyperplasia in the murine carotid ligation model. The increased activity of MARCKS has been seen to positively correlated with the development of lung cancer in advanced stages (stage II-IV), lymph node metastatic status, and malignant phenotypes. In hepatocellular carcinoma cell line (HepG2), MARCKS protein was observed to have a close relationship with cell migration and invasion. This was concluded when knockdown of MARCKS significantly reduced HepG2 migration and invasion activities. MARCKS protein is a key regulator in the expression of micro-RNA 21 (miR-21) which found to enhance apoptosis resistance, motility, and invasion in prostate cancer cells. A study found that downregulation of MARCKS resulted in a possible anti-tumour effect at the metastatic site of colon carcinoma. S. asper treatment was found able to suppress the expression of MARCKS protein. This might potentially contribute to the reduction of metastasis and invasion activity of cervical cancer.

Another protein found to be altered by S. asper is tumour protein D52 (TPD52). TPD52 is a protein that is overexpressed in ovarian cancer. The expression is ranging from absent in benign tumour to overexpression in all invasive samples. TPD52 was also found overexpressed in prostate cancer. The knockdown of TPD52 was reported to lead to an inducement of apoptosis through a caspase-dependent pathway. Furthermore, transient overexpression of EGFP-TPD52 results in the increased proliferation rate of LNCap cells. In a different report, TPD52 has been witnessed as being overexpressed in all cases of colorectal cancer with significantly more than 3-fold change. Besides, a similar pattern of expression was also seen in breast cancer. The findings reported by previous literature are predominantly consistent with ours in which TPD52 expression is high in cancer cells. Upon treatment with S. asper, the expression was observed to decrease up to more than 2-fold change. However, the details about the target function associated with TPD52 overexpression remain unclear.

Folate receptor alpha (FRα) is a protein that is bound at the glycosylphosphatidylinositol (GPI) anchor of the cell membrane. It provides a high-affinity binding site for folate. Studies show that in the condition of folate deficiency, FRα tends to over-expressed. In accordance with that, constant folate deficiency increases the risk for cervical carcinogenesis. FRα has been proved to be overexpressed in epithelial ovarian cancer. This is supported by a more recent study which produces a similar result in metastatic foci and recurrent ovarian cancer. FRα was also found positively correlated in adenocarcinoma of non-small lung cancer. This finding accords with another study conducted by Nunez et al. published in the same year. Not just that, FRα was also demonstrated to be upregulated in 30% of breast cancer cases and 70-80% of stage VI triple-negative breast cancer cases suggesting it to be a promising therapeutic target. In addition, overexpression of FRα is
proved to be associated with poor outcome in breast cancer.\textsuperscript{92} The reduction of FR\textsubscript{a} expression in response to treatment in this study suggested that \textit{S.asper} may potentially implicate certain poorly understood mechanism related to the anti-folate receptor and cervical malignancy.

It was analysed that the positive expression of parathymosin may be associated with poor prognosis of squamous cell carcinoma and adenocarcinoma of the gallbladder.\textsuperscript{93} A study found that parathymosin was involved in promoting cell proliferation by regulating the level of glucocorticoids.\textsuperscript{94} In gastric epithelium, parathymosin expression was seen to be altered in tumour compared to normal gastric.\textsuperscript{95} Parathymosin level was also observed to be increased in human upper urinary tract transitional cell carcinoma.\textsuperscript{96} In a more recent report, parathymosin was suggested to be a potential predictor for early recurrence and poor prognosis of hepatocellular carcinoma.\textsuperscript{97} Our finding, on the other hand, demonstrated the ability of \textit{S.asper} to suppress parathymosin activity up to 3-fold change. This suggests that parathymosin may become one of the key therapeutic targets in \textit{S.asper} treatment mechanism of cervical cancer.

**CONCLUSION**

In the present study, we have presented several proteins that are identified and quantified from the \textit{S.asper}-treated and untreated samples of HeLa cervical cancer cells. Nine proteins were selected as they have been discussed in many past literatures to be associated directly or indirectly to cancers. Based on previous studies, seven of the proteins seem consistent to support our hypothesis while only two seem to be contradictory. Taken together, these findings would likely suggesting that \textit{S.asper} demonstrated significant anticancer effects on cervical cancer cell line through the regulation of several key proteins and pathways. Hence, an additional investigation needs to be conducted to confirm the involvement of each protein in the \textit{S.asper} treatment on cervical cancer. Besides, further study is also required to elucidate comprehensively the cellular and molecular mechanism which contribute to the treatment effects.

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**REFERENCES**


2. Torre, Lindsey A, Rebecca L Siegel, Elizabeth M Ward, and Ahmedin Jemal, ‘Global Cancer Incidence and Mortality Rates and Trends—An Update.’, Cancer Epidemiology, Biomarkers & Prevention/ : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 2016; 25: 16–27


4. Wright, Jason D., Jianduan Li, Daniela S. Gerhard, Zhengyan Zhang, Phyllis C. Huettner, Matthew A. Powell, and others, ‘Human Papillomavirus Type and Tobacco Use as Predictors of Survival in Early Stage Cervical Carcinoma’, Gynecologic Oncology, 2005; 98: 84–91


20. Priaulx, Jennifer, Harry J de Koning, Inge M C M de Kok, György Széles, and Martin McKee, ‘Identifying the Barriers to Effective Breast, Cervical and Colorectal Cancer Screening in Thirty One European Countries Using the Barriers to Effective Screening Tool (BEST)’, *Health Policy*, 2018


23. Overbeek, Annelies, Marleen H. van den Berg, Flora E. van Leeuwen, Gertjan J.L. Kaspers, Cornelis B. Lambalk, and Eline van Dulmen-den Broeder, ‘Chemotherapy-Related Late Adverse Effects on Ovarian Function in Female Survivors of Childhood and Young Adult Cancer: A Systematic Review’, *Cancer Treatment Reviews*, 2017; 53: 10–24


27. Chatterjee, Ranjit Kumar, Nigar Fatma,


35. Li, Lu-Qing, Jun Li, Yan Huang, Qiang Wu, Sheng-Ping Deng, Xiao-Jian Su, and others, ‘Lignans from the Heartwood of Streblus Asper and Their Inhibiting Activities to Hepatitis B Virus’, Fitoterapia, 2012; 83: 303–309.


48. Choufani, G, N Nagy, S Saussez, H Marchant,


Liu, Tuoen, Christopher K Daniels, and Shousong Cao, ‘Comprehensive Review on the HSC70 Functions, Interactions with Related Molecules and Involvement in Clinical Diseases and Therapeutic Potential.’, Pharmacology & Therapeutics, 2012; 136: 354–374


Cappello, Francesco, ‘HSP60 and HSP10 as Diagnostic and Prognostic Tools in the Management of Exocervical Carcinoma.’, Gynecologic Oncology 2003; 661


Smedts, F, F Ramackers, S Tranoyansky, M Pruszczynski, M Link, B Lane, and others, ‘Keratin Expression in Cervical Cancer.’, The American Journal of Pathology, 1992; 141: 497–511

Shao, Mu-Min, Siu Ki Chan, Alex M C Yu, Christopher C F Lam, Julia Y S Tsang, Philip C W Lui, and others, ‘Keratin Expression in Breast Cancers.’, Virchows Archiv/: An International Journal of Pathology, 2012; 461: 313–322


Ding, Shi-Jian, Yan Li, Ye-Xiong Tan, Man-Rong Jiang, Bo Tian, Ying-Kun Liu, and others, ‘From Proteomic Analysis to Clinical Significance’, Breast Cancer Research and Treatment, 11 2009; R39

Ding, Shi-Jian, Yan Li, Ye-Xiong Tan, Man-Rong Jiang, Bo Tian, Ying-Kun Liu, and others, ‘From Proteomic Analysis to Clinical Significance’, Molecular & Cellular Proteomics, 2004; 3: 73–81

and Cancer, 2000;29: 48–57
85. Zhao, Ronghao, and I David Goldman, ‘Folate and Thiamine Transporters Mediated by Facilitative Carriers (SLC19A1-3 and SLC46A1) and Folate Receptors.’, Molecular Aspects of Medicine, 2013;34: 373–385
89. Kalli, Kimberly R, Ann L Oberg, Gary L Keeney, Teresa J H Christianson, Philip S Low, Keith L Knutson, and others, ‘Folate Receptor Alpha as a Tumor Target in Epithelial Ovarian Cancer.’, Gynecologic Oncology, 2008; 108: 619–626