Electronic supplementary materials
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Anti-migratory effects and microtubule targeting properties of *Piper betle* leaf aqueous extract on cancer cells *#

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Materials and methods

PB leaf aqueous extract and cell culture

PB leaf were sampled at Simpang Morib, Selangor, Malaysia. Plant was identified and verified by the Plant and Herbs Herbarium of Forest Research Institute of Malaysia (FRIM). PB aqueous extraction was then performed as previously described (Ng et al. 2014).

Human lung adenocarcinoma (A549) cells and human colorectal adenocarcinoma (HT29) cells (American Tissue Culture Collection, Manassas, VA) were cultured in RPMI-1640 medium and McCoy's medium, respectively. Media was supplemented with 10 % (v/v) fetal bovine serum (FBS) and incubated in 5 % carbon dioxide (CO_2) at 37°C.

IC_{50} and Sub-toxic concentration determination by MTT assay

A total number of 10,000 cells per well was seeded in a 96-well plate and incubated at 37°C with 5% CO_2 for 24 hours. The media was then removed before treatment solution were added. A series of concentration ranged from 20- 300 μg/mL for PB, 3-50 μM for 5-FU and 2.5 -50.0 μg/ml for paclitaxel were added into the wells and cells were treated for 24 hours. After 24 hours, 10 μL of MTT solution (Sigma-Aldrich, USA) was added into each well and incubated for 3 hours. The mixture was then removed and 100 μL of DMSO (Sigma-Aldrich, USA) was added. Mixture was left for 30 minutes to dissolve the purple crystals. Absorbance was then read at 527 nm using Epoch 2 microplate spectrophotometer (BioTek, USA).

Percentage of cell viability was then calculated. IC_{20} and IC_{30} were determined for cell migration assay. IC_{50} was used for microtubules structure and network observation experiment.

Treatments

A549 cells were treated with subtoxic level of PB and 5-FU. Cell migration was then observed by real-time xCELLigence migration assay (ACEA Biosciences, USA) for 20 hours. HT-29 cells were treated with IC_{50} of PB, 5-FU and paclitaxel. Immunocytochemistry analysis was then performed on treated HT29 cells. Cell morphology and tubulin networks were observed under the inverted microscope (Nikon, Japan) and fluorescence microscope with DAPI and FITC filter set (Nikon, Japan), respectively.

Cell Migration Assay

Cell migration was observed in six different groups, i.e. A549 cells (1) in media with chemo attractant, FBS (positive control); (2) in serum free media (negative control); (3) treated with IC_{20} 5-FU, 4 μM; (4) treated with IC_{30} 5-FU, 12.5 μM; (5) treated with IC_{20} PB, 20 μg/mL and (6) treated with IC_{30} PB, 100 μg/mL.

Cell Invasion and Migration (CIM) plate consists of two chambers. Lower chamber was prepared by adding 160 μl of pre-warmed media into each well. Negative control wells were filled with serum free media. The remaining wells were added with media supplemented with 10% FBS. FBS was used as chemo-attractant in this experiment.

For the upper chamber, a total number of 30,000 cells in serum free media per well was prepared. A total of 100 μL of cells (equivalent to 30,000 cells) with each treatment concentration was pipetted into each well. Sixty (60) μL of serum free media was then added to each well, making the total volume of 160 μL per well. Lower and upper chambers were then assembled together and the plate was left in an incubator for 1 h to allow cell attachment. The impedance value of each well was automatically monitored by the xCELLigence real time cell analyser (ACEA Biosciences, USA) for 20 h and expressed as a cell index (CI) value. CI is the impedance of electric flow which caused by the adherent cells.

Immunocytochemistry of tubulin

Cells on coverslips after respective treatments were fixed in 10% formalin for 20 minutes. Cells were then washed and subjected to permeabilization by 0.5% Triton X-100 for 15 minutes. Blocking step was performed using 1% serum albumin for 30 minutes. Cells were then subjected to primary antibody (mouse anti-human alpha tubulin IgG) for overnight incubation at 4°C. Cell were then incubated with secondary antibody (goat anti-mouse IgG conjugated Dylight 488) for 3 hours. Nuclei of the cells were counterstained with DAPI (1 µg/ml) for 40 minutes. After washed with PBST, the coverslips were mounted with DPX (Merck Millipore, Germany) on slides. The slides were dried for 4 hours before the observation under the fluorescent microscope (Nikon, Japan) equipped with DAPI and FITC filter set.