Article

Significant Decreased Expressions of CaN, VEGF, SLC39A6 and SFRP1 in MDA-MB-231 Xenograft Breast Tumour Mice Treated with *Moringa oleifera* Leaves and Seed Residue (MOLSr) Extracts

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Abstract: *Moringa oleifera* is a miracle plant with many nutritional and medicinal properties. Chemopreventive value of the combined mixture of moringa leaves and seed residues (MOLSr) at different ratios (M1S9, M1S1 and M9S1) were investigated. MOLSr extracts were subjected to phytochemical screening, antioxidant assays, metabolite profiling and cytotoxic activity on various human cancer cell lines including primary mammary epithelial cells (PMEC). The MOLSr ratio with the most potent antiproliferative activities were used in xenograft mice injected with MDA-MB-231 cell lines for *in vivo* tumourigenicity study as well as protein and gene expression studies. M1S9, specifically comprised of saponin and amino acid, retained the lowest antioxidant activity and highest glucosinolates content as compared to other ratios. Cell viability decreased significantly in MCF-7 breast cancer cells and PMEC after treatment with M1S9. Solid tumour from MDA-MB-231 xenograft mice was inhibited up to 64.5% at third week after treatment with high-dose M1S9. High-dose M1S9 significantly decreased the expression of calcineurin (CaN) and vascular endothelial cell growth factor (VEGF) proteins as well as the secreted frizzled-related protein 1 (SFRP1) and solute carrier family 39 member 6 (SLC39A6) genes. This study provides new scientific evidence for the chemoprevention properties of MOLSr in breast cancer model.

Keywords: moringa leaves and seed residue, chemoprevention, MCF-7, MDA-MB-231, gene expression profiling

1. Introduction

According to the World Health Organization (WHO), cancers of the lung, colorectal, stomach, liver and breast contributed to approximately 9.6 million of global deaths in 2018 [1]. Although chemotherapy is widely used treatment for cancers, the threatening problem is multi-drug resistance of chemotherapy, leading to ineffective outcomes [2]. Those dreading side effects and complications of conventional chemotherapy had triggered the scientific community to venture on

cancer prevention, chemoprevention. Chemoprevention is a crucial step that modulate specific pathway in carcinogenesis to reverse, suppress or prevent cancer progression to invasive cancers by using natural, synthetic or biological agents to improve the prognosis with milder and negligible side effects [3]. It is desirable to incorporate chemoprevention strategy as part of precision health. However, more work is required to realize this. Plants with anti-cancer properties have been investigated as potential sources of chemopreventive agents. This includes *Moringa oleifera* Lam. (*M. oleifera*) [4,5].

Moringa (MO) is a plant family Moringaceae, mostly found in tropical and subtropical regions with at least 13 species. Various parts of *M. oleifera*, including leaves, seed, root and bark extracts had been reported to possess anti-cancer activities by interfering with oncogenesis, cancer cell growth and progression [6-9]. Moringa seeds (MOS) comprises up to 40% of oil and high-quality fatty acid composition and is extensively used for human consumption and commercial purposes like cosmetics, biodiesel and others [10,11]. Part remains after oil seed extraction (with minimal production costs), moringa seed residues (MOSr) also known as press cake, seed cake or cake residue retain mainly the positively charged protein [12]. MOSr serve as a scavenger for heavy metals removal, as a natural coagulant for wastewater treatment and also found to possess anti-bacterial properties [13,14].

In this study, we evaluated the cytotoxic effects of the combined moringa leave and seed residues (MOLSr) using *in vitro* model. We also investigated the potential of chemopreventive properties the MOLSr extract using an *in vivo* model for delineation of the mechanistic pathways.

2. Materials and Methods

2.1. Preparation of moringa mixtures

M. oleifera leaves (MOL) and seed residue (MOSr) were obtained from The Borneo Moringa Sdn. Bhd. (Malaysia) in Tenom, Sabah, Malaysia with voucher identification number Bm_mo_191012_1. The ethanolic extracts of the MOL and MOSr were provided by MitoMasa Sdn. Bhd. in freeze-dried format. Three different ratios of the extracts of MOL and MOSr mixtures were freshly prepared in water with the final concentration of 10 mg/mL. M9S1 consisted of 9 mg of MOL extract and 1 mg of MOSr extract. M1S1 consisted of 5 mg of MOL extract and 5 mg of MOSr extract. M1S9 consisted of 1 mg of MOL extract and 9 mg of MOSr extract.

2.2. Phytochemical screening, total flavonoid, phenolic contents and antioxidant assays

Moringa mixtures were subjected to phytochemical analysis following methods described by Harborne (1998) and Kokate (2005) based on visual observation of colour changes or the formation of precipitates [15,16]. Quantitative analysis on total flavonoid content (TFC) and total phenolic content (TPC) of moringa mixtures were determined spectrophotometrically by aluminium calorimetric method (quercetin as the reference standard) and Folin-Ciocalteu method (gallic acid as the reference standard) with minor modifications, respectively. The antioxidant capacity of moringa mixtures was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay (Trolox as the reference standard) and ferric reducing antioxidant power (FRAP) assay (Trolox and ascorbic acid as the reference standard and positive control, respectively). These antioxidant assays were carried out according to Yang *et al.* (2011) with slight modifications [17]. Detailed steps were previously described by Abdul Hisam *et al.* (2018) [18]. All the experiments

were conducted in triplicates.

2.3. Metabolite profiling

Liquid Chromatography Mass Spectrometry/Quadrupole Time of Flight (LC-MS/QTOF) was used to identify bioactive compounds in moringa leave (MOL), moringa seed residue (MOSr) and moringa mixtures at different ratios: M1S9, M1S1 and M9S1. The stock solutions were purified by solid phase extraction (SPE) procedure. Two microliters of each sample were injected and analyzed by LCMS-QTOF (model 6520 Agilent Technologies, SA, USA). The chemical entities of each sample were resolved using a ZORBAX Eclipse Plus C18 column (100 mm x 2.1 mm x 1.8 μm, Agilent Technologies, SA, USA) maintained at 40°C. The flow rate of 0.25 mL/min with solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) was used. A linear gradient was developed over 36 minutes from 5% to 95% of mobile phase (B). The total run time was 48 minutes for each analysis. Electrospray ionization (ESI) source was set with a V Cap 4000 V, skimmer 65 V and fragmentor 125 V. The nebulizer was set at 45 psig and the nitrogen drying gas was set at a flow rate of 12 L/min. The drying gas temperature was maintained at 350ºC. Data was collected in positive ESI ionization mode and in full scan mode from 100 to 1000 m/z. During the analysis, two reference masses of 121.0509 m/z ($C_5H_4N_4$) and 922.0098 m/z ($C_{18}H_{18}O_6N_3P_3F_{24}$) were continuously injected to allow correction for accurate mass.

2.4. Cell culture growth conditions

Human primary mammary epithelial cells (PMEC) (ATCC® PCS 600-010™) was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and maintained in Mammary Epithelial Cell Basal Medium (ATCC® PCS-600-030™) supplemented with mammary epithelial cell growth kit (ATCC® PCS-600-040™) and incubated at 37°C in a humidified 5% CO_2 atmosphere according to manufacturer's guideline. Non-malignant Chang's liver (ATCC® CCL-13), human hepatocellular carcinoma (HepG2) (ATCC® HB-8065™), cervical adenocarcinoma (HeLa) (ATCC® CCL-2™) and human breast adenocarcinoma (MCF-7) (ATCC® HTB-22™) cell lines were maintained in Eagle's Minimum Essential Medium (ATCC® 30-2003™), respectively (Sigma Aldrich, USA). Human colorectal carcinoma (HCT-116) cell line (ATCC® CCL-247™) was cultured in McCoy's 5A (ATCC® 30-2007™). These cultures were supplemented with 10% fetal bovine serum (Biowest, South America) and incubated at 37°C in a humidified 5% CO_2 atmosphere. When the cells reached 70-80% confluency, the cells were detached with phosphate buffer saline (Sigma Aldrich, USA), trypsinized with 3 mL of trypsin-EDTA (Nacalai Tesque, Japan) and seeded in either 96-well plate or sub-cultured.

2.5. Cell viability assessment

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a mitochondrial-based cytotoxic test on cell viability. A total of 1×10^5 of cells were seeded overnight and then treated with tamoxifen (TAM) (positive control) and moringa mixtures (M1S9, M1S1 and M9S1) with two-fold series dilution from 100 μg/mL to 6.25 μg/mL, at three time points (24, 48 and 72 hours), respectively. At each time points, 20 uL of 5 mg/mL dimethyl-thiazole-tetrazolium (MTT) (Sigma Aldrich, St Louis, MO, USA) in phosphate buffer saline (Sigma Aldrich, St Louis, MO, USA) was added and further incubated at 37°C for 3 hours. The crystallized MTT was solubilized with

DMSO before the concentration was determined by optical density at 570 nm using a microplate reader (BMG POLARstar Omega, Germany). Wells with untreated cells and only DMSO solution were served as negative (100% cell viability) and background controls (for blank subtraction), respectively. The percentages of viable cells were plotted against concentrations to determine cell viability (%) and the IC_{50} value. All experiments were conducted in triplicates.

2.6. Xenograft mice model

Animal study was approved by the UiTM Committee on Animal Research and Ethics [Ethic approval number: UiTM CARE 278/2019 (05/04/2019)]. All animal experiments were conducted in accordance with standard ethical guidelines. Six- to eight-week-old female SPF NOD/ShiJic-SCID mice were obtained from Nomura Siam International (Bangkok, Thailand). All mice were housed in the pathogen-free environment under controlled conditions (temperature 20-26°C, humidity 40-70%, light/dark cycle 12h/12h) and received water and food *ad libitium*. To evaluate the tumourigenesis in animal model, the highly aggressive MDA-MB-231 cell line was injected into SCID mice. MDA-MB-231 cell line (ATCC® HTB-26™) was grown in Leibovitz's medium (L15) (HyClone, USA) in a humidified incubator at 37°C without CO_2 with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Beyotime Biotechnology, China). Mice were subcutaneously injected with $3 \times 10^6/100$ µL MDA-MB-231 cells into the right flank region in the SCID mice to allow tumour grow up to 100–150 mm³.

2.7. In vivo tumourigenicity study

After tumour volume had reached 100–150 mm³, the xenograft mice were randomized into five groups (n = 6) and received M1S9 orally (250 mg/kg, 500 mg/kg, 750 mg/kg), normal saline orally (negative control) and 30 mg/kg TAM subcutaneously (positive control) for 60 consecutive days or until the reduction of tumour size was 40-60% (Figure S1). Dose and route used for positive control (tamoxifen 30 mg/kg) is based on the method described by Yoneya *et al.*, (2010) [19]. Body weight and tumour volume were measured weekly up to two months. The tumour-growth-inhibition rate (I.R.) was calculated using the following formula: I.R. (%) = {[(Average tumour volume of the control – average tumour volume of the treated group)/Average tumour volume of the control]*100%}. After two months, serum blood was collected for biochemical analysis. Subsequently, all organs (liver, kidney, spleen, heart and lung) and tumours were excised and weighted as relative organ weight (expressed as the percentage of organ weight to body weight). The tumours were snap-frozen in liquid nitrogen for later gene expression analysis.

2.8. Protein and gene expression analysis

Serum blood was subjected for biochemical analysis on calcineurin (CaN), estrogen (ES) and vascular endothelial cell growth factor (VEGF) using ELISA kits (FineTest, Wuhan, China), following manufacturer's instructions. The Human Breast Cancer RT² Profiler PCR array (Rotor-Gene® Format) (PAHS-131ZR, Qiagen® GmbH, Hilden, Germany) was used to profile the expression of 84 genes associated with key human breast cancer pathways. Briefly, total RNA were isolated from pulverized tumour tissues of non-treated (negative control), TAM-treated (positive control) and high-dose M1S9-treated mice using RNeasy® Lipid Tissue Mini kit (Qiagen® GmbH, Hilden, Germany). Three biological replicates were prepared from each group. Eluted RNA was

quantitated and purity-checked using UV-absorbance-based NanoDrop® (Thermo Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer RNA 6000 Nano chip (Agilent, Palo Alto, CA), respectively. A total of 1000 ng of RNA sample was converted to cDNA using RT² First Strand Kit (Qiagen® GmbH, Hilden, Germany) according to manufacturer's protocol. The cDNA was then mixed with RT² SYBR Green ROX FAST Mastermix and RNase-free water before dispensing into the array and then subjected to Rotor-Gene® Q real time PCR cycler (Qiagen® GmbH, Hilden, Germany) for analysis. Threshold cycle (C_T) of each gene was imported and analysed for $\Delta\Delta C_T$ after normalization using housekeeping genes in a web-based RT² Profiler Data Analysis Software (https://www.qiagen.com/my/shop/genes-and-pathways/data-analysis-center-overview-page/). Gene expression was considered significantly regulated by TAM and M1S9 when the mean fold regulation was greater than ± 2.

2.9. Gene function and gene network analysis

The web-based Database for Annotation, Visualization and Integrated Discovery (DAVID) gene annotation tool (https://david.ncifcrf.gov) was used to enrich the up- and down-regulated genes (fold regulation more than ±2) to understand the biological effects (mechanism) of TAM and M1S9 towards MDA-MB-231 tumours. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to classify those genes based on their cellular functions and biological pathways involved. The *Homo sapiens* database was selected as the background in the enrichment analysis. The GO biological process and KEGG pathway enrichment analyses were considered significant with *p*-value less than 0.05 and enrichment gene count greater than or equal to 3. Subsequently, gene network analysis was performed using Qiagen's Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, CA, USA) software. IPA database contains all known interactions and was used to elucidate the biological interpretation of genes in regulating the MDA-MB-231 cells with TAM and M1S9 extract.

2.10. Statistical analysis

The data were expressed as the mean ± SEM values (n = 3), using one-way ANOVA followed by Dunnett's t-test (*p*<0.05). All statistical analyses were performed using statistical software package, IBM® SPSS® Statistics Version 22.

3. Results

3.1. M1S9 extract detected the presence of saponins, amino acids and high glucosinolates content but with low antioxidant activity

The qualitative phytochemical screening revealed the presence of tannins, flavonoids, phenols, proteins, fats and fixed oils as well as carbohydrates in M1S1 and M9S1 (Table 1). However, flavonoid was not detected in M1S9 but saponins and amino acids were detected instead. M1S9 extracts possessed the lowest level of total flavonoid content (TFC), total phenolic content (TPC) and antioxidant activity. For metabolite profiling (Figure S2), a total of 17 compounds were identified in different extracts composition (Table 2). Compounds found uniquely in MOL including flavonoids, such as isoquercetin, quercetin, astragalin, isorhamnetin and kaempferol) and in MOSr including glucosinolates (glucosinalbin and glucotropaeolin), glycoside (niazirin), phenolic acid (p-coumaric acid) and other natural occurring compounds (benzoic acid, vanillin and heptadecane). Although

other studies showed that glucosinolate is predominantly found in the MOL and MOSr, this study showed no glucosinolate was found in the MOL, mainly due to different preparation method [20].

Table 1. Moringa mixture analysis: **(a)** Qualitative phytochemical analysis; **(b)** Quantitative phytochemical analysis; **(c)** Antioxidant assays (DPPH and FRAP) of combined mixture of moringa leaves and seed residue (MOLSr) extracts at different ratios (M1S9, M1S1 and M9S1).

	Control		MOLSr		
	Negative	Positive	M1S9	M1S1	M9S1
(a) Qualitative phytochemical analysis					
Tannins	-	++++	+	+++	+++
Triterpenoids	-	++++	-	-	-
Flavonoids	-	++++	-	+	++
Saponins	-	+++	++	-	-
Anthraquinone glycosides	-	++++	-	-	-
Phenols	-	++++	+	+++	++++
Steroids	-	++++	-	-	-
Alkaloids	-	++++	-	-	-
Proteins	-	+	+	+	+
Amino acids	-	++++	+	-	-
Fats and fixed oils	-	++++	+++	++	++
Carbohydrates	-	++++	+++	++	++
(b) Quantitative phytochemical analysis					
TFC (μg QE/mg)	NR	NR	0.88 ± 0.09	2.58 ± 0.11	4.72 ± 0.06
TPC (μg GAE/mg)	NR	NR	57.7 ± 1.03	68.0 ± 0.53	82.2 ± 4.57
(c) Antioxidant assays					
DPPH (IC_{50}, μg/mL)	1.2±0.10*	-	113.43±25.45	12.50±3.67	11.65±4.99
FRAP (μg Trolox/mg)	-	172.41±12.6$^\$$	1.37±0.56	2.04±0.67	5.60±0.73

(a) For qualitative phytochemical analysis, (-) means the absence of the phytochemical; (+) means the presence of the phytochemical, amount based on the colour intensity from lowest to highest, (+) < (++) < (+++) < (++++); (NR) means non-related. (b) For quantitative phytochemical analysis, total flavonoid content (TFC) and total phenolic content (TPC) are presented as mean ± 2 standard deviations (n=3) with units of μg QE/mg (μg of quercetin equivalent per mg extract) and μg GAE/mg (μg of gallic acid equivalent per mg extract), respectively. Note: NR-non-relevant. (c) For antioxidant assays, FRAP value was presented as mean ± 2 standard deviation (n=3) with unit of μg Trolox per μg sample dry weight (μg Trolox/mg). Note: *-positive control using Trolox; $^\$$-positive control using ascorbic acid.

Table 2. List of compounds identified in moringa leave (MOL), moringa seed residue (MOSr) and combined mixture of moringa leaves and seed residue (MOLSr) extracts at different ratios (M1S9, M1S1 and M9S1) according to retention time, molecular weight and metabolite classification.

Compound group	Compound name	Retention time	Molecular Formula	Molecular weight	Percentage of relative abundance (%)				
					MOL	MOSr	M1S9	M1S1	M9S1
Glucosinolate	Glucosinalbin	1.351	$C_{14}H_{19}NO_{10}S_2$	425.0419	ND	0.107	0.046	0.029	ND
Glucosinolate	Glucotropaeolin	1.395	$C_{14}H_{19}NO_9S_2$	409.0502	ND	0.016	0.004	ND	ND

Miscellaneous	Cinnamic acid	1.667	$C_9H_8O_2$	148.0516	0.090	0.020	0.060	0.060	0.090
Miscellaneous	Quinoline	2.501	C_9H_7N	129.0575	0.007	ND	0.004	0.005	0.008
Miscellaneous	Benzoic acid	5.913	$C_7H_6O_2$	122.037	ND	0.010	0.007	ND	ND
Glycoside	Niazirin	8.212	$C_{14}H_{17}NO_5$	279.1121	ND	0.009	0.009	0.006	0.005
Flavonoid	isoquercetin	10.422	$C_{21}H_{20}O_{12}$	464.0956	1.030	ND	0.630	0.930	1.230
Flavonoid	Quercetin	10.442	$C_{15}H_{10}O_7$	302.0436	0.560	ND	0.350	0.510	0.680
Flavonoid	Astragalin	11.478	$C_{21}H_{20}O_{11}$	448.1009	0.370	ND	0.240	0.340	0.510
Steroid	Strophanthidin	11.703	$C_{23}H_{32}O_6$	404.2181	0.050	0.01	0.020	0.030	0.040
Flavonoid	Isorhamnetin	11.772	$C_{16}H_{12}O_7$	316.0584	0.040	ND	0.020	0.030	0.040
Flavonoid	Kaempferol	12.291	$C_{15}H_{10}O_6$	286.0479	0.060	ND	0.020	0.040	0.060
Glycoside	Niazimicin	14.802	$C_{16}H_{23}NO_6S$	357.1252	0.050	ND	0.030	0.040	0.050
Phenolic acid	p-coumaric acid	20.79	$C_9H_8O_3$	164.0475	ND	0.005	0.005	ND	ND
Miscellaneous	Vanillin	15.878	$C_8H_8O_3$	152.0479	ND	0.010	0.008	ND	ND
Miscellaneous	Heptadecane	26.47	$C_{17}H_{36}$	240.2828	ND	0.003	ND	ND	0.002
Miscellaneous	Palmitic acid	34.17	$C_{16}H_{32}O_2$	256.2414	0.004	ND	ND	0.003	0.003

Note: ND-non-determined.

3.2. M1S9 extract inhibited the proliferation of MCF-7 breast cancer cell line

Among the cell lines, the IC_{50} values of tamoxifen (TAM) (serves as positive control) were relatively low, ranging from 3-15.5 μg/mL, suggesting that it inhibited cell growth not only in normal cells, but also in cancer cell lines at dose- and time-dependent manner (Table 3). Once characterized in PMEC, M1S9 and M1S1 induced toxic effect towards these cells while no obvious cytotoxic effect was observed in M9S1-treated cells. M1S9 extract exhibited the highest anti-proliferative activities towards MCF-7 breast cancer cells at IC_{50} values equal to 38.5 μg/mL (72 hours). Previous study found that MCF-7 cells were less tumourigenic as they are slow growing primary tumours in mammary fat pad and no metastases were observed in mouse model [21]. Therefore, the highly aggressive MDA-MB-231 cell line was injected into SCID mice to establish the *in vivo* model for elucidation of the anti-cancer effects using M1S9 extract.

Table 3. The IC_{50} values of tamoxifen (TAM) (positive control) and combined mixture of moringa leaves and seed residue (MOLSr) extracts at different ratios (M1S9, M1S1 and M9S1) after 24, 48 and 72 hours of treatment with respective cell lines.

Cell lines	Time points (hours)	IC₅₀ (μg/mL)*			
		TAM	M1S9	M1S1	M9S1
Primary mammary	24	4.0	70.0	82.0	-
epithelial cells	48	4.0	33.5	78.5	-
(PMEC)	72	3.0	17.5	51.0	-
Non-malignant	24	4.0	-	-	-
Chang's liver cells	48	6.0	93.5	-	-
	72	3.0	-	-	-
Hepatocellular	24	7.0	-	-	-
carcinoma	48	4.0	-	-	-
(HepG2)	72	3.0	-	-	-

Colorectal carcinoma	24	9.0	-	-	-
cells (HCT-116)	48	5.5	-	-	-
	72	4.5	-	-	-
Cervical	24	5.0	91.0	-	-
adenocarcinoma cells	48	3.5	75.0	-	-
(HeLa)	72	3.0	81.0	91.0	-
Breast	24	15.5	97.5	-	-
adenocarcinoma cells	48	7.5	53.0	-	-
(MCF-7)	72	8.0	38.5	57.0	-

3.3. High-dose M1S9 extract suppressed tumour growth in MDA-MB-231 xenograft tumour

At the end of M1S9 treatment, no significant difference in body weight changes were observed in xenograft mice treated with M1S9 and TAM up to eight weeks (Figure 1a). However, the tumour volume of MDA-MB-231 showed a significant reduction in mice treated with M1S9 (all three doses) and TAM, compared to the control mice ($p<0.05$) up to six weeks (Figure 1b). The low-dose and medium-dose M1S9 were not able to inhibit tumour growth effectively after 6th week and 7th week, respectively, while the effect of high-dose M1S9 sustained throughout the experimental period. The maximum percentage of tumour growth inhibition were 64.5% (at week 3), 55.6% (at week 4) and 54.8% (at week 4), respectively for the high-, moderate- and low-doses of M1S9 extracts (Figure 1c). The relative organ weights for the liver, heart, spleen, lung and kidney were unaffected by different dosages of M1S9 extract and TAM ($p>0.05$) when compared to the control group (Figure 1d), suggesting the extract did not induce any toxic effect on these organs [22,23].
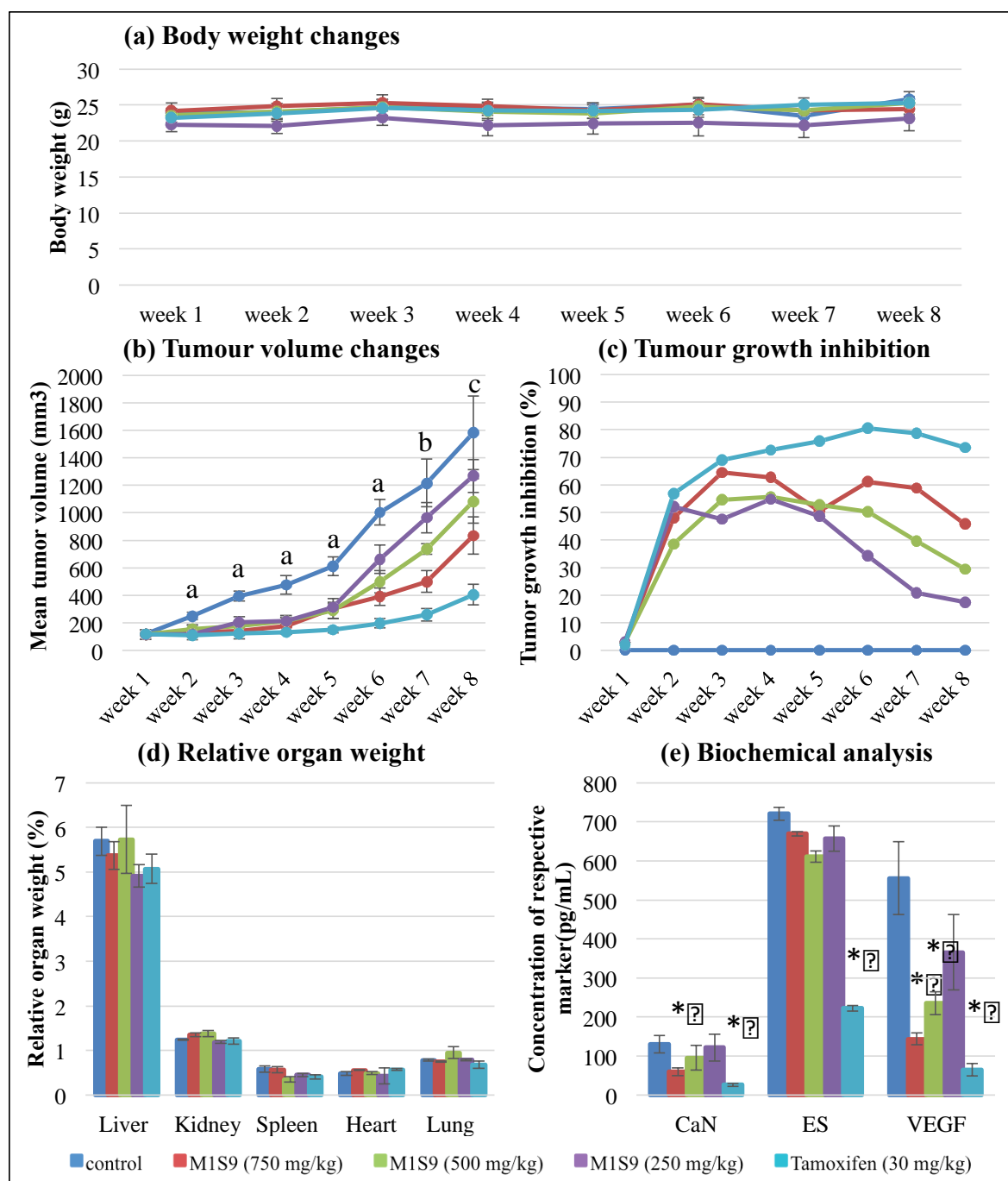
Figure 1. The effects of M1S9 extract in the breast tumour growth using MDA-MB-231 xenograft animal model: **(a)** Body weight changes (g); **(b)** Tumour volume changes (mm³); **(c)** Tumour growth inhibition (%); **(d)** Relative organ weight (%); **(e)** Biochemical analysis (pg/mL) over eight weeks after treatments three doses of M1S9 extracts, normal saline (negative control) and tamoxifen 30 mg/kg (positive control). Values are in mean ± S.E.M. In tumour volume changes, a, b and c indicate significant differences ($p<0.05$) with the control at the same week as determined by Dunnett test, a non-parametric test. "a" was significant different in all treatment groups, "b" was significant different in all treatment groups except low dose M1S9 (250 mg/kg) and "c" was significant different in all treatment groups except low dose M1S9 (250 mg/kg) and medium dose M1S9 (500 mg/kg). In biochemical analysis, asterisk (*) means significant differences from control group ($p<0.05$). Abbreviations: CaN-calcineurin; ES-estrogen; VEGF-vascular endothelial cell growth factor.

3.4. High-dose M1S9 extract down-regulated the expression of CaN and VEGF proteins as well as SFRP1 and SLC39A6 genes in MDA-MB-231 xenograft tumour

The biological effects mediated by high-dose M1S9 over the non-treated groups were investigated at the protein level (Figure 1e) and transcript level (Figure 2). Both CaN and VEGF levels in the mice treated with high-dose M1S9 showed significant reductions while in TAM-treated cells, CaN, ES, and VEGF proteins were suppressed significantly. For gene expression study, AR, ESR1, IL6, MYC, NR3C1 and THBS1 genes were induced significantly ($p<0.05$) and only CTNNB1 gene was suppressed significantly ($p<0.05$), respectively in TAM-treated cells. After M1S9 treatment, only two genes showed down regulation with significant differences, including SFRP1 and SLC39A6. The overview of functional role of each differential expressed gene was tabulated in Table S1. TAM-treated group participated actively in steroid-receptor mediated, glucocorticoid and classical WNT signalling. Significantly down-regulated CTNNB1 gene was involved in epithelial-to-mesenchymal transition (EMT), angiogenesis and cell adhesion molecules process. For M1S9-treated group, only classical WNT signalling and apoptosis process were regulated significantly by SFRP1 gene.
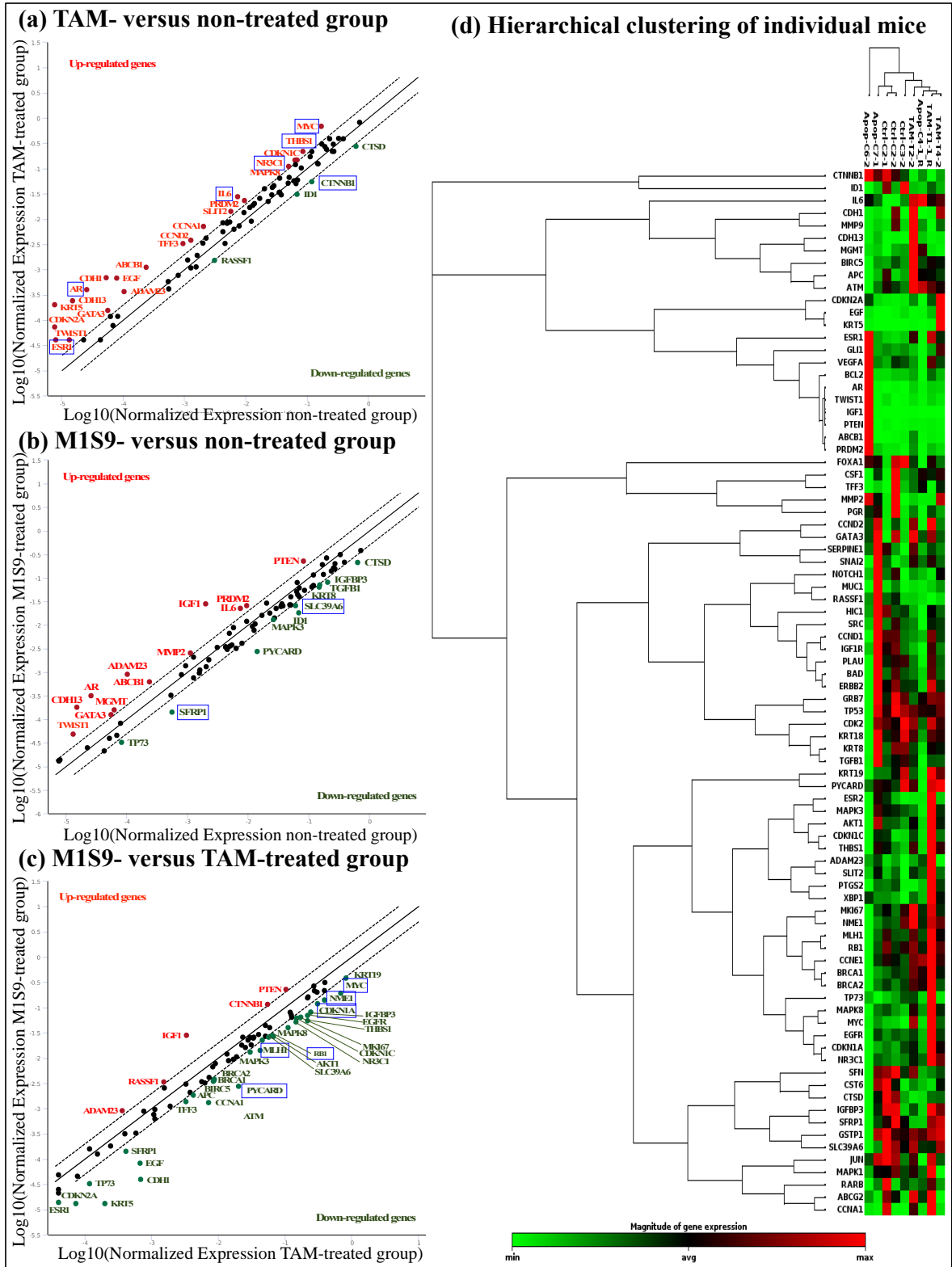
Figure 2. Effect of M1S9 on the expression levels of 84 key human breast cancer genes in MDA-MB-231 xenograft tumour: **(a)** TAM-treated versus non-treated group; **(b)** M1S9-treated versus non-treated group; **(c)** M1S9-treated versus TAM-treated group; **(d)** Hierarchical clustering of individual mice. Up-regulated genes (red characters) and down-regulated genes (green characters) have fold changes ≥ ±2. Blue bracket indicates genes with significant different at $p < 0.05$. Abbreviation: TAM-tamoxifen.

3.5. High-dose M1S9 regulated genes enriched in specific biological process, pathways and gene networks

Both groups were significantly enriched in the biological transcriptional regulation processes, which are important to understand the role of TAM and M1S9 in the pathogenesis of breast cancer (Table S2-4). GO terms enriched specifically in M1S9-treated group including regulation of MAPK, ERK1 and ERK2 cascade, response to ethanol, viral process and protein phosphorylation. For KEGG pathway enrichment analysis, both treatments participated in pathways of cancer, proteoglycans in cancer and several signalling pathways including P13K-Akt, FoxO, and Hippo. Gene networks were built to illustrate the correlation between differentially expressed genes (Figure 3, Table S5). For M1S9-treated MDA-MB-231 cells, related diseases and functions that overlap with TAM-treated cells were cell cycle, cell morphology and connective tissue development and function. However, unique diseases and functions related to M1S9-treated cells were cell death and survival, cellular growth and proliferation, hematological system development and function, immunological disease, skeletal and muscular system development and function as well as tissue development.
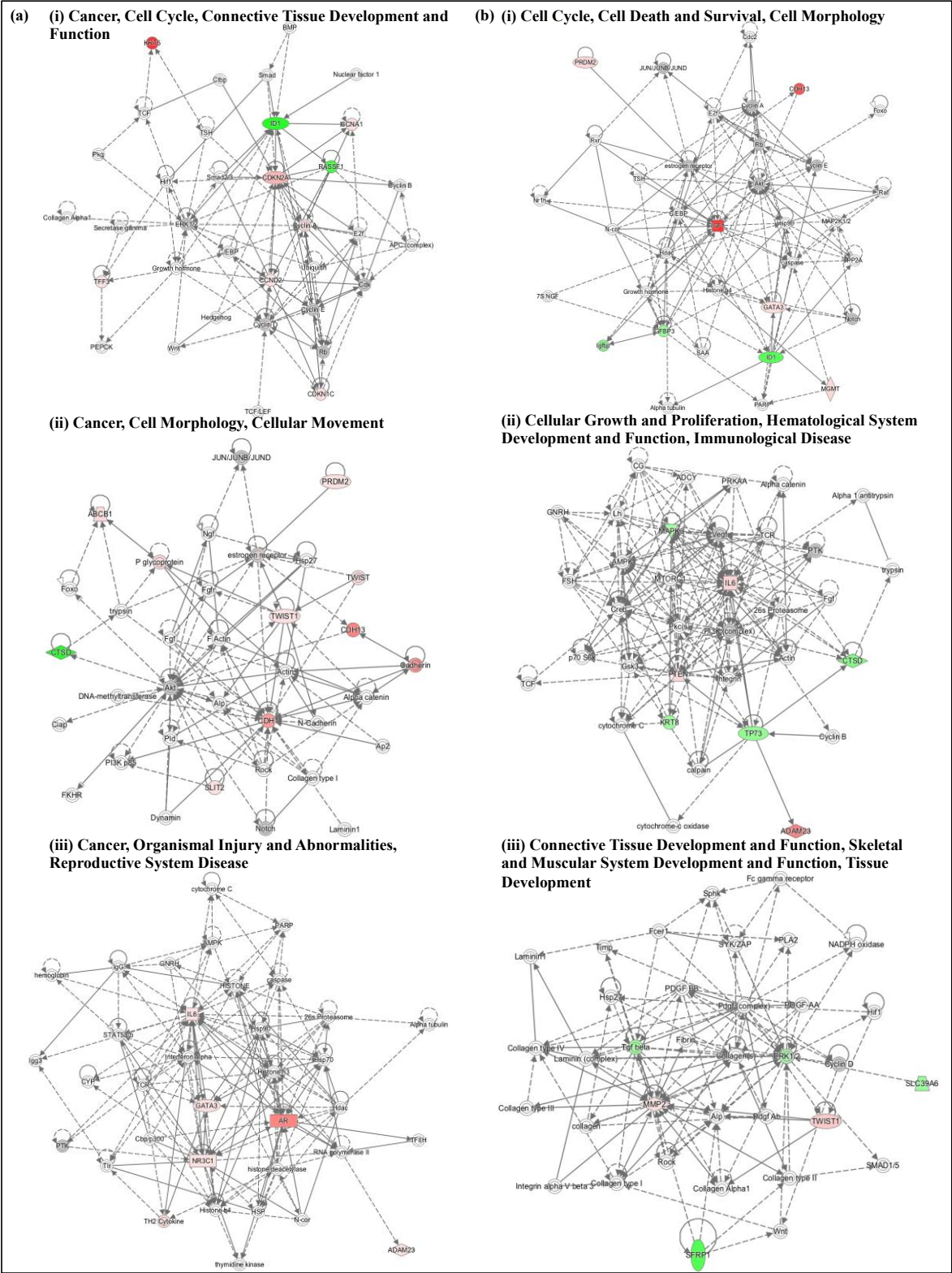
Figure 3. Top three enriched networks in **(a)** TAM-treated; **(b)** M1S9-treated MDA-MB-231 xenograft tumour. Up/down-regulated genes (fold changes ≥ ±2) are coloured in red and green, respectively, while the colour intensities are relatively to fold change. Nodes without colour were neither expressed nor assessed in this study. Abbreviation: TAM-tamoxifen.

4. Discussion

All the different parts of moringa (MO) plant contain valuable compounds with anti-cancer activity including ethanolic extract of moringa leave (MOL) and moringa seed (MOS) [6-9]. Since both MOL and MOS are potent anti-cancer agents, the moringa seed residue (MOSr) (after oil repletion with minimal production costs) was evaluated for its combined effect with moringa leaves (MOL) at different ratios. In the phytochemical analysis, the specific bioactive compounds in M1S9 include saponins and amino acids, but were not found in other combinations, suggesting these compounds mainly derived from the MOSr. Previous study showed that *Camellia oleifera* and *Camelina sativa* seed cakes (herein referred to seed residue) have a specific group of saponin compound, i.e. triterpenoid saponins that possess anti-tumour activity [24]. Although after oil repletion, the MOSr still retain the nutritive properties of amino acids, which were only reported in moringa seed (MOS) but not in MOL [25]. A recent study showed that the water extract of *M. oleifera* seed kernel possesses potent antioxidant activity due to high total phenolic, flavonoid and tannin contents [26]. However, antioxidant activity of MOS had diminished after oil repletion, probably related to low flavonoid and phenolic contents. Glucosinalbin (a glucosinolates) was the most abundantly found compound solely in MOSr, in which MOS having 8% higher glucosinolates level as compared to other parts of moringa [4,27]. Moringa-derived glucosinolates possess chemoprotective potential through cytoprotective, anti-carcinogenic, anti-oxidant effects and induced apoptosis in cancer cell [28]. Therefore, the combined mixture of MOL and MOSr could demonstrate combined effects for chemopreventive response via biological process other than antioxidant process.

According to the American National Cancer Institute guideline for plant screening, any extract that have IC_{50} value equal or less than 20 μg/mL in *in vitro* cytotoxic test, is considered as a promising anti-cancer potential [29]. M1S9 exerted profound suppressive effect on the proliferation of the MCF-7 cell lines (IC_{50} = 38.5 μg/mL) as well as the PMEC (the normal breast cells) (IC_{50} = 17 μg/mL) after 72 hours of treatment. Araújo *et al* (2013) had determined that the aqueous extracted MOS was potentially cytotoxic to the human peripheral blood mononuclear cells (IC_{50} = 34.3 μg/mL) [30]. This might due to the presence of isothiocyanate (ITC) in the MOS that are toxic to the normal cells [31]. The ethanolic extracts of MOL and bark exhibited anti-cancer properties while MOS extract showed no remarkable anti-cancer properties in both breast MDA-MB-231 and colorectal HCT-8 cancer cells [6,32]. On the other hand, dichloromethane-fraction of MOS is highly toxic to MCF-7 (IC_{50} = 26 μg/mL) but limits its toxicity to normal cancer cells, MCF 10A (IC_{50} > 400 μg/mL) [8]. Taken together, different extraction solvents might lead to the variation in phytochemical compositions that determine its respective medicinal properties. Although M1S9 demonstrated the lowest antioxidant activity among the moringa mixtures, it possesses the anti-cancer effect, highlighting the mechanism underlying anti-cancer effects are not governed by antioxidant properties. To this end, the role of M1S9 on chemoprevention warrants further investigation using *in vivo* model mimicking human body responses.

Due to less tumourigenic and slow growing of MCF-7, more aggressive MDA-MB-231 cell line was used to induce solid tumour in xenograft mice and examine its effect with M1S9 treatment. At the third week of treatment, percentage of tumour growth inhibition for high-dose M1S9 and tamoxifen (TAM) were 64.5% (the highest rate throughout eight weeks) and 69.1%, respectively. TAM-treated mice showed the highest inhibition rate at the sixth week, up to 80.5%. In Ehrlich's solid tumour implanted mice model (a breast cancer model), high-dose MOL (500 mg/kg) was

ingested orally for successive 14 days and exhibited tumour growth inhibition up to 34.98% [33]. Therefore, we postulated that M1S9 possesses better anti-cancer ability in killing the MDA-MB-231 breast cancer cell than MOL alone. Moreover, there was no statistically significant difference found in the relative weights of the liver, heart, spleen, lung and kidney organs, indicating the safe usage of M1S9 for up to eight weeks in current investigation. Both *in vitro* and *in vivo* models exerted anti-cancer potential in breast cancer cells and further delineation of underlying pathways/mechanisms of action were investigated.

Angiogenesis plays an important role in the development of breast cancer. Angiogenesis is characterized by the development of a vascular supply, which is a critical factor to spread the cancer cells to other organs, termed metastases. Vascular endothelial growth factor (VEGF) plays an important marker for angiogenesis and the main pro-angiogenic factor in the angiogenesis of breast cancer [34]. M1S9-treated mice showed a significant reduction in VEGF proteins, suggesting that M1S9 inhibits endothelial cell proliferation or blocks the effects of VEGF upon endothelial cells. There is evidence that the overexpression of VEGF is correlated with an adverse prognosis, at least in certain tumour. Tumour that expressed VEGF is particularly attractive as a target for anti-cancer therapy because its angiogenesis-promoting activity is at the level of the endothelial cell and, compared with agents that directly target tumour cells, tumour penetration is less critical for VEGF inhibitors [34]. Vascular endothelial growth factor receptor 2 (VEGFR2) mediated calcineurin/nuclear factor of activated T-cells (NFAT) pathway plays leading roles in the angiogenesis of breast cancer. Calcineurin (CaN) is a target enzyme in this pathway. In endothelia, VEGFR2 activates CaN, triggers NFAT translocated into nucleus, and up-regulates angiogenic factors [35].

From metabolite profiling, M1S9 extract possesses flavonoid-based compounds, such as isoquercetin, quercetin, astragalin, isorhamnetin and kaempferol. These flavonoid-based compounds have been reported to act as inhibitor of CaN, which block CaN/NFAT pathway, and consequently suppresses angiogenesis [35]. We hypothesize that flavonoid-based compounds in M1S9 can act as inhibitor of CaN. CaN/NFAT pathway promotes growth, invasion, migration and angiogenesis in cancers. NFATc3 activation in endothelial is important for angiogenesis in breast cancer *via* secreted frizzle-related protein 2. The tyrosine kinase of VEGFR2 pathway activates phospholipase Cγ, releases calcium *via* inositol-1,4,5-triphosphate (IP3), and regulates CaN *via* calmodulin in endothelia chronologically. Activated CaN regulates NFAT dephosphorylation, consequently NFAT enters into nuclear and induces VEGFA, VEGFR, and cyclooxygenase-2 (COX-2) expression by endothelial cells. COX-2 catalyzes the synthesis of prostaglandin E2 (PGE2), which binds to PGE2 receptors on endothelial cells, stimulating their proliferation migration and vessel formation. Furthermore, tumour cells secreted VEGF into extracellular matrix, which binds to VEGFR on endothelia, activating NFAT signalling cascade to form a vicious cycle (Figure 4) [35].
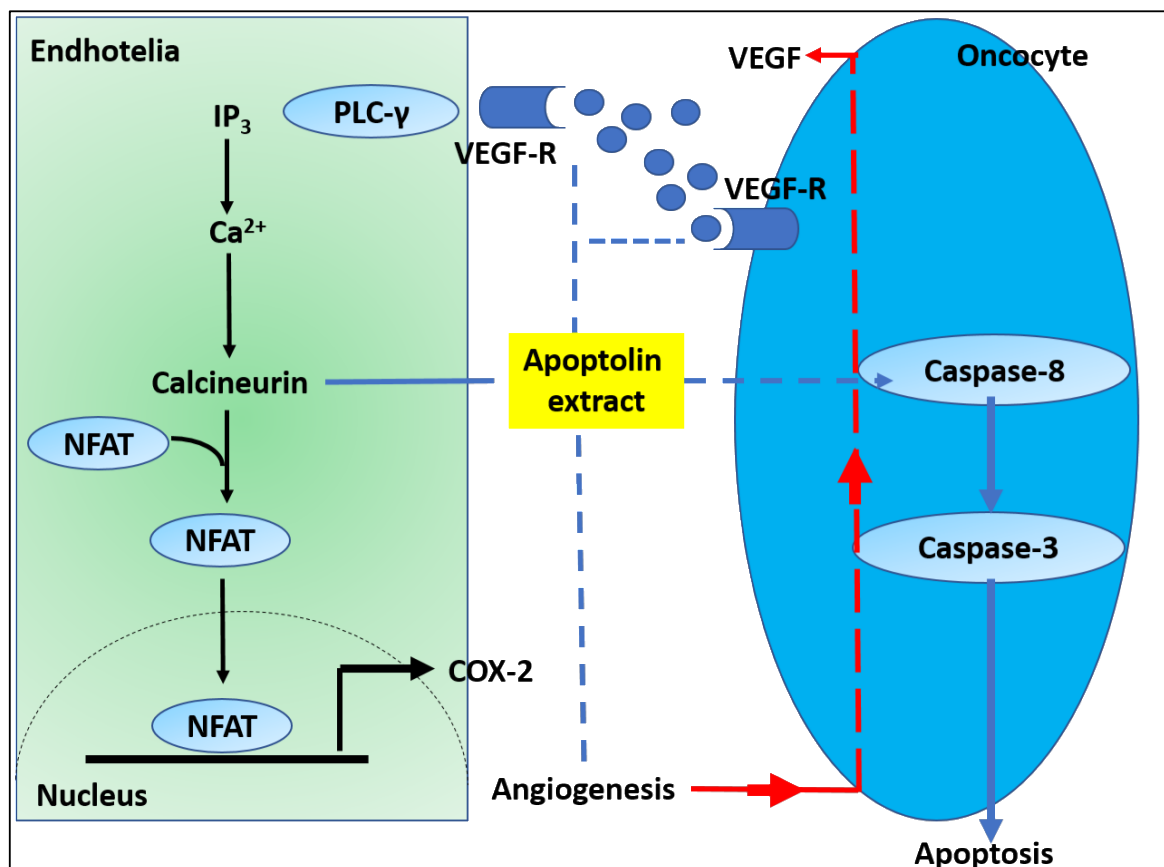
Figure 4. Anti-angiogenesis of M1S9 extract via inhibiting calcineurin activity. (1) The tyrosine kinase of VEGFR pathway activates phospholipase Cγ (PLCγ), releases calcium via inositol-1,4,5-triphosphate (IP3), and activates calcineurin via calmodulin. Activated calcineurin regulates NFAT dephosphorylation, consequently NFAT entering nuclear to induce the expression of cyclooxygenase-2 (COX-2), which stimulate angiogenesis. (2) Oncocytes secrete VEGF into the extracellular matrix, which binds to VEGFR on endothelial cells, leading to the activation of NFAT signalling cascade to form a vicious cycle. (3) M1S9 inhibits calcineurin activity in NFAT pathway and limits VEGF releasing from oncocytes. (4) M1S9 activates caspase-8 of apoptosis pathway in oncocytes.

At the molecular level, only SLC39A6 and SFRP1 genes were down-regulated significantly in M1S9-treated MDA-MB-231 tumour. SLC39A6 (Solute carrier family 39, member 6), also known as LIV-1, is a zinc transporter involves in the metabolism of protein, nucleic acid, carbohydrate and lipid and also regulate the gene transcription, growth, development and differentiation [36]. Deregulated expression of zinc transporters could perturb the regulation of zinc in cellular processes, leading to the initiation or progression of breast cancer [37]. Overexpression of SLC39A6 increases the cell proliferation and regulates metastasis in KYSE30 and KYSE450 cells (oesophageal cancer cells) as well as HeLa cells (cervical cancer cells) while knockdown of this gene suppressed the cell proliferation and reduced lymphatic metastasis [38]. Cheng *et al.* (2017) proposed that the role of SLC39A6 is to enhance carcinogenesis process, participating in pathways of AKT and ERK that may involve in proliferation, invasion and metastasis [38]. These studies together demonstrated that the down-regulation of SLC39A6 gene in M1S9-treated MDA-MB-231 tumour

could be associated with lower tumour volumes than non-treated mice, probably by restricting cancer cell proliferation. In the context of protein level, Kasper *et al*. (2005) showed that down-regulation of LIV-1 protein is correlated with a more aggressive tumour (higher grade, stage and size) while less aggressive tumour showed no significantly difference in LIV-1 protein expression as compared with normal tissue [39]. This study showed different expression patterns as compared to molecular level. It is yet to elucidate its protein expression levels and pathways involved to further strengthen current finding. SFRP1 (secreted frizzled-related protein 1) plays an important role in Wnt signalling and apoptosis [40]. In MDA-MB-231 xenograft model, SFRP1 suppresses tumour growth by blocking the Wnt signaling and inhibits cancer cell adhesion and migration by blocking the thrombospondin-1 [40-42]. In patient cohort with triple negative breast cancer (TNBC), SFRP1 gene was significantly overexpressed in MDA-MB-231 cell lines and non-TNBC subtypes (SKBR-3 and MCF-7) [43]. Subsequently, loss-of-function test on SFRP1 in TNBC modified the tumourigenic properties of the cells through pro-apoptotic and migratory pathways, but do not rely on Wnt signalling. In gastric cancer, overexpressed SFRP1 gene is associated with the activation of transforming growth factor-beta (TGFβ) signalling pathway that induces cell proliferation, epithelial-mesenchymal transition (EMT) and invasion [44]. In other words, SFRP1 promotes tumourigenesis and metastasis leading to a more aggressive phenotype. In laryngeal carcinoma cells, 5-aza-2'-deoxycytidine (5-aza-dC) exposure increased the gene expression of SFRP1 that can suppress cell proliferation *in vitro* model and tumour growth *in vivo* model [45]. In this study, it was found that exposure of MDA-MB-231 tumour to M1S9 extract significantly decreased SFRP1 gene expression, suggesting the cells was responding to the treatment with reduction in the cell growth.

M1S9 extract was found to regulate the apoptotic process (through the up/down-regulation of certain genes), activate the MAPK cascade (through the up-regulation of AR, IL6 and IGF1 genes) and the ERK1/ERK2 cascade (through the down-regulation of MAPK3, PYCARD, TGFB1), which might be involved in cancer chemoprevention. Gene network analysis suggests that M1S9 extract might trigger cell cycle, cell death and survival in MDA-MB-231 cancer cells. Anti-cancer potential is mainly attributed by the glucosinolates in M1S9 extract that have been hydrolyzed into active metabolite, isothiocyanate (ITC). Hydrolysis of glucosinolate is catalyzed by myrosinase from plant source in small intestine or bacterial myrosinase in colon at neutral pH [46]. There are two types of glucosinolates found in M1S9 extract that are primarily from MOSr, i.e. glucosinalbin and glucotropaeolin compounds, which can be converted into 4-hydroxybenzyl ITC and benzyl ITC, respectively [46]. Benzyl ITC has been previously found to display anti-proliferative, anti-cancer and anti-metastatic properties [47]. Jurkowska *et al.* (2018) reported that 4-hydroxybenzyl ITC from white mustard seeds possesses anti-proliferative effect on human neuroblastoma (SH-SY5Y) and glioblastoma (U87MG) [48]. These implied that M1S9 could potentially demonstrate anti-tumour activity with the presence of benzyl ITC and 4-hydroxybenzyl ITC and its underlying mechanism remained to be elucidated.

Although M1S9 extract possesses anti-tumour activity, its effectiveness was lower than TAM-treated model and was toxic to PMEC. Gong *et al.* (2018) showed that lutein (a type of carotenoid) inhibited MDA-MB-468 and MCF-7 breast cancer cell growth without affecting the growth of PMEC [49]. This suggests that removal of certain phytochemical compounds presence in M1S9 might improve the treatment efficiency to not exert toxic effect to PMEC. Several studies have

been conducted to investigate the combined effects of herbal products with chemotherapeutic drug. It is because phytochemical compounds in moringa plant possess anti-tumour function with low toxicity, low side effects and low tumour resistance [5]. The combined effect of ethanolic extract of MO and doxorubicin showed enhanced cytotoxic effect by triggering apoptosis against HeLa cervical cancer cells [50]. In pancreatic cancer cells, aqueous extract of MOL not only inhibited the growth of cancer cells through NF-kB signalling, but also improved the efficiency of chemotherapy when combined with cisplatin (a drug for pancreatic cancer) [51]. In breast cancer, the combination of a bioactive subfraction of *Srobilanthes crispus* and tamoxifen demonstrated the anti-cancer response on MCF-7 and MDA-MB-231 cell lines and did not show any cytotoxic side effect of the drug on non-malignant MCF-10A cells [52].

In order to achieve better cytotoxic effect with low side effect against normal cell, ratio adjustment between MOL and MOSr would be beneficial to identify a moringa mixture that is enriched with all identified compounds (especially for those with anti-cancer ability). It might exhibit more profound selectively of anti-cancer ability on cancer cells *in vivo* and *in vitro* model, without killing the non-cancer cells. Moreover, the combined effect with tamoxifen also can be investigated to gain insight on whether it will enhance anti-tumour function with low toxicity, low side effects and low tumour resistance.

5. Conclusions

Moringa mixture, M1S9 exhibited cytotoxic activities towards PMEC and MDA-MB-231 cell lines without relating to the antioxidant activity. We hypothesized that M1S9 extract inhibited breast cancer angiogenesis via targeting angiogenic proteins such as VEGF and calcineurin. M1S9 exhibits the anti-cancer potential by interfering the signal transduction cascade responsible for cancer proliferation and progression. Although the mechanism governed by M1S9 extract is not completely delineated at the molecular level, this study is impactful as it added value of the moringa seed residue (MOSr) into a usable product in pharmacological aspect, especially in the treatment of aggressive breast cancer.

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