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# Morinda citrifolia Leaf Extract Suppressed Metastasised Cancer Progression via EGFR and MAPK Pathways

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**Supporting information** HPLC and LC-MS profiles of the extract standardised to scopoletin and epicatechin and a listing of bioactive chemical contents of M. citrifolia leaf are available online at http://www.thieme-connect.de/products.

## ABSTRACT

Morinda citrifolia leaf has anti-inflammatory and immune enhancing effects against lung cancer. The effects of the extract on metastasised lung and liver cancer tissues were compared to Erlotinib (an anticancer drug) for cancer aggression, proliferation, and angiogenesis. Forty Balb/c mice were induced to develop metastatic lung and liver tumours via xenograft subcutaneous injection of non-small cell lung cancer A549 cells ($2 \times 10^7$ cells/mouse) into their backs. The extract (150 and 300 mg/kg body weight) and Erlotinib (50 mg/kg body weight) were fed to the mice for 21 days, and the microstructure and mRNA expressions of the tumour tissues were analysed. The extract dose-dependently down-regulated RAC-alpha serine/threonine-protein kinase, B cell leukaemia/lymphoma 2, mitogen-activated protein kinase kinase kinase 14, mitogen-activated protein kinase 1, and vascular endothelial growth factor alpha in these tissues. The scopoletin (coumarin) and epicatechin (flavonoid) standardised extract also mitigated the cancerous tissues microstructure changes, and suppressed the tissue remodeling enzyme (matrix metallopeptidase 9) and angiogenesis biomarkers (epidermal growth factor receptor and integrin). The 300 mg extract/kg body weight was more effective than the 50 mg Erlotinib/kg body weight in suppressing the lung and liver tumoor metastasis. The extract inhibited the cancer aggression by interfering with epidermal growth factor receptor and mitogen-activated protein kinase carcinogenesis pathways, and suppressing proliferation, tissue remodelling, and angiogenesis without any observable side effects at the given dose.

## Abbreviations

| | |
|---|---|
| AKT1 | RAC-alpha serine/threonine-protein kinase |
| BCL2 | B cell leukaemia/lymphoma 2 |
| CXCL | chemokine (C-X-C motif) ligand |
| ECM | extracellular matrix |
| EGFR | epidermal growth factor receptor |
| H&E | haematoxylin and eosin |
| IHC | immunohistochemistry |
| IL | interleukin |
| MAP3K14 | mitogen-activated protein kinase kinase kinase 14 |
| MAPK | mitogen-activated protein kinase |
| MMP9 | matrix metallopeptidase 9 |
| NFκB | nuclear factor kappa-light-chain-enhancer of activated B |
| NOAEL | no observed-adverse-effect level |
| NSCLC | non-small cell lung cancer |
| PI3K | phosphatidylinositol 3-kinase |
| RAF | RAF proto-oncogene serine/threonine-protein kinase |
| VEGF | vascular endothelial growth factor |
| VEGFA | vascular endothelial growth factor alpha |

## Introduction

Cancer progression relies upon malignant cell proliferation and the generation of new blood vessels (angiogenesis) to sustain survival and invasion (metastasis). Lung and liver cancers are amongst the leading causes of cancer-related deaths worldwide (about 2 million new cases annually). Erlotinib is an FDA approved EGFR tyros-

ine-kinase inhibitor for treating locally advanced or metastatic NSCLC [1]. In a phase III study, Erlotinib significantly improved the overall survival, relative to the supportive care for refractory stage IIIB/IV NSCLC [2, 3]. However, Erlotinib often causes side effects such as weakness, diarrhoea, rash, shortness of breath, cough, fever and loss of appetite, dry eyes, unusual eyelash growth, swollen cornea, extreme tiredness, and nausea. Sometimes it causes more serious side effects such as interstitial lung disease, liver and kidney damage, gastrointestinal perforation, blistering and skin peeling, and bleeding and clotting problems, which may lead to a heart attack, stroke, and death [4].

The leaves of Morinda citrifolia L. (Rubiaceae) or noni (America)/mengkudu (Malaysia) are often used as vegetables or salads. The M. citrifolia leaves contain epicatechin and scopoletin, purported to have immune-modulating [5], antioxidant, liver protective and wound healing effects without any acute, subacute, and subchronic oral toxicity [6]. The NOAEL of oral M. citrifolia leaves ethanolic extract is 1 000 mg/kg [7]. M. citrifolia is beneficial for wound infections, pain, arthritis, swellings, and homeostasis. Morinda fruit has been reported to have immunostimulant, antioxidant, anticancer, and anti-inflammatory properties [8–10].

Patients and caregivers often use diets as complementary therapy to the prescribed anticancer drugs. This report investigated the mechanisms (antiproliferation, anti-metastasis and possibly antiangiogenesis) by which M. citrifolia leaf extract (standardised to scopoletin and epicatechin) could prevent the spread of cancer in lung and liver metastasised tumours in vivo.

## Results and Discussion

The control cancer-induced mice (▶ **Fig. 1a**) displayed two adenomatous growths in the pulmonary parenchyma of the lungs with large and medium-sized pseudostratified tumour cell clusters (black arrow) together with goblet-like granules within their cytoplasm (yellow arrow). They also showed two irregular tumour invasive glandular structures consisting of pseudostratified lepidic growth (cells) with a pink amorphous proteinaceous secretion within the glandular lumen (▶ **Fig. 1b**, black arrow). The light arrow shows free red blood cells within the alveolar lumen and pulmonary parenchyma. The cancer-induced mice treated with 300 mg extract/kg body weight had smaller tumours, with almost similar lung morphology to the control normal mice, and indicated a 41 % better antimetastatic effect than the 50 mg Erlotinib/kg body weight treatment.

The metastasised liver tumours of the control cancer-induced mice (▶ **Fig. 1c**) showed 60 % tumour infiltration (metastasis), which is indicated by poorly differentiated tumour cells and some areas of necrosis. After 3 weeks, the metastasised tumours in the control cancer-induced mice were ~290 $mm^3$, while tumours in the mice treated with 150 and 300 mg extract/kg body weight and 50 mg Erlotinib/kg body weight were significantly smaller (50, 95, and 87 % smaller, respectively). The mice treated with 150 mg extract/kg body weight showed tumour cell infiltration to the liver and metastatic foci formation, while the Erlotinib-treated mice showed hyperchromatic nuclei and hepatocytes cytoplasmic vacuolisation. The mice treated with 300 mg extract/kg body weight showed no such changes.

▶ **Fig. 2** shows the control cancer-induced mice overexpressed the EGFR in the metastasised tumours (for both lung and liver). The mice treated with 300 mg extract/kg body weight suppressed the EGFR expression more effectively than those treated with 50 mg Erlotinib/kg body weight and 150 mg extract/kg body weight. The lung tumours expressed MMP9, while the liver tumours expressed integrin-β1 (▶ **Fig. 3**). These expressions were less severe in the extract- and Erlotinib-treated groups. The mice treated with 300 mg extract/kg body weight had downregulated expression of MMP9 and integrin-β1 in the tumours to near normal healthy levels. Integrin-β1 is the most copiously expressed integrin in NSCLC [11]. High MMP9 expression is an indicator for aggressive tumour growth in NSCLC [12].

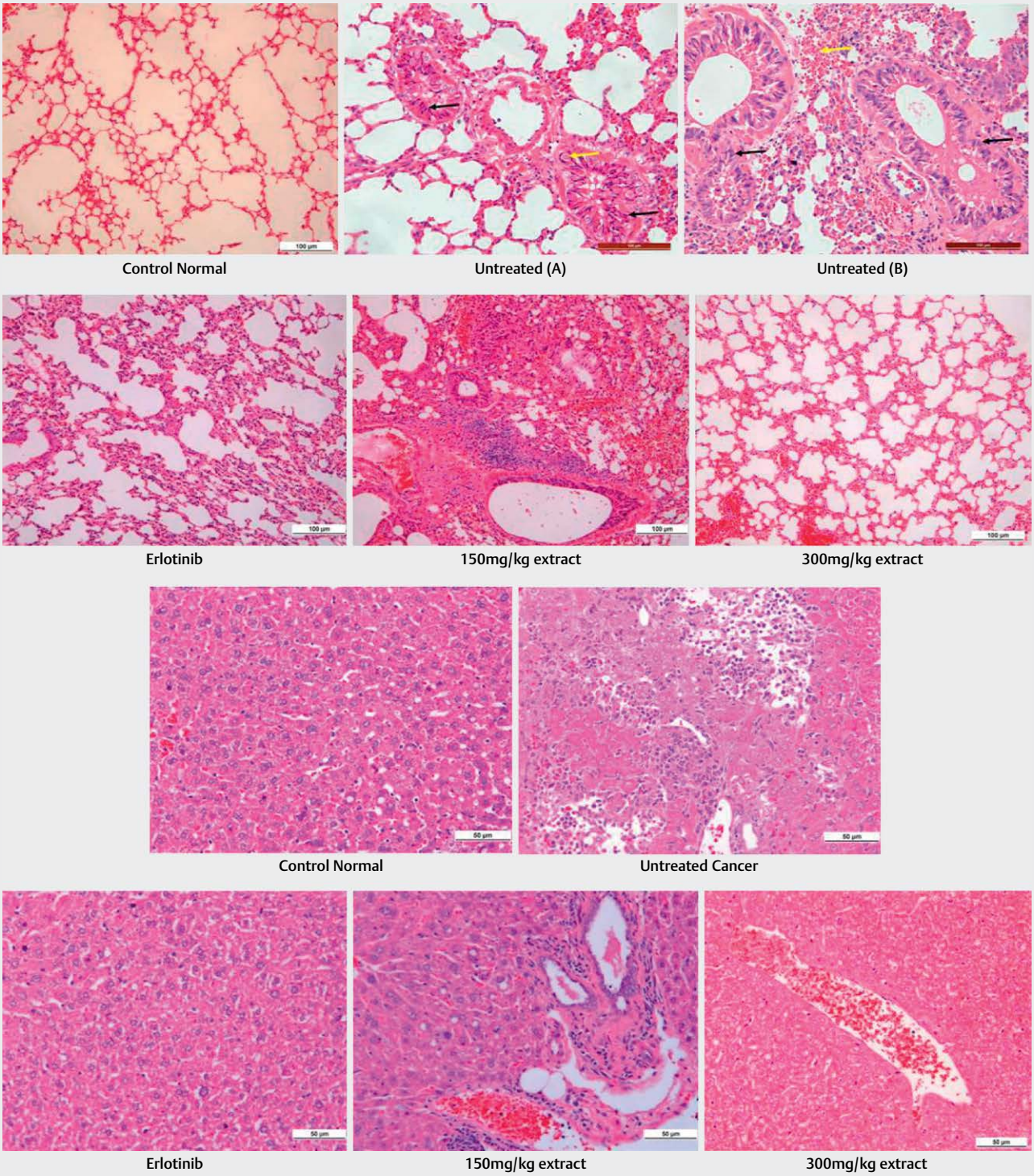
The 300 mg extract/kg body weight treatment effectively downregulated the BCL2, AKT1, VEGFA, and MAP3K14 expressions in the lung tumours by more than threefold (▶ **Fig. 4a**). The 50 mg/kg Erlotinib treatment only significantly downregulated the BCL2, AKT1, and MAPK1 expressions (but not VEGFA or MAP3K14), while the 150 mg/kg extract treatment only downregulated BCL2 and MAP3K14 expressions (relative to the untreated control cancer group). In the liver, the 300 mg/kg extract treatment also significantly ($p < 0.05$) downregulated BCL2, AKT1, VEGFA, and MAPK1 (▶ **Fig. 4b**) more effectively than the 50 mg/kg Erlotinib treatment.

This vegetable extract showed no adverse changes in the mice behaviour, body, or food and water intake at the given dose, with a reported oral NOAEL of 1 000 mg/kg [7], unlike most cancer chemotherapy. The 300 mg/kg extract dose is equivalent to consuming about 100–125 g of fresh leaves daily for a 50-kg adult (FDA animal dose to human conversion guidelines).

Epicatechin and scopoletin are common compounds in edible leaves and may be a potential dietary therapy against carcinogenesis. Scopoletin or epicatechin when used alone produces weak cytotoxic activities towards various cancer cell lines. The $IC_{50}$ of scopoletin for A549 lung cancer was above 100 µM [13]. Scopoletin was reported to boost the apoptosis of human prostate tumour PC3 cells [14] and the human leukaemia cell line HL-60 [15], while (−)-epicatechin could inhibit growth and induced apoptosis in SW480 human colon cancer cells [16]. When combined, epicatechin and scopoletin may synergistically suppress cell proliferation. Scopoletin possesses antiangiogenic properties by inhibiting (a) endothelial cells growth and migration, (b) extracellular signal-regulated kinase (ERK) 1/2 activation, (c) tube formation, and (d) VEGF expression through NFκB [13]. Scopoletin, which is a coumarin, also helped activate protein kinase C (PKC) to induce normal T lymphocytes cell proliferation without mitogen stimulus [17].

(−)-Epicatechin enhanced curcumin apoptotic effects towards human lung cancer cells [18]. It also impaired angiogenesis, arrested metastasis through metalloproteinases inhibition, and helped reverse multidrug resistance [19]. Erlotinib's antiangiogenic properties were demonstrated by its ability to inhibit human umbilical vein endothelial cells (HUVECs) growth and xenograft vessel density [20]. Kaempferol in the leaf extract can also inhibit cancer [5].

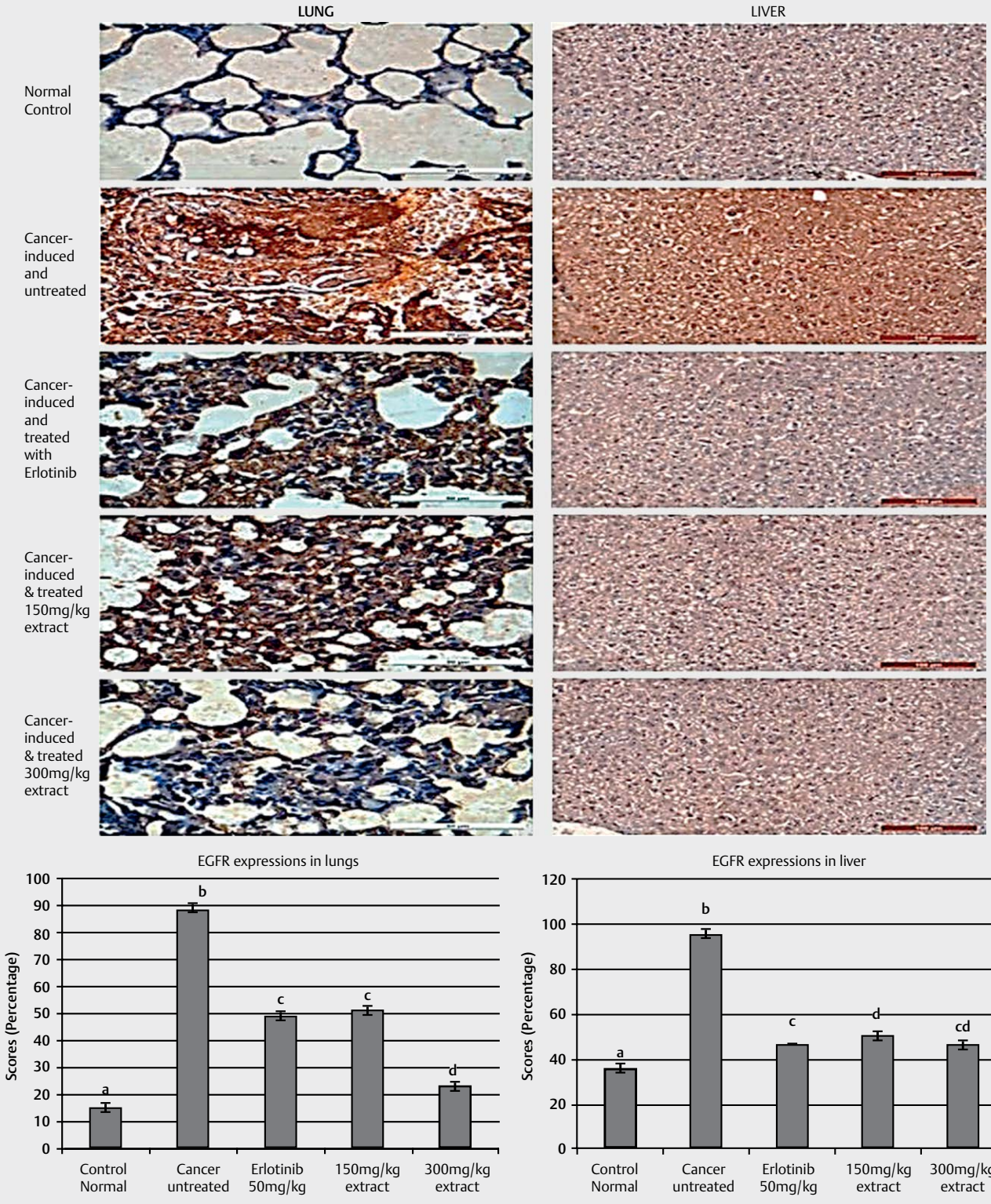
The VEGF/VEGF-receptor pathway involving the PI3K/AKT and RAS/RAF/MAPK pathways is important for cancer cell proliferation, angiogenesis, migration, and invasion [21]. The VEGF and PI3K/AKT/MTOR pathway is critical for the fibronectin-integrin effects on proliferation. The control cancer-induced mice overexpressed




▶ **Fig. 1** The histological images of the metastasised cancers in the lungs and livers. [H&E; x100; x200 (Untreated cancer **a** and **b** only)]. The adenomatous growth-like patterns in the lung were seen in the untreated control cancer group. The poorly differentiated metastasised tumour cells in the liver tumours were seen in the untreated control cancer group; (H&E; x200).

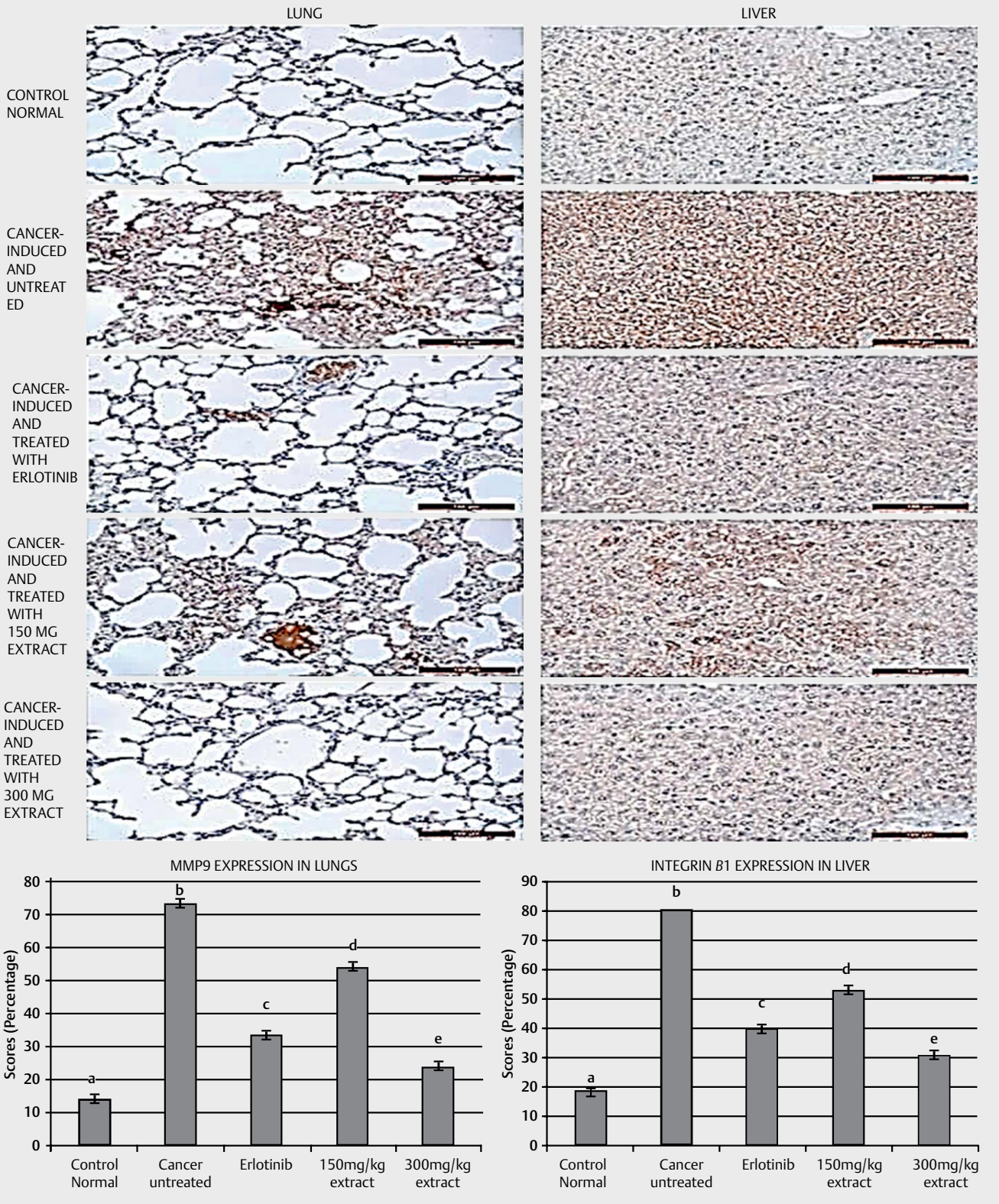
EGFR in both lung and liver tumours (brown IHC staining and strong positive signal by DAB visualisation). The leaf extract dose-dependently restored the mice tumours towards normal healthy conditions by suppressing cancer cell proliferation and metastasis through the suppression of EGFR, MMP9, and integrin-β1 activities, and by inhibiting the VEGF/EGFR/NFκB signalling pathway.

The VEGFA activates endothelial cells to produce MMPs that break down the stroma and ECM proteins [22] for angiogenesis and

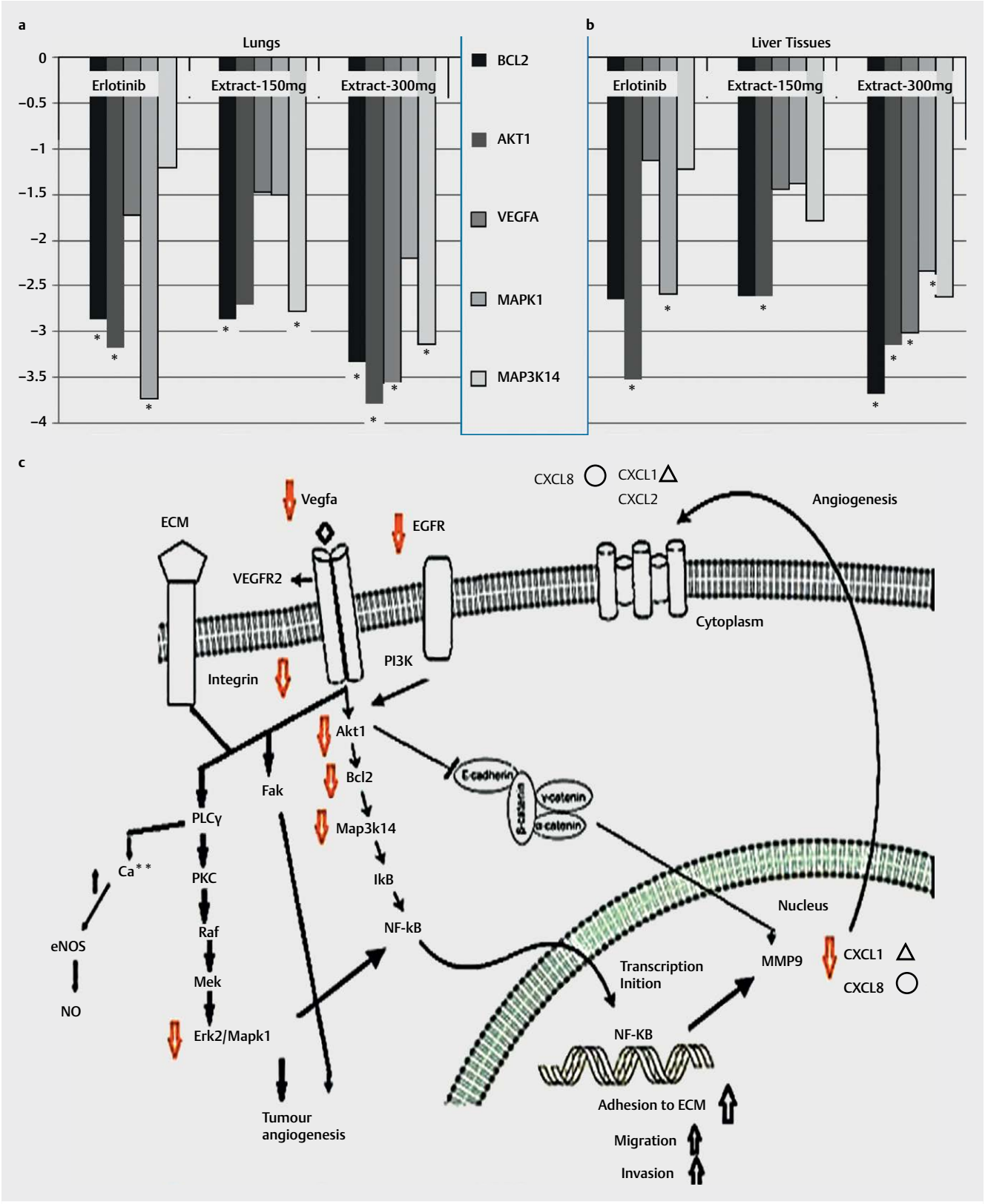



▶ **Fig. 2** Immunohistochemical staining for EGFR in the metastasised tumours in the lungs and liver (lung IHC x200; liver IHC x100). Means with different superscript letters within the graph are significantly different ($p < 0.05$).

metastasis. The MMP9 are activated by fibronectin via the PI3K/AKT or RAS/RHO/MAP pathway [23]. The vegetable extract dose-dependently suppressed angiogenesis-related mRNA expressions (MMP9, VEGFA, and MAPK1), indicating reduced ECM degradation and remodelling. MMP9 is linked to the vascular remodelling and aggressive invasion of lung cancers [21]. The 300 mg extract /kg body weight treatment downregulated MAP3K14 (or NFκB-inducing kinase; NIK) that consequently helped inhibit downstream




▶ **Fig. 3** Immunohistochemical staining for MMP9 in the metastasised tumours in the lungs and integrin-$\beta$1 staining in the livers (x100). Means with different superscript letters within the graph are significantly different ($p < 0.05$).




▶ **Fig. 4** Mouse mRNA expressions in the lung **a**, liver **b**, and tumours **c**, and schematic representation of the signalling pathways involved in the inhibition of angiogenesis and metastasis of lung adenocarcinoma by the Morinda leaf extract. * Significant difference ($p < 0.05$) between control and treatment groups. Adapted from https://www.qiagen.com/my/shop/genes-and-pathways/pathway-details/.

metastasis genes including MMP9, VEGF, and the urokinase-type plasminogen activator receptor (uPAR) [24]. The 50 mg Erlotinib/kg body weight treatment did not show this effect.

The leaf extract also dose-dependently suppressed BCL2 and AKT1 expressions. The VEGF induces BCL2 expression. The upregulated BCL2 from the endothelial cells subsequently initiated the nuclear factor of kappa light polypeptide gene enhancer in the B cells inhibitor (IκB)/NFκB-dependent pathway, which elevated proangiogenic IL8 and CXCL1 expressions [25]. High AKT1 activation in all NSCLC subtypes induced endothelial cell migration via nitric oxide signalling [26]. AKT regulated tumour angiogenesis through downstream targets such as the mammalian target of rapamycin (mTOR)/p70S6K1 signalling axis, Forkhead box O (FOXO) inhibition, VEGF mRNA upregulation, nitric oxide synthase induction, and/or glycogen synthase kinase 3 beta (GSK3β) inhibition [27].

The extract dose-dependently downregulated integrin-β1 expression, which is necessary for angiogenesis and metastasis. Integrins are transmembrane signal transduction receptors for attachment from the ECM to the cells. The lung cancer cells are protected against apoptosis by the activation of integrin-β1 via ECM proteins (including fibronectin) [20]. In NSCLC, overexpression of integrin α5β1 was negatively associated with patient survival [28]. Activation of integrin-β1 on endothelial cells would trigger the transcription of a gene repertoire related to angiogenesis [heparin-binding epidermal growth factor-like growth factor (HB-EGF), IL8, CXCL1], adhesion [vascular cell adhesion molecule (VCAM), E-selectin], signal transduction (NFκB), and coagulation (tissue factor) [29]. Integrins modulate the angiogenesis-related cell signalling pathways of transmembrane protein kinases, such as receptor tyrosine kinases (RTK) [30].

The M. citrifolia (Noni) leaf extract was shown to inhibit proliferation and induced apoptosis in A549 cells ($IC_{50}$ = 23.47 µg/mL) and mouse Lewis (LL2) lung carcinoma cells ($IC_{50}$ = 5.50 µg/mL) in vitro by arresting the cancer cell cycle at G0/G1 phases and significantly increasing caspase-3/-8 without changing caspase-9 levels [31]. The A549 is an NSCLC cell line, which is the most common lung adenocarcinoma subtype (with high mortality rate).

The vegetable previously demonstrated anticancer properties by increasing the proapoptotic (TRP53) genes, downregulating the pro-tumourigenesis genes (BIRC5, JAK2/STAT3/STAT5A), increasing anti-inflammatory biomarkers [IL4, IL10 and glucocorticoid receptor (NR3C1)], enhancing the antioxidant NFE2L2 [nuclear factor (erythroid-derived 2)-like 2]-dependent responses against oxidative injuries, modulating the immune responses (increasing blood lymphocytes, spleen tissues B cells, T cells, and natural killer cells), enhancing the tumour suppressor gene PTEN (phosphatase and tensin homolog), inhibiting cellular tumour growth genes (MDM2, RAF1, MTOR) [5], inducing G0/G1 cell cycle arrest and the extrinsic apoptosis pathway, reducing inflammatory markers cyclooxygenase 2 (COX2), increasing inflammatory cells clearance, enhancing the efflux of inflamed tissues, suppressing oedema accumulation, and inhibiting oxidative stress [31]. The vegetable extract was not cytotoxic on MRC5 normal lung cells ($IC_{50}$ > 100.00 µg/mL).

These results demonstrated the Morinda leaf effects on metastasised cancer tissues microstructure, indicating the suppression of cancer cell proliferation and vascular and tissue remodelling, which were confirmed by mRNA expressions. The 300 mg extract /kg body weight treatment appeared more effective than the 50 mg Erlotinib /kg body weight treatment for most of the parameters measured.

## Materials and Methods

Human lung adenocarcinoma (A549) cell lines were cultured in Kaighn's Modified Ham's F-12 (F-12K) medium (ATCC) [5] containing 10 % fetal bovine serum (PAA) and 1 % of 100 µg/mL penicillin and streptomycin (Biowest) in a humidified incubator at 37 °C with 5 % carbon dioxide.

### Extraction and chemical analysis

The M. citrifolia leaves were identified and authenticated by the Biodiversity Unit, Institute of Bioscience, UPM (Voucher No. SK2322/14). The leaves were dried and mixed in a ratio (w/v) of 1:5 with 50 % ethanol in water. The total yield after extraction three times was 13.61 %. The extracts were analysed using HPLC (Waters 2996) with an Atlantis C18 column (4.6 mm × 250 mm; 5 µm, Waters Corp.) maintained at 25 °C. HPLC grade MeOH, acetonitrile (MeCN), and analytical grade trifluoroacetic acid (TFA) were obtained from Merck. The mobile phase consisted of three solvents: A: MeCN, B: MeOH, and C: 0.1 % TFA in $H_2O$ (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 $min^{-1}$ with a 50-µL sample injection volume and a UV spectra detector set at 210 and 450 nm. Pure standards [scopoletin, and (−)-epicatechin] were purchased from Sigma-Aldrich. The extract was standardised to scopoletin (2.2 %, retention time, Rt = 12.02 min) and epicatechin (3.4 %, Rt = 9.17 min) as the main compounds, which were qualitatively and quantitatively identified via the retention times and calibrated standard plots. Spiking with scopoletin and epicatechin produced sharp extended peaks at the specified retention times and their presence was further confirmed with LC-MS (**Fig. 1S, Supporting Information**). The epicatechin isomer in the extract was not determined because the HPLC retention times between isomers are usually either very close to each other or they may overlap, and LC-MS only confirmed the molecular weight of the parent and daughter molecules. There have not been many reports on the difference in biological activities between the epicatechin isomers. The isomer of the epicatechin may be determined in the future, since the scope of this work was on the animal studies and biological activities. Known chemical compounds in the M. citrifolia leaf have been reported and are shown in **Table 1S, Supporting Information**.

### Animal studies

Male Balb/c mice (6 weeks old, weighing 19–20 g) from Faculty of Veterinary Medicine, University Putra Malaysia, were given standard chow and water, and kept in a 12-h light/12-h dark cycle [5]. The study was approved by the Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R016/2013). The A549 cells ($2 \times 10^7$ in 100 µL PBS) were injected subcutaneously into the mice backs [32]. When the metastasised lung tumour size reached 100 $mm^3$, 14 days after implantation, the mice were grouped (n = 10) accord-

ingly: (1) Control healthy, (2) Cancer-induced untreated control (saline vehicle only), (3) Cancer-induced and treated with 50 mg Erlotinib/kg body weight (orally gavaged daily), (4 and 5) Cancer-induced and treated with 150 or 300 mg extract/kg body weight. After 21 days, they were sacrificed via intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (10 mg/kg) and the tumour volume was measured [5]. The lung and liver tissues were snap frozen in liquid nitrogen for gene expression analysis, while some were fixed in 10 % formalin and embedded in paraffin for H&E and IHC examination [5]. The IHC primary antibody kits (ChemMate DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse) were for (a) anti-EGFR (ab15669 from Abcam), (b) anti-MMP9 (Dako Corporation), and (c) anti-integrin (Novus) [31].

The tissue mRNA was isolated using Trizol (Invitrogen) and analysed quantitatively for VEGFA, AKT1, BCL2, MAP3K14, and MAPK1. The Custom RT$^2$ Profiler PCR Array (CAPM11988), RT$^2$ SYBR Green qPCR Mastermix, RT$^2$ First Strand Kit, RNase-Free DNase Set (SuperArray Bioscience Corporation), and Data Analysis version 3.5 (SABiosciences) were used, with heat shock protein 90 alpha (cytosolic), class B member 1 (HSP90AB1; NM_008302), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_008084) as housekeeping genes for mRNA analysis [5].

All data are the mean ± standard deviation and were analysed by one-way analysis of variance (ANOVA) and Duncan's test for significant differences ($p < 0.05$) using IBM SPSS Statistics 21 software.

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### Conflicts of Interest

The authors declare no conflict of interest.

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