Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
E-mail: jzus@zju.edu.cn

Correspondence:

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

Mee Lee LOOI†[1], Alwyn Khai Howe WONG[2], Shelly Anne GNAPRAGASAN[2], Anis Zafirah JAPRI[2], Aiysvariyah RAJEDADRAM[2], Kar Yong PIN[3]

[1]*Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia*
[2]*School of Biosciences, Taylor's University, Lakeside Campus, Subang Jaya, Selangor Darul Ehsan 47500, Malaysia*
[3]*Forest Research Institute Malaysia, Kepong, Selangor Darul Ehsan 52109, Malaysia*
†E-mail: meelee.looi@um.edu.my

https://doi.org/10.1631/jzus.B2000278

Piper betle (PB), also known as "betel" in Malay language, is a tropical Asian vine. PB leaves are commonly chewed by Asians along with betel quid. It contains phenols such as eugenol and hydroxychavicol along with chlorophyll, β-carotene, and vitamin C (Salehi et al., 2019). Extracts from PB leaves have various medicinal properties including anticancer, antioxidant, anti-inflammatory, and antibacterial effects (Salehi et al., 2019). Previous research has shown that PB induces cell cycle arrest at late S or G2/M phase and causes apoptosis at higher doses (Wu et al., 2014; Guha Majumdar and Subramanian, 2019). A combination of PB leaf extract has also been shown to enhance the cytotoxicity of the anticancer drug, 5-fluorouracil (5-FU), in cancer cells (Ng et al., 2014).

The ability of cancer cells to disseminate to distant regions is partly the result of microtubule dynamics. Microtubules are part of the cell cytoskeleton, contribute to shape and dynamics, and are essential for cell movement and directionality (Ganguly et al., 2012; Mukhtar et al., 2014). As a result of rapid cancer cell division, anti-mitotic drugs that potentially target microtubule dynamics have been suggested to be the best cancer therapeutic agents (Mukhtar et al., 2014). The potential of PB in targeting cancer cell migration was investigated in this study. The modulation effect of PB on microtubule structure and networks was also observed in comparison to a standard microtubule inhibitor, paclitaxel.

The sub-toxic levels of PB and 5-FU were used to ensure no cell cytotoxicity in the cell migration analysis. After 24 h of respective treatment, values of IC_{20} (20% inhibitory concentration) and IC_{30} (30% inhibitory concentration) recorded for PB on human lung adenocarcinoma (A549) cells were 20 and 100 μg/mL, respectively, whereas IC_{20} and IC_{30} for 5-FU on A549 cells were determined at 4.0 and 12.5 μmol/L, respectively. The anti-migration effects of PB and 5-FU were observed real-time on A549 cells for 20 h using xCELLigence (ACEA Biosciences, USA). A high cell index (CI) value indicates a positive migration effect as more cells adhere to the gold microelectrodes, causing high impedance. The negative control group (cells with serum-free media) showed CI values less than 0.5 (Fig. 1). This implies that cells did not migrate throughout the experiment. However, after 20 h, CI was found to exceed 3.0 for the positive control group (cells in media with chemoattractant). Data showed that A549 cells migrated from the upper chamber (cells in serum-free media) to the lower one (media containing fetal bovine serum (FBS)) in response to the FBS chemo-attractant. Significant differences in CI values were also found between the negative and positive control groups (P<0.05).

* Project supported by the Taylor's Research Grant Scheme (No. TRGS/MFS/2/2013/SBS/003)
# Electronic supplementary materials: The online version of this article (https://doi.org/10.1631/jzus.B2000278) contains supplementary materials, which are available to authorized users
ORCID: Mee Lee LOOI, https://orcid.org/0000-0001-5415-6973

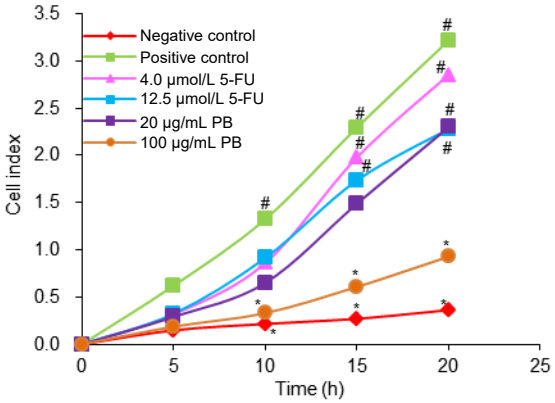

Fig. 1 Cell index (CI) values of respective treatment groups on A549 cells at different time points (5th, 10th, 15th, and 20th hours)

5-FU: 5-fluorouracil; PB: *Piper betle*. * $P<0.05$, compared with the positive control group; # $P<0.05$, compared with the negative control group

After 5 h of respective treatments, no significant differences in CI values were seen between groups ($F=3.107$, $P=0.060$). However, significant differences were observed between treatment groups after 10 h ($F=6.114$, $P=0.008$), 15 h ($F=10.984$, $P=0.001$), and 20 h ($F=10.555$, $P=0.001$). Indeed, after 10 h, cells in the positive control wells migrated towards the chemoattractant, leading to high CI values (Fig. 1). Data showed that low concentrations of PB (20.0 μg/mL) and 5-FU (4.0 and 12.5 μmol/L) had minimal effects in inhibiting cell migration; cells treated with 100 μg/mL of PB had significant anti-migration effects ($P=0.034$). This result implies that 100 μg/mL PB inhibited cell migration from the upper chamber to the lower one. 5-FU has potential in obstructing cancer cell migration as previously shown (Shakibaei et al., 2015). PB exerted greater anti-migratory effect compared with 5-FU (Fig. 1).

We further observed cell morphology, structure, and the tubulin network in PB-, paclitaxel-, and 5-FU-treated human colorectal adenocarcinoma cells (HT29). We observed apoptotic cells in HT29 treated with high-dose 5-FU (Fig. 2e) and noted a population of round cells in paclitaxel- and PB-treated cancer cells (Figs. 2c and 2g). Round cells are a feature of the M phase as previously reported (Théry and Bornens, 2008; Cadart et al., 2014). Thus, based on our morphological observations of PB-treated cancer cells (round shaped), we hypothesize that the possible mechanism of cell death triggered by PB is likely via microtubule polymerization. We therefore then included paclitaxel for comparison purposes and found both PB- and paclitaxel-treated cancer cells have similar morphologies. Paclitaxel is a microtubule inhibitor that can cause M phase cell-cycle arrest (Perez, 2009); it stimulates polymerization which prevents or suppresses, microtubule disassembly, leading to cell-cycle arrest in the G2/M phase and ultimately apoptosis (Perez, 2009). We therefore further investigated microtubule structure in respective treatment groups; results showed that PB had a similar long distorted spindle appearance compared with

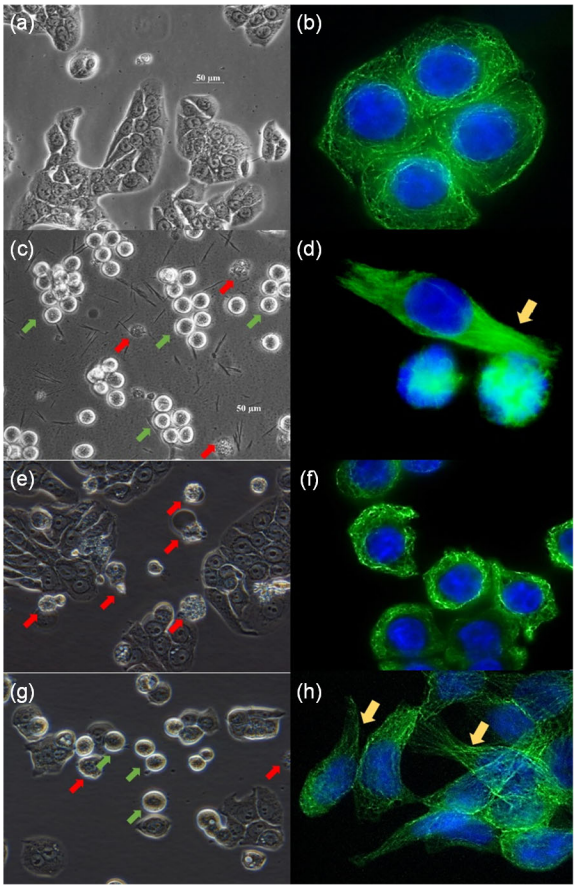

Fig. 2 Cell morphologies and microtubule networks of untreated, 10 μg/mL paclitaxel-, 130 μmol/L 5-FU-, and 200 μg/mL PB-treated HT29 cells after 24 h

(a, b) Untreated; (c, d) 10 μg/mL paclitaxel; (e, f) 130 μmol/L 5-FU; (g, h) 200 μg/mL PB. Cell morphologies were observed under 400× magnification. Red arrows indicate apoptotic cells, while green arrows indicate M phase cells. Microtubule networks were observed under 1000× magnification. Tubulin was stained with anti-tubulin antibody at 1:1000 dilution followed by goat anti-mouse IgG conjugated with DyLight 488 at 1:1000 dilution (green signal). Nuclei were counterstained with DAPI (blue signal). Paclitaxel- and PB-treated HT29 cells have long spindles (yellow arrows). 5-FU: 5-fluorouracil; PB: *Piper betle*; DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride

paclitaxel-treated cells (Figs. 2d and 2h). Distorted spindles are a defect on microtubules and can prevent cells from dividing (Ganguly et al., 2015). We proposed that microtubule network disruption might represent the possible mechanism by which PB triggers cancer cell death.

It has previously been reported that microtubule-targeting drugs inhibit cell motility, triggering both anti-migratory and anti-angiogenic effects (Belotti et al., 1996; Hayot et al., 2006; Ganguly et al., 2015). Microtubules are an integral part of the cytoskeletal system of all eukaryotic cells. Indeed, because of their dynamic behavior, these structures play an important role in numerous biological processes including mitosis, cellular motility, and cytoplasmic transport. Cancer cell death can be induced via minor alterations of microtubule dynamics that engage the spindle checkpoint and cell cycle arrest (Blajeski et al., 2002). As previously reported, different concentrations of microtubule-targeting drugs exert varied effects on cancer cells (Hayot et al., 2006; Ganguly et al., 2015; Gandalovičová et al., 2017). A high drug concentration is needed to cause microtubules defects (Ganguly et al., 2015), while a low concentration is capable of inhibiting cell migration (Hayot et al., 2006). In this study, PB in higher concentrations (200 μg/mL) triggered a microtubule polymerization response, while lower concentrations (20 and 100 μg/mL) inhibited cell migration. We have demonstrated the potential use of PB as a microtubule-targeting agent which also exhibits anti-migratory effects on cancer cells.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Contributors

Mee Lee LOOI contributed to the study design, data analysis, writing and editing of the manuscript. Alwyn Khai Howe WONG, Shelly Anne GNAPRAGASAN, Anis Zafirah JAPRI, Aiysvariyah RAJEDADRAM, and Kar Yong PIN performed the experimental research and data analysis. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Acknowledgments

We thank Taylor's University and Forest Research Institute Malaysia (FRIM), Malaysia, for the laboratory facilities.

Compliance with ethics guidelines

Mee Lee LOOI, Alwyn Khai Howe WONG, Shelly Anne GNAPRAGASAN, Anis Zafirah JAPRI, Aiysvariyah RAJEDADRAM, and Kar Yong PIN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Belotti D, Vergani V, Drudis T, et al., 1996. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res*, 2(11):1843-1849.

Blajeski AL, Phan VA, Kottke TJ, et al., 2002. G_1 and G_2 cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *J Clin Invest*, 110(1):91-99. https://doi.org/10.1172/JCI13275

Cadart C, Zlotek-Zlotkiewicz E, le Berre M, et al., 2014. Exploring the function of cell shape and size during mitosis. *Dev Cell*, 29(2):159-169. https://doi.org/10.1016/j.devcel.2014.04.009

Gandalovičová A, Rosel D, Fernandes M, et al., 2017. Migrastatics—anti-metastatic and anti-invasion drugs: promises and challenges. *Trends Cancer*, 3(6):391-406. https://doi.org/10.1016/j.trecan.2017.04.008

Ganguly A, Yang HL, Sharma R, et al., 2012. The role of microtubules and their dynamics in cell migration. *J Biol Chem*, 287(52):43359-43369. https://doi.org/10.1074/jbc.M112.423905

Ganguly A, Cabral F, Yang HL, et al., 2015. Peloruside A is a microtubule-stabilizing agent with exceptional anti-migratory properties in human endothelial cells. *Oncoscience*, 2(6):585-595. https://doi.org/10.18632/oncoscience.169

Guha Majumdar A, Subramanian M, 2019. Hydroxychavicol from *Piper betle* induces apoptosis, cell cycle arrest, and inhibits epithelial-mesenchymal transition in pancreatic cancer cells. *Biochem Pharmacol*, 166:274-291. https://doi.org/10.1016/j.bcp.2019.05.025

Hayot C, Debeir O, van Ham P, et al., 2006. Characterization of the activities of actin-affecting drugs on tumor cell migration. *Toxicol Appl Pharmacol*, 211(1):30-40. https://doi.org/10.1016/j.taap.2005.06.006

Mukhtar E, Adhami VM, Mukhtar H, 2014. Targeting microtubules by natural agents for cancer therapy. *Mol Cancer Ther*, 13(2):275-284. https://doi.org/10.1158/1535-7163.MCT-13-0791

Ng PL, Rajab NF, Then SM, et al., 2014. *Piper betle* leaf extract enhances the cytotoxicity effect of 5-fluorouracil in inhibiting the growth of HT29 and HCT116 colon cancer cells. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 15(8):692-700. https://doi.org/10.1631/jzus.B1300303

Perez EA, 2009. Microtubule inhibitors: differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. *Mol Cancer Ther*, 8(8):2086-2095.

https://doi.org/10.1158/1535-7163.Mct-09-0366
Salehi B, Zakaria ZA, Gyawali R, et al., 2019. *Piper* species: a comprehensive review on their phytochemistry, biological activities and applications. *Molecules*, 24(7):1364. https://doi.org/10.3390/molecules24071364
Shakibaei M, Kraehe P, Popper B, et al., 2015. Curcumin potentiates antitumor activity of 5-fluorouracil in a 3D alginate tumor microenvironment of colorectal cancer. *BMC Cancer*, 15:250. https://doi.org/10.1186/s12885-015-1291-0
Théry M, Bornens M, 2008. Get round and stiff for mitosis. *HFSP J*, 2(2):65-71. https://doi.org/10.2976/1.2895661
Wu PF, Tseng HC, Chyau CC, et al., 2014. *Piper betle* leaf extracts induced human hepatocellular carcinoma HEP3B cell death *via* MAPKS regulating the p73 pathway *in vitro* and *in vivo*. *Food Funct*, 5(12):3320-3328. https://doi.org/10.1039/C4FO00810C

List of electronic supplementary materials

Materials and methods

中文概要

题　目：槟榔叶水提液对癌细胞的抗迁移作用及其微管靶向性的研究

概　要：本文研究了槟榔（*Piper betle*，PB）水提取物对癌细胞转移能力的调节作用及其对微管蛋白结构和网络的影响。采用亚毒剂量对 PB 和 5-氟尿嘧啶（5-FU）处理的癌细胞进行抗迁移研究，观察细胞迁移 20 小时。在所有处理组中，100 μg/mL PB 处理的癌细胞均显示出最大的抗迁移作用（P=0.016）。总体上，PB 对癌细胞的抗迁移作用高于 5-FU。通过细胞形态观察发现，PB 处理的细胞表现出与标准微管抑制剂（紫杉醇）相似的细胞特征，并在 PB 和紫杉醇处理的癌细胞中发现 M 期细胞群。通过对微管结构和网络进一步研究，我们发现 PB 和紫杉醇处理的癌细胞表现出长期破坏的纺锤体。因此，我们认为 PB 具有对癌细胞的抗迁移作用，并可能改变蛋白结构和网络。

关键词：槟榔；细胞迁移；微管；微管蛋白网络