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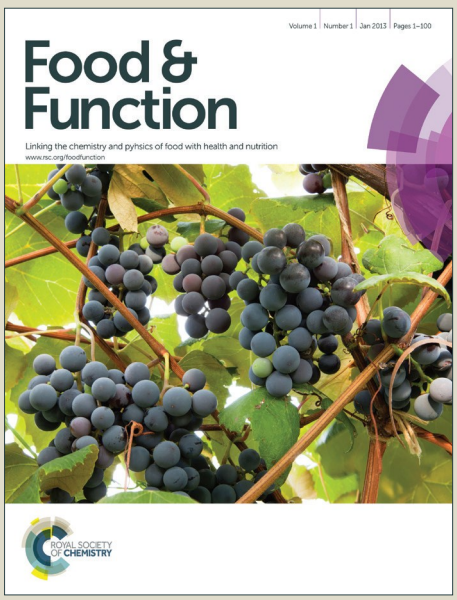

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ARTICLE

Morinda citrifolia edible leaves extract enhanced immune response against lung cancer

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Abstract

Lung cancer causes 1.4 million deaths annually. In the search for functional food as complementary therapy against lung cancer the immuno-stimulatory properties of the vegetable *Morinda citrifolia* leaves were investigated and compared with the anti-cancer drug Erlotinib. Lung tumour-induced BALB/c mice were fed with 150mg/kg, 300mg/kg body weight of the leaf extract or Erlotinib (50 mg/kg body-weight) for 21 days. The 300mg/kg body-weight extract significantly (and dose-dependently) suppressed lung tumour growth, more effectively than the 50 mg/kg body-weight Erlotinib treatment. The extract significantly increased blood lymphocytes counts, spleen tissues B cells, T cells and natural killer cells, and reduced the epidermal growth factor receptor (EGFR) which is a lung adenocarcinoma biomarker. The extract also suppressed the cyclooxygenase 2 (COX2) inflammatory markers; and enhanced the tumour suppressor gene (phosphatase and tensin homolog, PTEN). It inhibited tumour growth cellular genes (transformed mouse 3T3 cell double minute 2 (MDM2), V-raf-leukemia viral oncogene 1 (RAF1), and mechanistic target of rapamycin (MTOR)) mRNA expressions in the tumours. The extract is rich in scopoletin and epicatechin as the main phenolic compounds. The 300 mg/kg *Morinda citrifolia* leaves 50% ethanolic extract showed promising potential as a complementary therapeutic dietary supplement which was more effective than the 50 mg/kg Erlotinib in suppressing lung adenocarcinoma. Part of the mechanisms involved enhancing immune responses, suppressing proliferation and interfering with various tumour growth signalling pathways.

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, resulting in 1.6 million new cases and 1.4 million deaths per year. Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases, with the 5-year survival rate of only 16%[1]. Chemotherapy is relatively ineffective for patients with advanced NSCLC and the response rate is only 20% to 35% with a median survival of 10 to 12 months[2]. In a phase III study, the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) drugs such as Erlotinib significantly improved the overall survival relative to supportive care for refractory stage IIIB/IV NSCLC. However, Erlotinib use is limited because of several serious side effects[3] and the emergence of cancer mutations which confer drug resistance. The common Erlotinib side effects are weakness, diarrhoea, rash, shortness of breath, cough, and loss of appetite, fatigue (feeling tired), and nausea. Erlotinib may cause more serious side effects such as lung problems (shortness of breath, cough, and fever); interstitial lung disease, liver and kidney problems; blistering and skin peeling; gastrointestinal perforation; bleeding and clotting problems which may lead to heart attack stroke, dry eyes, unusual eyelash growth, or swelling of the cornea; harm to an unborn baby and even death.

The inflammatory process and cyclooxygenase2 (COX2) is an important contributor to lung cancer development and pathogenesis. NSCLC is often accompanied by increased COX2 expressions[4], which catalyzes arachidonic acid conversion into the unstable intermediate prostaglandin H2, to form other prostanoids, prostaglandin (PG)-E2, prostacyclin and thromboxane (Tx)-A2. The COX2 expression correlates positively with poor prognosis stage I disease and increased COX2 mRNA levels foretell a low survival rate in NSCLC[5].

Morinda citrifolia (Rubiaceae) leaves are commonly consumed as vegetables after blanching by the Polynesians of South East Asia. The *M. citrifolia* leaves also have some healing properties and are traditionally used to treat wound infections, pain, arthritis, swellings, and similar conditions. *M. citrifolia* leaves has antioxidant, liver-protective and wound restorative effects[6] without any acute, sub-acute and sub-chronic oral toxicity[7]. An oral intake of 1000 mg/kg of *M. citrifolia* leaves ethanolic extract has been reported as the no observed-adverse-effect level (NOAEL)[8]. This study attempts to investigate the possible immune-stimulatory and tumour suppressive effects of *M. citrifolia* edible leaves as complementary therapy against lung cancer and compare it with Erlotinib.

Experimental

Plant Materials

The *M. citrifolia* leaves (Voucher No. SK2322/14, identified by Biodiversity Unit, Institute of Bioscience, University Putra Malaysia, Serdang) were collected from Institute of Bioscience, University Putra Malaysia, State of Selangor, Malaysia. The leaves were dried and mixed in the ratio (w/v) of 1:5 with the 50% ethanol in water. The yield was 13.61%. The extracts were analyzed by high-performance liquid chromatography, HPLC (Waters 2996, Milford, MA) [9]. HPLC grade methanol (MeOH), acetonitrile (MeCN) and analytical grade trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany).

Chemical characterisation and standardisation of the 50% ethanolic leaf extract.

The leaf extract were analysed using High pressure liquid chromatography equipped with an Atlantis C18 column (4.6 mmX250 mm; 5µm, Waters Corporation, Milford, MA, USA), maintained at 25°C. The mobile phase consisted of three solvents: A; MeCN, B; MeOH, and C; 0.1 TFA% in H2O (v/v), programmed consecutively in linear gradients as follows: 0 min, 10% A, 10% B, and 80% C; 15 min, 20% A, 20% B, and 60% C; 26 min, 40% A, 40% B, and 20% C; 28-39 min, 50% A, 50% B, and 0% C; and 40-45 min, 10% A, 10% B, and 80% C. The elution was run at a flow rate of 1.0 mL/min with 50µL sample injection volume, and UV spectra detector set at 210 and 450 nm. The extract contains 2.19% scopoletin (Retention time, Rt=12.02 min) and and 3.41% epicatechin (Rt=9.17 min) as the main compounds, qualitatively and quantitatively identified via the retention times and calibrated standard plots. Spiking with scopoletin and epicatechin produced sharp extended peaks at the specific retention times which qualitatively confirmed their presence.

Cell culture

Human lung adenocarcinoma (A549) cell lines and Kaighn's Modification of Ham's F-12 (F-12K) medium were obtained from American Type Culture Collection (ATCC). A549 NSCLC cells were cultured in F-12K medium, which supplemented with 10% fetal bovine serum (PAA, Austria) and 1% of 100μg/mL penicillin and streptomycin (Biowest, USA). Cells were grown in a humidified incubator at 37°C with 5% CO^2.

Animals

Male BALB/C mice (6 weeks old, weighing 19–20g) were purchased from Faculty of Veterinary Medicine, University Putra Malaysia. Mice were given standard chow and water and kept on a 12-h light/12-h dark cycle. The animal studies were performed in strict accordance with the Guidelines of the Institutional Animal Care and Use Committee (IACUC) University Putra Malaysia (protocol approval number: UPM/IACUC/AUP-R016/2013).

***In vivo* tumour xenograft model**

Ten mice were selected randomly and assigned as the control healthy Group (1). A549 NSCLC cells ($2x10^7$) resuspended in 100µL PBS were injected subcutaneously into the backs of forty mice (Lu et al., 2009). When the tumour size reached approximately 100 mm^3, after 14 days of implantation, the mice were randomly assigned to the following experimental groups and administered daily by oral gavage for 21 days: (II) saline; (III) Erlotinib (50 mg/kg) (Clark et al., 2008); (IV) 150 mg/kg and (V) 300 mg/kg of *M. citrifolia* leaves. The tumour volume was measured every 3 days by using the following formula:-

Tumour Volume, mm^3 $[a(b^2)/2)]$, a=largest diameter; b=smallest diameter

Mice were sacrificed via intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (10 mg/kg). Tumours were excised and some were snap frozen in liquid nitrogen for gene expression, while others were fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin (H&E) and immunohistochemical (IHC) examination. Blood samples were collected in EDTA coated collection tubes for differential counts. Blood cells were stained using ABBOT reagent and analyzed in ABBOT Celldyn 3700 (GeligaSistemSdnBhd, Petaling Jaya, Malaysia).

Immunohistochemistry Staining

The immunohistochemistry (IHC) staining evaluated the EGFR expression in the tumour foci of the A549-induced tumour using the light microscope. IHC kits (ChemMate™ DAKO EnVision™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse) were purchased from Dako, Denmark. The primary antibody was anti-EGFR antibody ab15669 (Abcam; Cambridge, United Kingdom). The tumour tissues were embedded in paraffin blocks and microtome cut into 4 µm sections, deparaffinized and consequently incubated in xylene, absolute ethanol, 90%, 70% and 50% ethanol for 3 minutes each. They were then treated with sodium citrate (pH 6) and boiled for 10 minutes for de-masking. The slides were rinsed with washing buffer (dissolved 5 PBS tablets in 1L distilled water and added 1ml Tween-20), followed by blocking with 300µl of H_2O_2 for 5 minutes. After washing, the processed tissue sections were incubated with primary antibodies (EGFR) overnight at 4°C. Subsequently, slides were treated for 1 h with secondary, goat anti-mouse anti-rabbit antibodies conjugated with horseradish peroxidase and developed with 3,39-diaminobenzidine. Finally, the tissue sections were rinsed in distilled water, counterstained with Mayrri hematoxylin and mounted with DPX mounting medium for microscopic examination. Immunohistograms were taken with a phase contrast microscope (Leica, Wetzlar, Germany). Immunohistochemical staining of tissue sections were performed in triplicate.

Immunophenotyping Analysis of Inflammatory Cytokines

The isolated lymphocytes from the spleens (the primary site for platelet-reactive T and B cells activation [12] of A549-bearing mice after 21 day treatment were analyzed by flow cytometry. Immunophenotyping was performed to assess lymphocyte population on a Becton Dickinson (BD) FACS Calibur (BD, Franklin Lakes, New Jersey) using fluorochrome conjugated monoclonal antibodies (Serotec) directed against receptors to CD4+ (PerCP-Cy), CD8+ (APC), CD19+ (PE) and CD335+ (PerCP-Cy) antigens following the manufacturer's protocol (BD Bioscience). The collected spleen tissues were meshed through 70µm cell strainer with 5ml PBS-EDTA-BSA (phosphate buffered saline-ethylenediaminetetraacetic acid-bovine serum albumin). After spin down, the pellets were washed with 3ml PBS twice, followed by incubation with 2ml lysis buffer (NH_4Cl) at 4°C, 10 minutes. After washing, the number of cells should not be more than $2x10^6$ cells. The cells were incubated with 5µl/dye in ice for 90 minutes, then washing with PBS, and added 600µl of 1% paraformaldehyde. The cells were incubated at 4°C for 7 days. Cell pellet was suspended in PBS and analyzed by FACS using CellQuest analysis software (BD FACS Canto II, USA).

Gene expression

Tumour RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Custom RT² Profiler PCR Array (CAPM11988), RT² SYBR Green qPCRMastermix, RT² First Strand Kit and RNase-Free DNase Set were purchased from SuperArray Bioscience Corporation (Frederick, MD). Quantitative RT-PCR array for differentially expressed genes was performed utilizing RT² Profiler PCR Array Data Analysis version 3.5 (SABiosciences; Fredrick, MD, USA), which normalized to HSP90AB1 (NM_008302) and GAPDH (NM_008084). RT-PCR data is represented as the average relative mRNA gene expression of each experimental group (n=3). Fold change (2^(-ΔΔCt)) is defined as the normalized gene expression (2^(-ΔCt)) in the test sample, divided by the normalized gene expression (2^(-ΔCt)) in the control sample. Fol

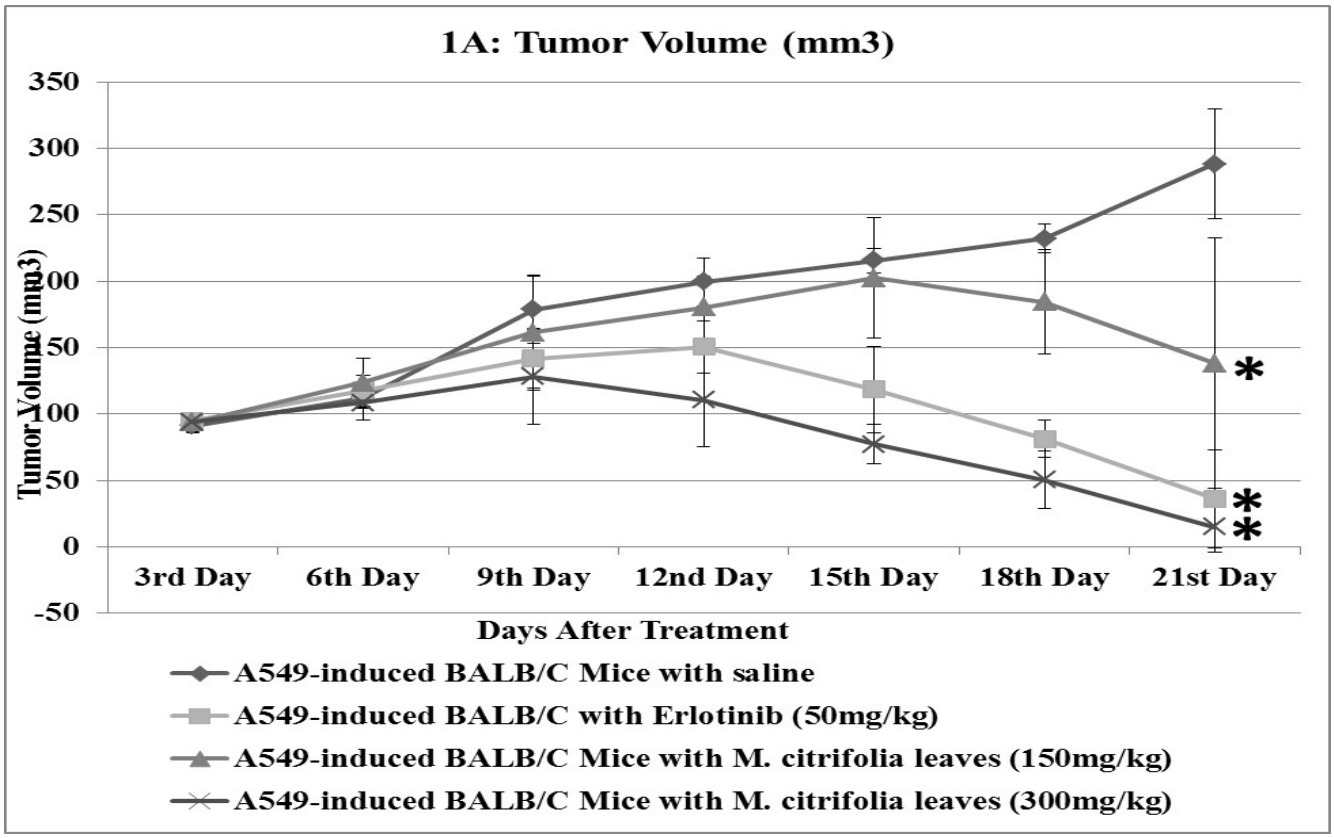
regulation represents fold-change as biologically meaningful results. Fold change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. The p values are calculated based on a t-test of the replicate 2^(-ΔCt) values for each gene in the control group and treatment groups.

Statistical Analysis

Data was expressed as mean ± standard deviation (mean±SD) of at least three independent experiments; significant differences ($p<0.05$) using one-way analysis of variance (ANOVA) and Duncan test using statistical analysis IBM SPSS Statistics 21 software. The Student's t-test was used where values of $p<0.05$ were considered significant.

Results

Fig. 1A show the tumour volume measured every 3 days were most highly suppressed in animals receiving 300mg/kg of extract (14.81±19.06mm^3), followed by the 50 mg/kg Erlotinib (36.26±37.00mm^3), and 150mg/kg extract (138.28±93.91mm^3) which were significantly smaller compared to the control tumour-induced untreated animals (288.21±41.53mm^3). The 300mg extract /kg improved the body weight increases to be insignificantly different to the healthy control mice by the 21st day (Fig 1B).



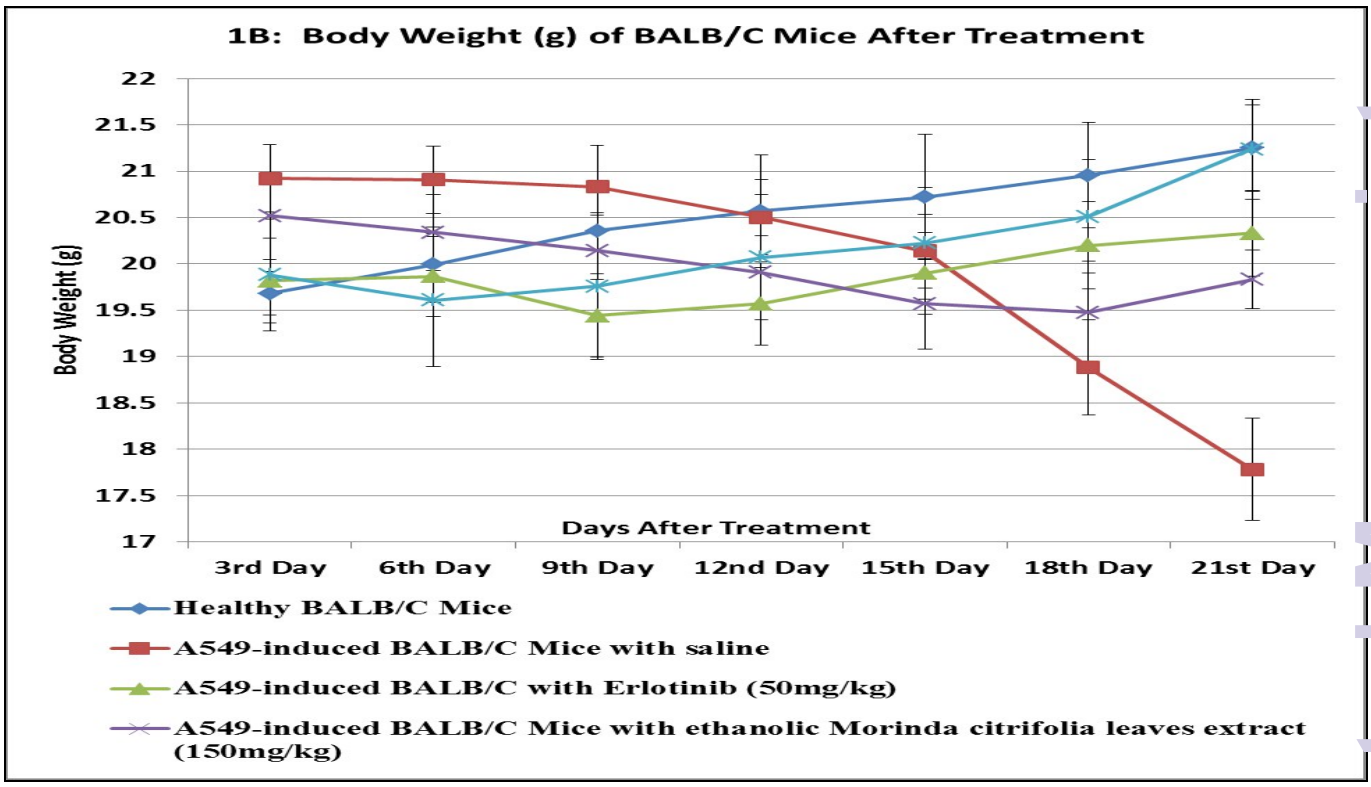


Fig. 1. Effect of *M. citrifolia* leaves extract on the tumour volumes (tumourigenicity) and body weights of NSCLC-induced mice by comparing untreated and extract-treated or Erlotinib-treated mice (mean ± SD).

Fig. 2 shows a good correlation between the tumour EGFR expressed by the A549 NSCLC and their IHC EGFR status, in line with reports by other researchers [13]. Fig 2A demonstrates the different orientation directions (visible as small aggregated clusters or cord like growth) and forms (variable sized and shaped hyperchromatic nuclei) of the cancer cells, & some unknown cells. The cancer cells secreted copious extracellular matrix to expedite their growth, which appeared as unstructured pinkish proteinacious constituents. The proliferated fibroblastic-like tumour cells are disseminated in a twisted whirlpool-like pattern. Fig 2B displays the EGFR immunohistochemistry stain of a fibrosacormas growth mass extracellular matrix to the antibody, and their dispersed orange-chocolate coloured affirmative responses.

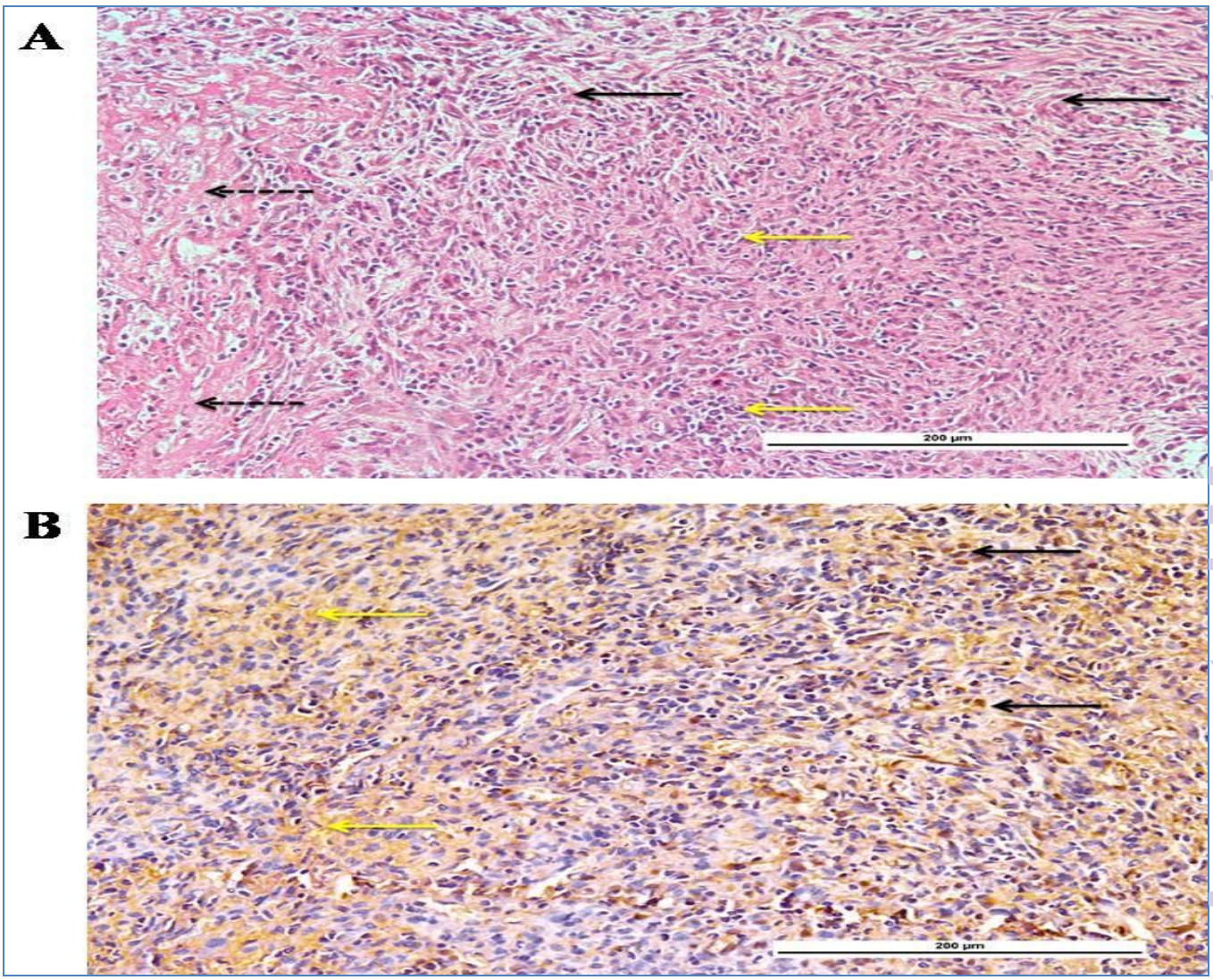


Fig 2A shows the tumour cells oriented in different directions and forms, like small aggregated clusters or cord like growth (yellow arrow) with variable sized and shaped hyperchromatic nuclei & unidentified cells. The abundant extracellular matrix secreted by the tumour cells to facilitate their spread, appeared as amorphous pinkish pool of proteinacious materials (dashed black arrow). The multiplied fibroblastic-like cancer cells are distributed in a warped whirlpool-like shape (black arrow).
Fig 2B shows the immunohistochemistry staining of the extracellular matrix ground substance of a fibrosacormas like tumour mass to EGFR antibody, and the disseminated golden-brown positive reaction to the antibody in the tissue section background (yellow arrow). Some of the tumour mass cells showed positive reactions to the EGFR is indicted by their deep brown color (black arrow).

Fig. 2. Photomicrograph of the tumour of mice at the end of the treatment, representative of three different experiments (A) H&E, (B) IHC, X200)

Fig. 3 shows the untreated tumour induced mice had significantly lower lymphocytes ($3.14\pm0.14x10^9$/L) counts than healthy mic ($6.08\pm0.10x10^9$/L). Treatment with 300mg/kg of extract significantly increased the total white blood cell (WBC, $9.93\pm0.42x10^9$/L) levels and lymphocyte ($7.98\pm0.43x10^9$/L) counts in the tumour-induced mice. The 50 mg/kg Erlotinib and 150mg/kg extract treatment also produced significantly lower effects than the 300mg/kg extract. Similarly, Fig. 4 shows a highly significant increase in the immune cells (T helper cells, monocytes, macrophages, and dendritic cells), and CD4 markers by 42.45±0.15%; co-receptors for the T cell receptor CD8 biomarker by 27.13±0.39%; B cells (CD19 biomarker) by 26.89±0.49% and natural killer cells (CD335 biomarker) levels by 2.34±0.14% with the 300mg/kg extract treatment compared with the untreated tumour-induced mice.

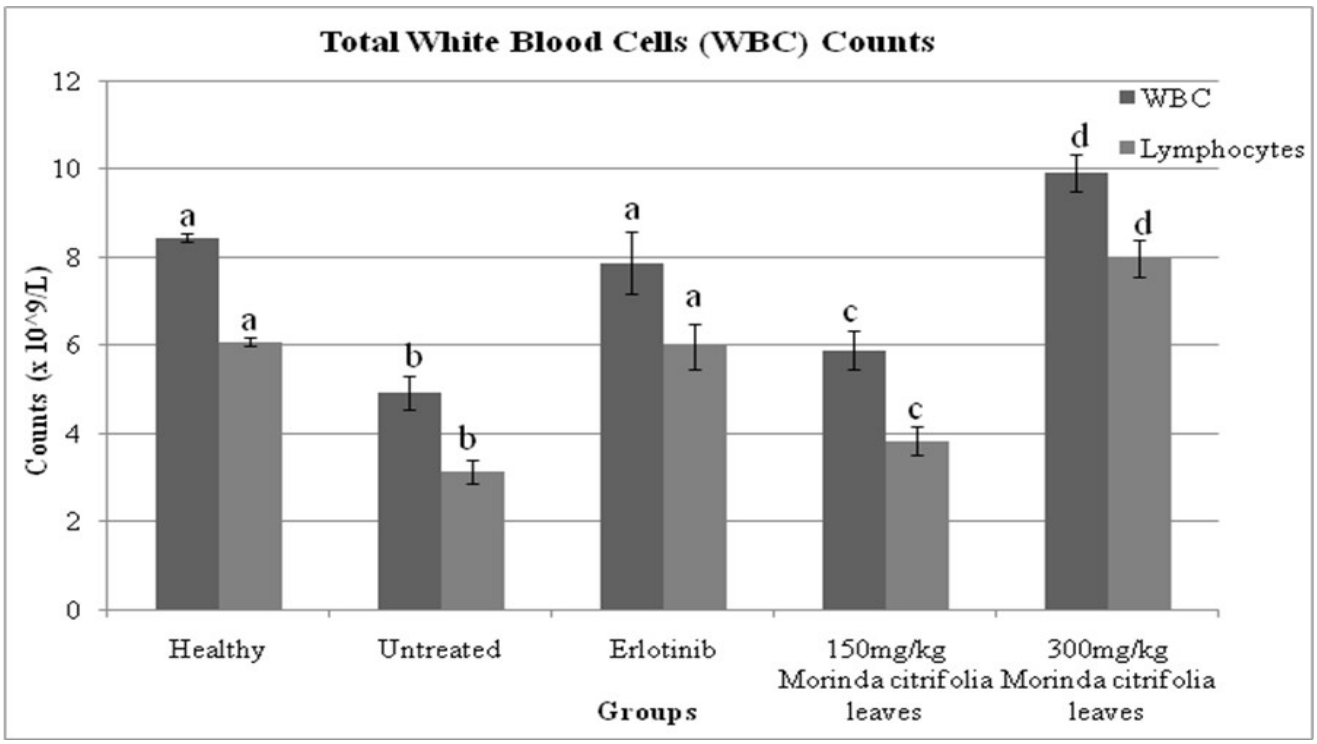
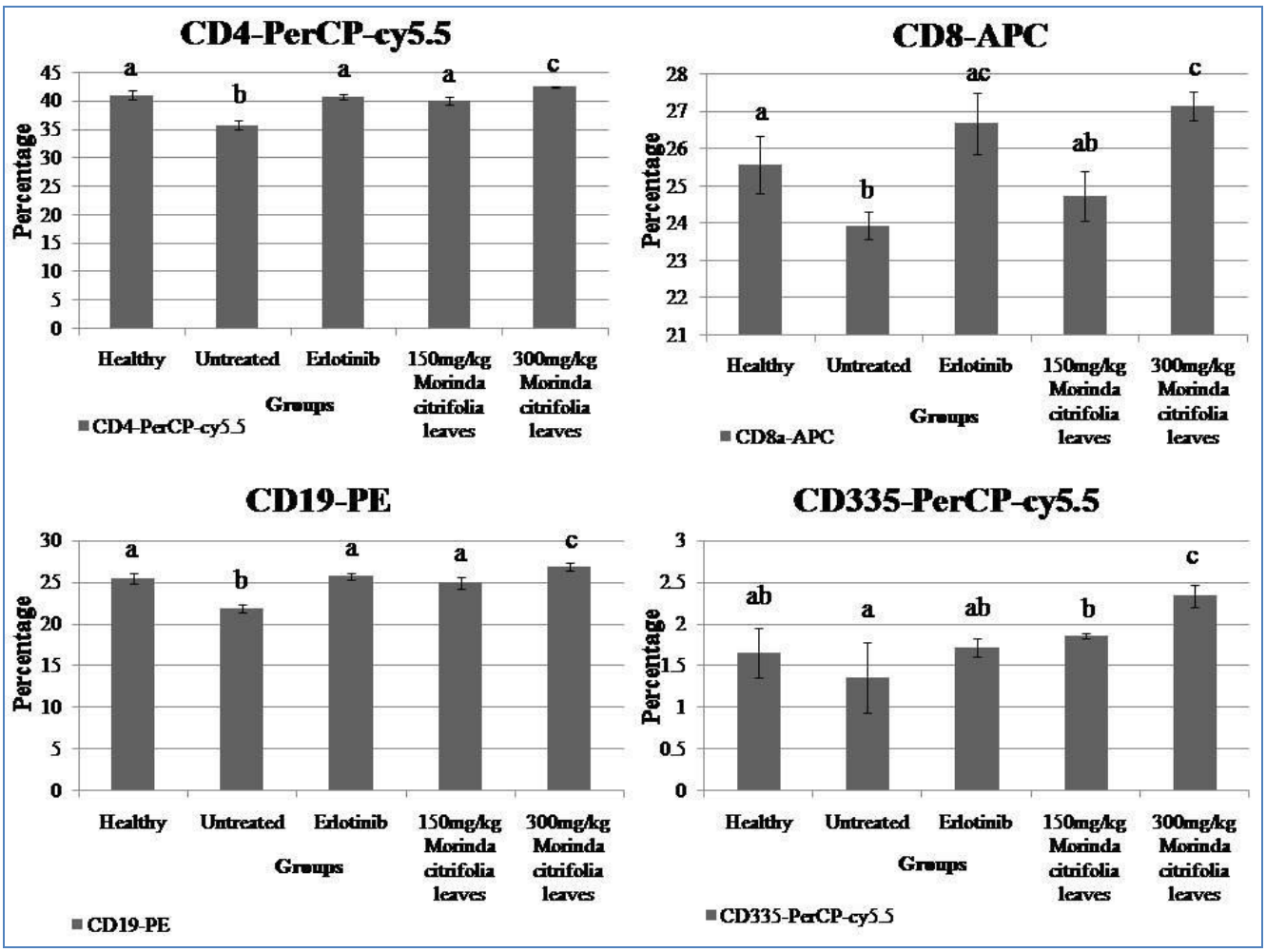


Figure 3. White blood cell counts in various groups. Values are expressed as mean ± standard deviation (n = 3). Means with different superscript letters within the same graph are significantly different ($p<0.05$).



Values are expressed as mean ± standard deviation (n = 3). Means with different superscript letters within the graph are significantly different ($p<0.05$).

Fig. 4. Immuno-phenotype analyses of B cells, T cells and natural killer markers in spleen tissue.

Mouse RT-PCR Arrays were performed in the tumour tissues. Quantitative RT-PCR analysis was performed using the comparative threshold cycle method to calculate fold change in gene expression normalized to Gapdh and Hsp90ab1 as the reference housekeeping gene. Table 1 shows the extract significantly ($p<0.05$) up-regulated the tumour suppressor gene (phosphatase and tensin homolog, PTEN) and down-regulated the tumour cells proliferation genes such as (i) Epidermal growth factor receptor (EGFR), (ii) MDM2 (transformed mouse 3T3 cell double minute 2), (iii) RAF1 (V-raf-leukemia viral oncogene 1), and (iv) MTOR (mechanistic target of rapamycin) mRNA expressions in the tumour-induced mice. Table 1 also shows the extract strongly and dose-dependently suppressed the pro-inflammatory COX2 (cyclooxygenase 2) expression (150mg/kg:-1.72-fold, p=0.17; 300mg/kg:-4.09-fold, p=0.05), while the 50 mg/kg Erlotinib did not.

Table 1: Effects of treatments on tumour-related genes expressions in the lung tissues

Gene	Accession Number	Description	Fold Change					
			Erlotinib (50mg/kg)	p value	*M. citrifolia* leaves extract (150mg/kg)	p value	*M. citrifolia* leaves extract (300mg/kg)	p value
MDM2	NM_010786	Transformed mouse 3T3 cell double minute 2	-1.25	0.53	-1.16	0.57	-2.96	0.04*
EGFR	NM_007912	Epidermal growth factor receptor	-3.62	0.01*	-1.90	0.09	-2.85	0.03*
RAF1	NM_029780	V-raf-leukemia viral oncogene 1	-2.51	0.00*	-1.50	0.13	-3.41	0.00*
PTEN	NM_008960	Phosphatase and tensin homolog	2.82	0.14	2.42	0.04*	4.23	0.05*
MTOR	NM_020009	Mechanistic target of rapamycin	-2.75	0.03*	-2.22	0.04*	-2.71	0.05*
COX2	NM_011198	Cyclooxygenase-2	-1.38	0.90	-1.72	0.17	-4.09	0.05*

Values represent fold change between control and treatment group, and differed significantly at $p < 0.05$, represented by "*".

Figure 5 shows the HPLC profile and analysis of the Morinda leaf extract together with the epicatechin and scopoletin standards run under similar conditions. The profile shows that the epicatechin (3.4%) and scopoletin (2.2%) may be the major bioactive compounds that may have contributed to the effects.

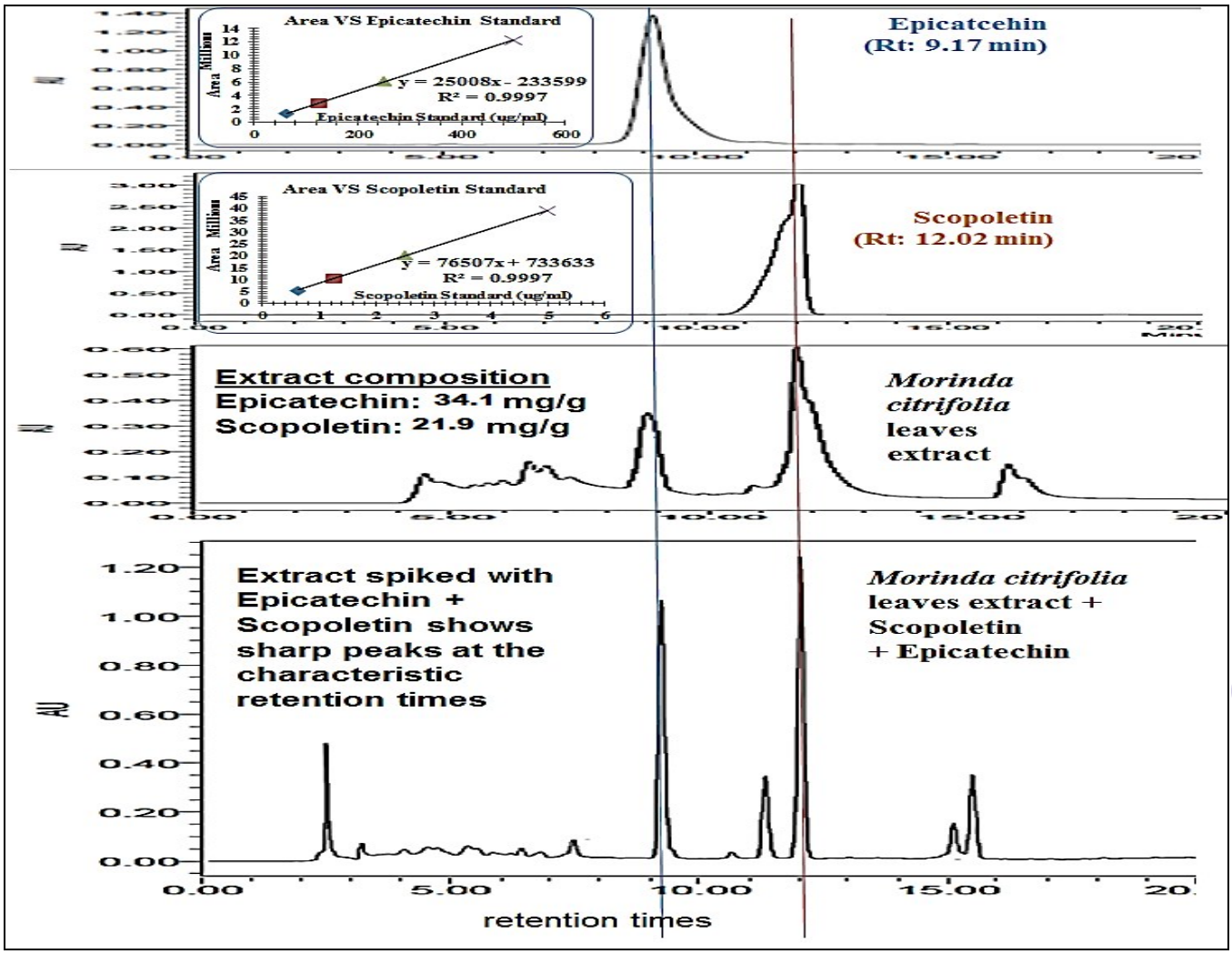


Figure 5. Qualitative and quantitative HPLC chemical analysis of *M. citrifolia* leaves 50% ethanol extract.

Discussion

The NSCLC has distinct morphological and molecular subtypes, usually with EGFR activation and the stimulation of (i) the rat sarcoma (RAS)-mitogen-activated protein kinase 1 (ERK), which controls gene transcription and cell proliferation, and (ii) the phosphoinositide-3 kinase (PI3K)-v-akt murine thymoma viral oncogene homolog 1 (AKT) axes, which induces pro-survival signals [14]. These signalling biomarkers are anticancer targets to induce apoptosis and/or inhibit tumour angiogenesis. However, lung cancer cells always stimulate multiple signalling pathways to trigger proliferation and growth.

Scopoletin have been demonstrated to possess weak cytotoxic activities towards various cancer cell lines including A549 lung cancer with IC_{50} higher than 100μM [15]. Epicatechin is also not known to be an anti-cancer agent. However, epicatechin may enhance the cytotoxic properties of scopoletin similar to reports that showed(−)-epicatechin enhanced curcumin apoptosis and growth suppression effects towards Human Lung Cancer Cells[16]. The presence of epicatechin together with scopoletin in the edible leaf extract may synergistically boost the anti-lung cancer effects of *M. citrifolia* leaves extract under this situation. Scopoletin was identified as one of the compounds that may be partly responsible for the *Morinda* leaf anti-angiogenic activity[17] for suppressing tumour growth**.**

The leaves were also reportedly contained Δ^5 Sterols β-sitosterol; $\Delta^{5,7}$ Sterol campesta-5,7,22-trien-3β-ol; (+)−Catechin; α-Ionone; β-Carotene; β-Ionone; β-Sitosterol; 1,2-Dihydro-1,1,6-trimethyl-naphthalene; 1,5,15-Trimethylmorindol; 2,6,10,14,18,22-Tetracosahexaene; 2-Methyl-3,5,6-trihydroxyanthraquinone; 2-Methyl-3,5,6-trihydroxyanthraquinone-6-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside; 3-Hydroxymorindone; 3-Hydroxymorindone 6-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside; 3-O-Acetylpomolic acid; 4-(3'(R)-Hydroxybutyl)-3,5,5,trimethyl-cyclohex-2-en-1-one; 5,6-Dihydroxylucidin; 5,6-Dihydorxylucidin 3-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside; 5,15-Dimethylmorindol; 5,15-DMM; 5-Methylfurfural; 5-Benzofuran carboxylic acid-6-formyl methyl ester; 6,10,14-Trimethyl-2-pentadecanone; 13-Epi-phaeophorbide a methyl ester; 13-Hydroxy-9,11,15-octadecatrienoic acid; 132(R)-Hydroxypheophorbide a methyl ester; 13(S)-Hydroxypheophorbide a methyl ester; 15^1(R)-Hydroxypurpurin-7 lactone dimethyl ester; 15(S)-Hydroxypurpurin-7 lactone dimethyl ester; Alanine; Arginine; Aspartic acid; Asperuloside; Asperulosidic acid; Aucubin; Barbinervic acid; Benzaldehyde; Benzeneacetaldehyde; Campesta-5,7,22-trien-3β-ol; Campesterol; Citrifolinin A; Citrifolinin A-1; Citrifolinin Ba; Citrifolinin Bb; Citrifolinoside A; Citrifolinoside B; Citrifoside; Clethric acid; Cycloartenol; Cysteine; Cystine; Deacetyl asperuloside; Deacetylasperulosidic acid (DAA); E-Phytol; Epicatechin; Geranyl acetone; Glutamic acid; Glycine; Hederagenin; Histidine; Isoleucine; Kaempferol; Kaempferol-3-O-α-l-rhamnopyranosyl-(1-6)-β-d-glucopyranoside Kaempferol 3-O-β-D-glucopyranosyl-(1→2)-α-Lrhamnopyranosyl-(1→6)-β-D-galactopyranoside; Ketosteroids stigmasta-4-en-3-one; Leucine; Linoleic acid; Lucidin; Lucidin 3-O-β-Dxylopyranosyl-(1-6)-β-D-glucopyranoside; Methionine; Methyl oleate; Methyl pheophorbide a; Methyl pheophorbide b; Methyl plamitate; Nicotifloroside; Oleanolic acid; oxalic acid; Palmitic acid; Peucedanocoumarin III; Phenylalanine; Pheophorbide a; Phytic acid; Phytol; Proline; Pteryxin; Quercetin; Quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-d-glucopyranoside; Quercetin 3-O-β-D-glucopyranoside; Quercetin 3-O-β-D-glucopyranosyl-(1→2)-α-Lrhamnopyranosyl-(1→6)-β-D-galactopyranoside; Roseoside II; Rotungenic acid; Rutin; Scopoletin; Serine; Stigmasta-4-22-dien-3-one; Stigmasta-4-en-3-one; Stigmasterol; Tannic acid; Threonine; Triterpene cycloartenol; Tryptophan; Tyrosine; Ursolic acid; and Valine[18,19]**.** Flavonoids such as Kaempferols are also to be cancer inhibitory.

There are many in vitro and in vivo reports on the anti-cancer properties of *M. citrifolia* fruit extract[20] but very few on the leaf extract and none (to our knowledge) towards lung cancer. There was an in vivo study report of the juice extract against S180 tumor cells or Lewis lung carcinoma(LL/2) cells but none were found on the NSCLC. The epicatechin and scopoletin rich *M. citrifolia* leaves extract (Fig 5) demonstrated immuno-stimulatory effects evidenced by the enhanced total WBC, immune cells CD4, T cells CD8, B cells CD19 and natural killer cells CD335 levels in the blood and spleen. Targeting a single cellular or physiological event alone is usually insufficient to control cancer growth. Additionally, the *M. citrifolia* leaves markedly inhibited cancer cell proliferation, inflammation and angiogenesis via mRNA signalling pathways through MDM2/p53, RAF/MEK/ERK, PI3K/AKT/MTOR, PTEN-dependent and COX2 genes expression. Usually no single component is fully responsible for the activity of a crude leaf extract. Scopoletin may produce anti-angiogenic effects via downregulating ERK1/2 to inhibit endothelial cells migration and tube formation [21]. Scopoletin may also induced cell proliferation on normal T lymphocytes without mitogen stimulus via protein kinase C (PKC) activation [22].

M. citrifolia leaves extract dose-dependently increased the total WBC, lymphocytes, T cells CD8 and CD4 levels in the tumour induced mice, demonstrating its strong immune-stimulating effects. The CD8 cytotoxic T cells is important for effective immune defence against cancer, and NSCLC patients with high CD8 T cells infiltration have a relatively better survival rate [23]. The CD8 T cells need adequate CD4 T cell to fully function in vivo [24]. *M. citrifolia* leaves extract may help increase the capacity of T cell specific proliferation, anti-tumour cytotoxicity by releasing perforin, granzymes, granulysin, and interferon gamma (IFNγ) [25] to strengthen CD8 T cell-mediated tumour elimination immune response.

The antitumour effects of CD4 T cells are dependent on cytokine signalling, especially IFNγ and tumour necrosis factors α (TNFα) [26]. IFNγ can up-regulate major histocompatibility complex (MHC) molecules; consequently, the tumour recognition is enhanced, resulting in greater tumour cell lysis. The FAS ligand interaction with FAS, a TNF family of receptors, on target cells activates caspases that initiate deoxyribonucleic acid (DNA) fragmentation and apoptosis of cancer cells. The CD4 T cells could induce tumour dormancy that prevents tumour escape [27]. All data implied that the *M. citrifolia* leaves extract positively affected the activated receptors on the T cells, to enhance the T cell-mediated immune responses of the host towards the unwanted tumour cells.

The *M. citrifolia* leaves also enhanced the CD19 and CD335 in the tumour-induced mice. The CD19 is a transmembrane protein expressed on B cells and follicular dendritic cells. The CD19 cell surface expression is lower relative to CD20, but it begins earlier and persists longer through B-cell maturation. The CD19 has been validated clinically as a B-cell malignancy target, and enhancing the immune effector functions would be expected to improve the therapeutic efficacy [28].

The CD335 immunophenotype is characteristic of the natural killer (NK) cells. This natural cytotoxicity receptor (NCR) family member is present in the human and murine NK cell marker for the bone marrow, blood and spleen. The higher CD335 expression correlates with stronger cytotoxicity against tumour cell lines [29]. The T cells can be selectively induced to express CD335, through a functional PI3K/AKT signalling on stimulation with γc cytokines and T cell receptor (TCR) agonists [30]. The adaptive immunity is coordinated by the antigen-specific T and B lymphocytes and directly kills the tumour cells via the cytotoxic T lymphocytes (CTLs) in combination with the cytokine- and antibody-mediated tumour cell lysis [31].

This study demonstrates that the epicatechin and scopoletin rich *M. citrifolia* leaves help suppress the epidermal growth factor receptor EGFR, which is critical in tumour proliferation, invasion and metastasis and may be useful as a complementary functional food against cancer. The A549 cells NSCLC over-express EGFR [32] (high brown scale IHC staining and strong positive signal by DAB visualization) and the epicatechin and scopoletin rich extract dose-dependently suppressed this in this mammalian model.

The EGFR and COX2 have related signalling pathways that can interact to affect cellular proliferation, migration and invasion [33]. *M. citrifolia* leaves extract significantly decreased the tumour tissues COX2 levels *in vivo*, as similarly reported for green tea extract *in vitro* (Lu et al., 2012). In cancer, COX2 overexpression is possibly via several mechanisms [35], such as, i) intrinsic peroxidase activity due to increased mutagens production; ii) enhanced prostaglandins production stimulated by epithelial cells proliferation; iii) increased anti-apoptotic proteins (B-cell lymphoma 2, BCL2) levels, iv) pro-angiogenic factors production; v) enhanced invasiveness by matrix metalloproteinases and urokinase plasminogen activation; and vi) immune suppression.

The epicatechin and scopoletin rich *M. citrifolia* leaves extract dose-dependently increased the tumour suppressor PTEN levels i the mice lung tumour, comparable to green tea extract in lung cancer cells [36]. The PTEN regulates cells survival, proliferation, invasion and angiogenesis by acting as a lipid phosphatase that antagonizes PI3K signalling via dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) back to phosphatidylinositol (4,5)-bisphosphate (PIP2). Reduced PTEN expression was associated with poor clinical outcome in NSCLC [37]. Loss of PTEN permits activated AKT to phosphorylate the intracellular protein BAD (Bcl-2-associated death promoter), resulting in the release of the anti-apoptotic protein BCL2, which then leads to cancer cell survival [38]. The epicatechin and scopoletin rich leaves extract may possibly be used alone or together with other chemotherapeutic agents for PTEN mutant cancers therapy.

The epicatechin and scopoletin rich *M. citrifolia* leaves extract dose-dependently suppressed MTOR, which is an attractive cancer therapy target because its inhibition could reduce possible side effects associated with the upstream PI3K/AKT signalling molecules inhibition. The PI3K/AKT pathway is involved in broader biological functions including glucose signalling [39].

The epicatechin and scopoletin rich *M. citrifolia* leaves extract also dose-dependently inhibited the cancer cells proliferation by suppressing MDM2 gene expression. The MDM2 negatively controls pro-apoptosis p53 stability, function and concentration by i) preventing p53 transcription through binding via protein-protein interactions to the p53 N-terminal transcription activation domain, ii) promoting p53 degradation by ubiquitin dependent proteasomal degradation and acting as an E3 ubiquitin ligase, and iii) causing nuclear export of p53 into the cell cytoplasm, away from its action site [40]. The p53-dependent apoptosis is induced by the caspase proteases and death receptors and functions through pro-apoptotic proteins including BAX (BCL2-associated X protein), NOXA (phorbol-12-myristate-13-acetate-induced protein 1), or PUMA (p53 upregulated modulator of apoptosis) [41]. The epicatechin and scopoletin rich *M. citrifolia* leaves extract further dose-dependently inhibit the MAPK pathway through the suppression of RAF1 marker to produce anti-proliferative effects, and suppressio of their downstream targets: MEK and ERK. The human small cell lung cancer (SCLC) growth is partially dependent on signalling by RAF1 (Clark & You, 2006). Thus, RAF1 inhibition by the extract may also inhibit SCLC. The disruption of RAF1 expression by the extract could help inhibit angiogenesis and pro-survival of the endothelial cells because the PKC/RAF/MEK/ERK signalling pathway is involved in the vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) dimerization and activation [43,40]. The

proposed model for the epicatechin and scopoletin rich *M. citrifolia* leaves extract mechanism of action for anti-proliferative and immune-stimulating effects against lung adenocarcinoma in vivo is summarised in Fig 6.

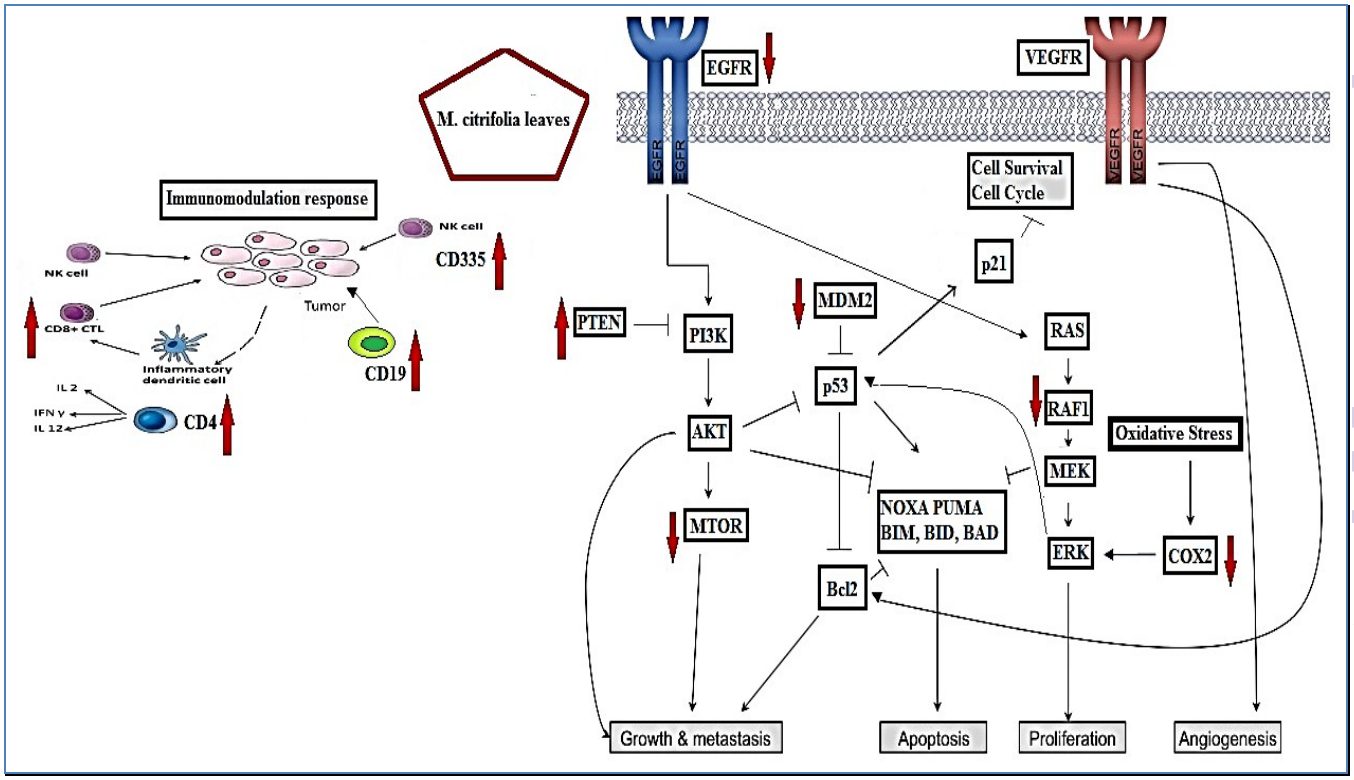


Fig. 6. Proposed model of *M. citrifolia* leaves extract mechanism of action for anti-proliferative and immunomodulation effects against lung adenocarcinoma in vivo.

Conclusion

The evidences demonstrated that the epicatechin and scopoletin rich *M. citrifolia* leaves extract may be used as a functional food and complementary therapy to suppress lung cancer by stimulating the immune responses and modulating multiple cancer cell gene signalling pathways against cancer cell proliferation and towards apoptosis, without producing detectable undesirable effects. The extract suppressed key inflammatory markers and inhibited various molecular and cellular markers involved in proliferation and angiogenesis, suggesting a mode of action targeting, as proposed in Fig. 5. The epicatechin and scopoletin rich *M. citrifolia* leaves may be a complementary/adjunct therapy or functional food to help fight lung cancer or adenocarcinoma.

Conflict of Interests

The authors declare that currently we have no conflict of interests in the research.

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Figure 1 Effect of *M. citrifolia* leaves extract on the tumourigenicity of A549-stimulated mice by comparing tumour volume in untreated and extract-treated mice.

Figure 2 H&E and IHC (EGFR) analyses on the tumour of A549-induced mice.

Figure 3 White blood cells counts in different treatment group.

Figure 4 Immunophenotyping analyses of B cells, T cells and natural killer markers on spleen tissue

Figure 5 Reverse-phase HPLC characteristics of standards and *M. citrifolia* leaves 50% ethanolic extract

Figure 6 Proposed model of *M. citrifolia* leaves extract mechanism of action for anti-proliferative and immunomodulation effects against lung adenocarcinoma in vivo.