RESEARCH ARTICLE

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Phyllanthus taxodiifolius Beille Disrupted N-cadherin, Vimentin, Paxillin and Actin Stress Fibers in Glioblastoma

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Abstract

Objective: Glioblastoma is the most aggressive and lethal brain tumor in adults with highly invasive properties. In this present study, we explored the effects of *Phyllanthus taxodiifolius* Beille extract on molecules known to be hallmarks of aggressive glioblastoma including N-cadherin and vimentin, mesenchymal markers, as well as paxillin, a major adaptor protein that regulates the linking of focal adhesions to the actin cytoskeleton. **Methods:** *P. taxodiifolius* were air-dried, powdered and percolated with methanol, filtered, concentrated and lyophilized to yield a crude methanol extract. C6 glioblastoma cell line was used in this study. The expression of N-cadherin and vimentin, as well as the activation of paxillin was determined using Western blot analysis. The effect of the extract on focal adhesions and actin cytoskeleton were investigated using immunofluorescence staining and confocal imaging. **Results:** In the presence of 40 µg/ml *Phyllanthus taxodiifolius* Beille extract, the expression of N-cadherin and vimentin were significantly decreased ($p<0.001$ and $p<0.05$, respectively). Activation of paxillin was also diminished as indicated by a reduction of phosphorylated-paxillin ($p<0.01$). Consequently, actin stress fibers in glioblastoma cells were abolished as evidenced by the decrease in focal adhesion ($p<0.001$) and stress fibers numbers ($p<0.001$). **Conclusion:** Our study demonstrates for the first time that *P. taxodiifolius* interferes with multiple key molecules related to pathological hallmarks of glioblastoma. These molecules are involved with cell contacts, focal adhesions, and the formation and stabilization of actin stress fibers, which are required for glioblastoma metastatic behavior. These results provide further evidence supporting the potential of *P. taxodiifolius* and its bioactive compounds as anti-cancer agents.

Keywords: glioblastoma- N-cadherin- vimentin- paxillin- focal adhesion- stress fibers

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Introduction

Glioblastoma is one of the most common and aggressive primary brain tumors in humans (Alexander and Cloughesy, 2017). Glioblastoma cells that migrate away from the primary tumor and invade the non-tumor cerebral parenchyma remain the major causes of death from glioblastoma. The current standard treatment cannot completely eliminate this invasive type of glioblastoma because the diffusely infiltrative properties of these tumor cells commonly result in cancer recurrence. Therefore, new treatments that can effectively eliminate glioblastoma cell invasiveness are required. Our previous study showed that crude extract of *Phyllanthus taxodiifolius* Beille, a plant widely distributed in many Asian countries, suppressed glioblastoma cell adhesion, migration, invasion and disrupted microtubule dynamics (Kwanthongdee et al., 2019). However, since glioblastoma migration and invasion is a multicellular process and can be regulated by several factors, further examination is needed to explore the effects of *P. taxodiifolius* extract on other cellular targets.

The up-regulation of N-cadherin is a hallmark of aggressive glioblastoma. N-cadherin, an adhesion molecule required for pre-migratory signaling and cell-cell adhesion between invasive cells, is a known marker for mesenchymal traits in which the cancer cells gain migratory and invasive properties. N-cadherin expression is positively correlated with glioma aggressiveness and can be used as a prognostic and predictive molecular

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biomarker for tumor grading and treatment (Iwadate, 2016; Chen et al., 2018). Moreover, decreased N-cadherin expression attenuated cancer cell migration and invasion (Shih and Yamada, 2012; Ciołczyk-Wierzbicka and Laidler, 2018).

Regulation of vimentin expression is another potential target for abolishing glioma cell invasion and metastasis. Vimentin, an intermediate filament protein, is abundantly expressed in glioblastoma. Its expression correlates with glioma aggressiveness and poor clinical outcome (Zhao et al., 2018; Nowicki et al., 2019). Vimentin forms intermediate filaments (IFs) that are transported along microtubules by microtubule-associated motor proteins (Liao and Gundersen, 1998; Helfand et al., 2002). Vimentin IFs also interact with actin filaments and focal adhesions (Strouhalova et al., 2020). Coordination of vimentin IFs, the actin network, and focal adhesions strengthens cell adhesion and promotes cancer cell migration and invasion, which make vimentin another known marker for mesenchymal traits (Burgstaller et al., 2010; Ostrowska-Podhorodecka et al., 2021).

Focal adhesion regulation plays a crucial role in cell migration and invasion. Focal adhesion kinase (FAK), an important focal adhesion signaling molecule, is upregulated in glioblastoma cells, which induces several signaling events that promote glioblastoma cell motility. An important signaling target of activated FAK is paxillin, a primary scaffolding protein in focal adhesions. Paxillin activation is required for the transduction of extracellular signals into intracellular responses that control cell motility. Specifically, activated paxillin recruits several proteins that promote the rearrangement of the actin cytoskeleton into stress fibers that contribute to focal adhesion formation and turnover (Turner, 2000).

In this study, using C6 glioblastoma cells, we demonstrate the effects of *P. taxodiifolius* extract on these key factors that influence glioblastoma cell migration, including N-cadherin, vimentin, and focal adhesion signaling. Our results strongly illustrate the potential of *P. taxodiifolius* to be further developed into a new treatment against glioblastoma invasion.

Materials and Methods

Plant material and extraction

P. taxodiifolius was collected from Amnartchareon, Thailand and deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment in Bangkok (voucher specimen BKF no. 127614). Crude extract of *P. taxodiifolius* was obtained following the methods previously described (Kwanthongdee et al., 2019). Briefly, the aerial parts were air-dried, powdered and percolated with methanol. The filtered methanolic extract was then concentrated using a rotary evaporator and lyophilized to yield a crude methanol extract. The crude extract was stored at –20° C in an air-tight, light-protected container.

High-Performance Liquid Chromatography (HPLC) analysis

The HPLC fingerprint profile of the crude extract was obtained using an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) UV/visible light detector. *Phyllanthus taxodiifolius* Beille (*P. taxodiifolius*) extracts were separated using a gradient elution in a Poroshell 120 EC-C18, 50 × 4.6 mm, 2.7 µm HPLC column (Agilent, Santa Clara, CA, USA). The gradient solvent systems used for elution were a mixture of 90% H_2O and 10% acetonitrile. Analyses of *Phyllanthus taxodiifolius* Beille (*P. taxodiifolius*) extracts were carried out using the following gradient elution: 4 min, 90% H_2O, 10% acetonitrile; 9 min, 100% acetonitrile; 15 min, 90% H_2O, 10% acetonitrile. The gradient elution was run for a total of 20 min at a flow rate of 0.5 ml/min. Peak detection was performed at 254 nm. All extracts were prepared at 1 mg/mL in methanol and injected into the system (5 µL) using an autosampler. Cleistanthin A was used as the standard compound (Supplementary Figure 1).

Cell culture

Rat glioblastoma (C6) cells were obtained from the American Types Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco CO., USA) containing 5% horse serum and 5% Fetal Bovine Serum (FBS) in the presence of 1% antibiotic-antimycotic (Thermo Scientific; Rockford, IL, USA). The cells were maintained in a humidified incubator at 37°C with 5% CO_2 and 95% air. Mycoplasma testing has been performed routinely to ensure no Mycoplasma contamination.

Immunocytochemistry and imaging

In brief, C6 cells were fixed in 4% paraformaldehyde at 37°C for 20 minutes, washed with PBS, and subsequently permeabilized in 0.2% Triton-X100/PBS. The permeabilized cells were then blocked in 10% BSA/PBS and incubated with anti-N-cadherin antibody (ab18203, Abcam), anti-FAK antibody (3285, cell signaling technology), anti-α-tubulin antibody (Millipore), or anti-vimentin antibody (ab92547, Abcam). After incubation with primary antibodies, cells were washed with 5% BSA/PBS, followed by incubation with an anti-rabbit secondary antibody conjugated to Alexa 488 (A32731, Gibco Co., USA.). Actin filaments were labeled with Alexa568-phalloidin (1:400; A12380, Invitrogen, USA). Nuclei were stained with DAPI (1:400; D9542, Sigma-Aldrich, St. Louis, MO, USA). The images from each condition were collected under a super-resolution laser scanning confocal microscope (Carl Zeiss LSM800) using a 63X oil-immersion objective lens (NA 1.4). All images were analyzed in ImageJ and SFEX 1.0 (Zhang et al., 2017). Figures were constructed using Adobe Photoshop.

Immunoblotting analysis

Cell lysis was performed using RIPA buffer (50 mM Tris pH 8.0, 0.1% SDS mM, NaCl, 1% TritonX-100, 1 mM EDTA) in the presence of freshly prepared protease inhibitor (Roche). BCA assay (Thermo Scientific;

Rockford, IL, USA) was used to measure total protein concentration. An equal amount of protein for each condition was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Polyvinylidene Fluoride (PVDF) membrane. Non-specific protein binding was blocked with 5% non-fat dry milk in TBS-T (0.1% tween-20mM TBS) at room temperature (RT) for 1 h. The membranes were incubated overnight at 4°C with primary antibodies. Anti-N-cadherin (ab18203) and anti-vimentin (ab92547) primary antibodies were purchased from Abcam (USA). Anti-GAPDH (AM4300, Ambion, Austin, TX, USA) was used as a loading control. Anti-FAK (3285), anti-paxillin (2542), anti-phospho-FAK (Tyr397) (3283), and anti-phospho-paxillin (Tyr118) (2541) antibodies were obtained from Cell Signaling Technology, USA. Membranes were washed 5x with TBS-T and incubated with secondary antibodies conjugated with horseradish peroxide at RT for 1 h. Finally, membranes were washed with TBS-T, incubated with enhanced chemiluminescent substrate (Millipore, Germany), and detected with hyperfilm (GE Healthcare, UK). ImageJ was used to analyze and quantify signal intensity.

Methanol (MeOH) and vinblastine were used as a vehicle control and a positive control, respectively.

Statistical Analysis

GraphPad Prism 8.4 was used for all statistical tests. One-way analysis of variance (ANOVA) followed by Dunnett's post-test comparison was applied to each dataset. A p-value of < 0.05 is considered significant. Data are expressed as means ± S.E.M. from at least three independent experiments. *$p < 0.05$, **$p<0.01$, and ***$p<0.001$ compared to control.

Results

P. taxodiifolius extract interfered with the distribution and expression of N-cadherin in C6 glioblastoma cells

We previously reported that *P. taxodiifolius* extract inhibited glioblastoma cell migration and invasion (Kwanthongdee et al., 2019). Here, we attempted to identify the molecular signals that underlie this inhibition by examining the effects of the extract on N-cadherin expression and localization. N-cadherin is an adhesion molecule that is highly expressed in aggressive glioblastoma and is required for cancer cell migration and invasion. High levels of N-cadherin are a known mesenchymal phenotype marker and indicative of migratory and invasive properties. Importantly, N-cadherin is up-regulated in C6 cells, which may explain their highly migratory and invasive behavior. To test the effects of the extract on N-cadherin expression, C6 glioblastoma cells were incubated with the *P. taxodiifolius* extract or vehicle control (methanol) for 24 hours. The concentrations of the *P. taxodiifolius* extract used here were obtained from the previous study (Kwanthongdee et al., 2019). We found that the expression level of the N-cadherin protein was significantly decreased in the presence of 16 and 40 µg/ml of *P. taxodiifolius* extract compared to the control (Figure 1a-b).

To determine N-cadherin localization, cell cultures were immunostained with an anti-N-cadherin antibody and imaged via confocal microscopy. In control cells, N-cadherin was largely localized to the cell membrane, in finger-like structures between cells (Figure 1c, control). These cadherin-rich fingers are membrane protrusions that have been observed in migrating cells (Hayer et al., 2016). In the presence of 16 and 40 µg/ml of *P. taxodiifolius* extract, these structures were absent and N-cadherin was predominantly distributed in the cytoplasm (Fig. 1c). Measurements of the relative intensity of N-cadherin across the cell revealed that N-cadherin was proportionally higher in the cytoplasm than at the membrane in cells treated with the *P. taxodiifolius* extract (Fig. 1d). These results suggest that the extract not only interferes with N-cadherin expression but also affects its localization and downstream signaling, which are consistent with our previous report showing that *P. taxodiifolius* disrupted glioblastoma cell migration and invasion (Kwanthongdee et al., 2019).

P. taxodiifolius extract disrupted vimentin filament formation and decreased vimentin levels in C6 glioblastoma cells

Vimentin is another important molecule highly expressed in glioblastoma, and its expression positively correlates with cancer aggressiveness. Vimentin, along with actin filaments and microtubules, is a critical cytoskeletal element that self-aggregates to form intermediate filaments (IFs). Importantly, in highly metastatic cancers, vimentin plays an important role in cancer cell migration and invasion (Ridge et al., 2022). Additionally, microtubules have been shown to be important for vimentin IF organization and transport (Liao and Gundersen, 1998; Prahlad et al., 1998; Helfand et al., 2002; Sakamoto et al., 2013). Our previous study demonstrated that *P. taxodiifolius* extract interfered with microtubule structure and dynamics. Here, we investigated whether the extract also affects vimentin IFs in C6 glioblastoma cells. Western blots from C6 glioblastoma cell lysates showed that vimentin protein, which is usually highly expressed in glioblastoma cells, was significantly decreased in cells treated with 16 and 40 µg/ml of *P. taxodiifolius* extract (Fig. 2a-b).

Figure 2c shows that in control C6 cells and MeOH treated cells, vimentin IFs formed dense networks located around the nucleus, elaborate the networks along the microtubules, and extend to the plasma membrane (Figure 2c, arrowheads). Strikingly, in the presence of the *P. taxodiifolius* extract (16 and 40 µg/ml), these extended filaments were found to be collapsed to areas surrounding the nucleus (Figure 2c, *P.tax*). These data suggest that the *P. taxodiifolius* extract disrupted both the expression of vimentin and the structure of vimentin IFs in glioblastoma cells.

P. taxodiifolius extract decreased FAK and paxillin activation, reduced focal adhesion, and disrupted actin stress fibers

Focal adhesions are critical for cancer cell adhesion, migration, and invasion (Corso and Bindra, 2016). In

motile cells, focal adhesion complexes act as molecular links between actin stress fibers and the extracellular matrix, resulting in traction forces that drive forward cell translocation. Focal adhesion kinase (FAK) is a major component and a key regulator for focal adhesion. Upon activation by extracellular signals, FAK phosphorylates multiple substrates including the multi-domain adaptor protein paxillin. Phosphorylation of paxillin by active FAK creates additional binding sites for several other proteins at the focal adhesion, which in turn activates downstream signaling cascades that induce actin filament reorganization and stress fiber formation, which ultimately promotes cell motility (Turner, 2000). Thus, we investigated whether the extract modulates FAK activity and paxillin phosphorylation in glioblastoma cells. The C6 cells were treated with either the extract, a vehicle control

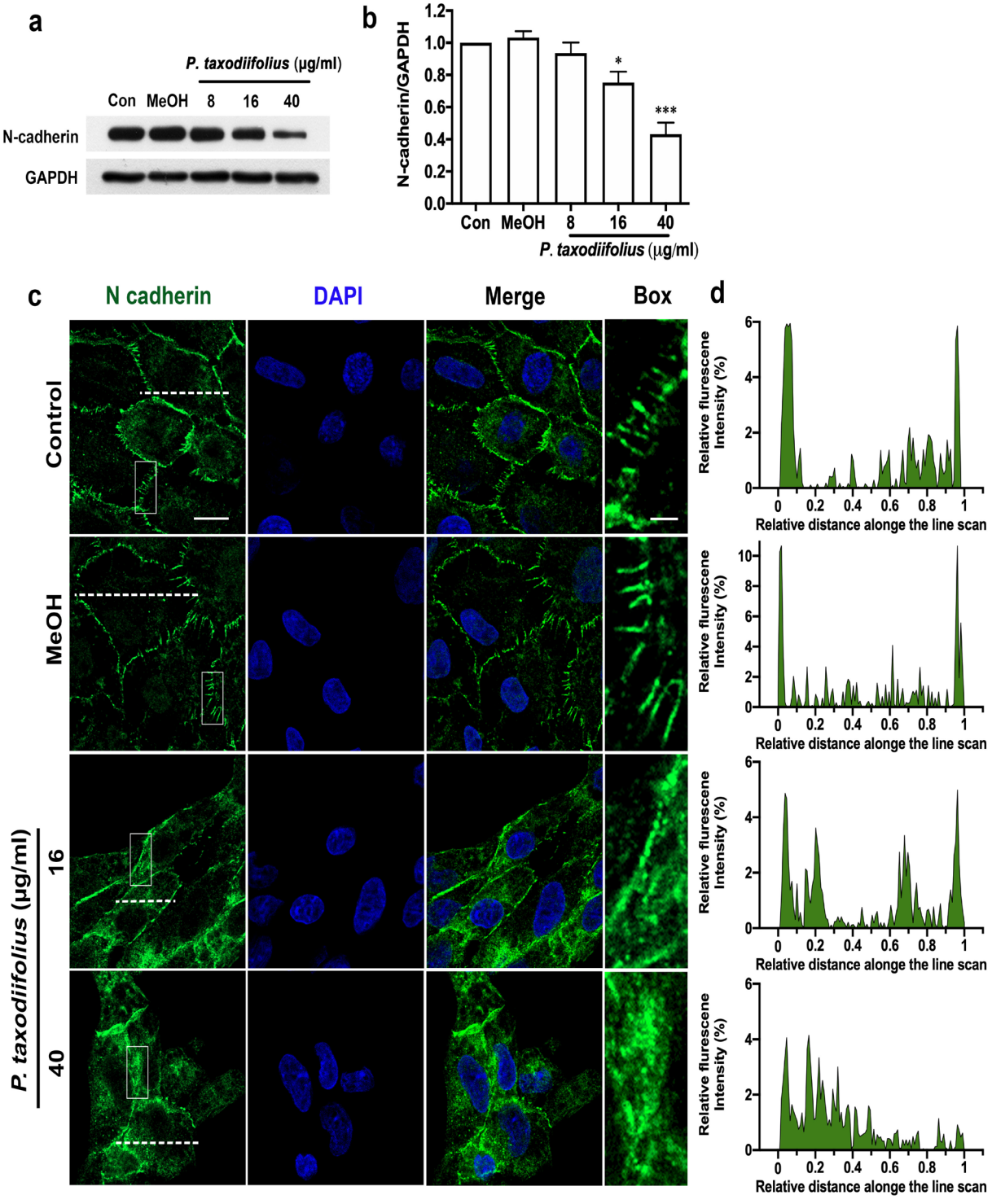

Figure 1. *P. taxodiifolius* Extract Decreased the Membrane Localization and Expression Levels of N-cadherin in C6 Cells.

(MeOH), or a positive control (vinblastine) for 24 h before measuring the level of FAK phosphorylation at Tyr397 (P-Tyr397 FAK) and paxillin phosphorylation at Tyr118 (P-Tyr118 paxillin). Consistent with our previous report, western blot analysis showed a decrease in P-Tyr397 FAK in the presence of the extract (Figure 3a and c). Paxillin activation was also significantly diminished after treating the C6 cells with the extract for 24 h (Figure 3b and d). Phosphorylation of FAK and paxillin results in the recruitment of other proteins to form the focal adhesion, which is required for the formation of actin stress fiber used to generate the force for cell migration and invasion (Etienne-Manneville, 2013). To further examine the effects of the extract on focal adhesion and stress fiber formation, we co-stained with an antibody against FAK and Alexa568-phalloidin to label focal adhesion and actin stress fibers, respectively, in the presence of the extract. As shown in Figure 3e, FAK labeling at the edge of the

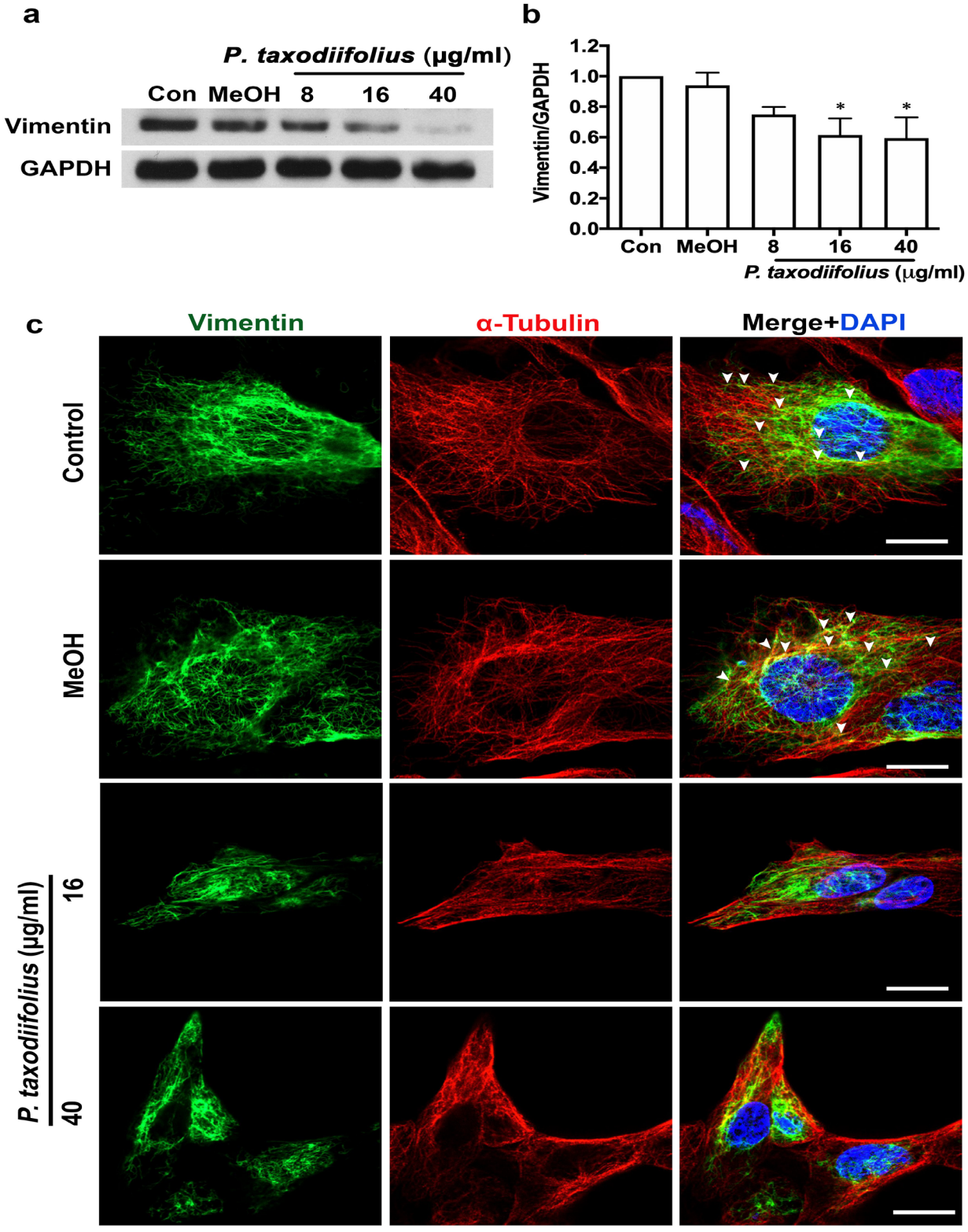

Figure 2. *P. taxodiifolius* Extract Disrupted Vimentin Filament and Vimentin Protein Expression in C6 Cells.

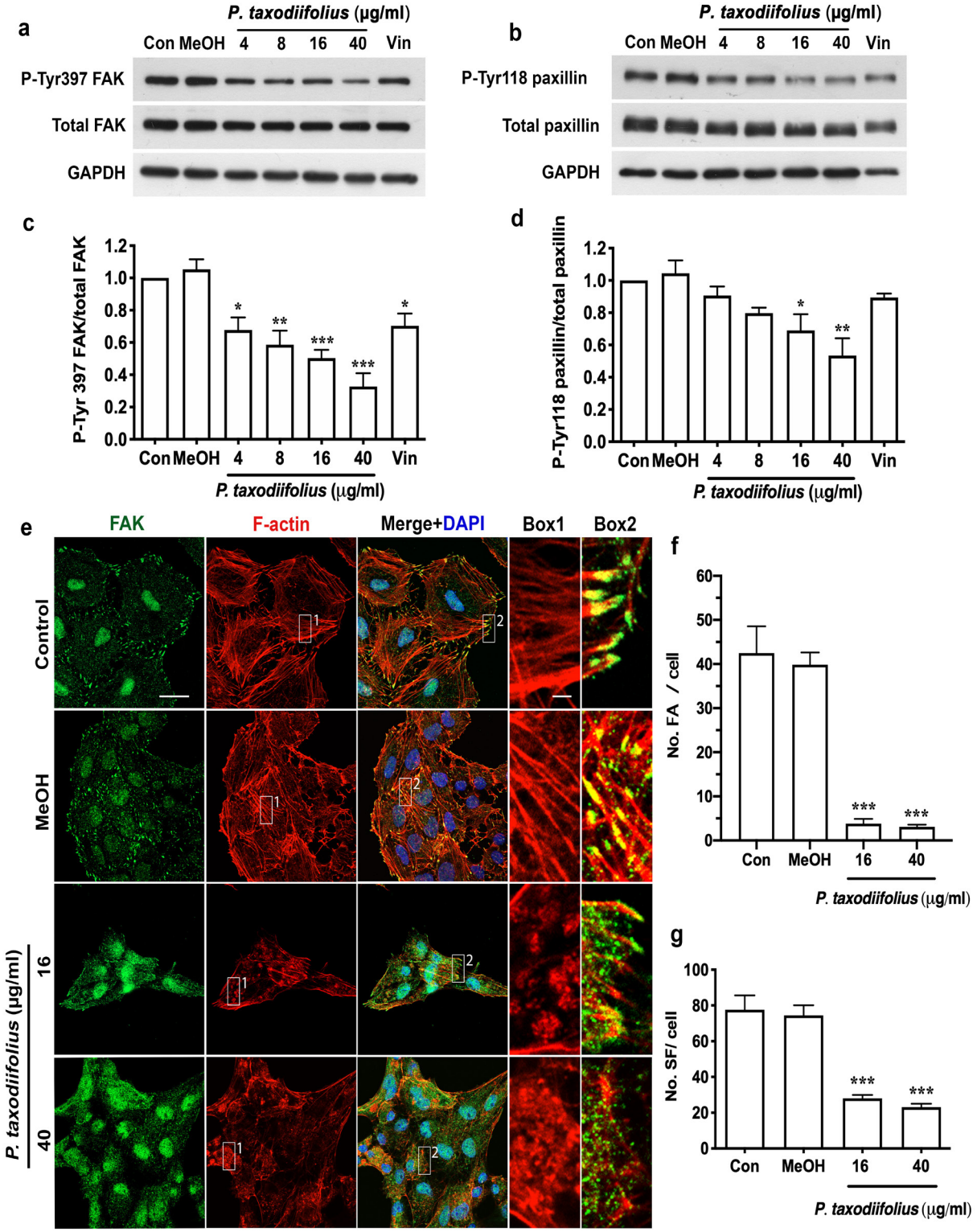

Figure 3. P. *taxodiifolius* Crude Extract Decreased the Number of Focal Adhesions, Paxillin Phosphorylation and Disrupted Actin Stress Fibers in C6 Cells.

C6 cells clearly colocalized with the actin stress fibers. Interestingly, the labeling of FAK at the focal adhesion and associated actin stress fibers was dramatically disturbed in the presence of the extract (Figure 3e). Specifically, the extract significantly reduced the number of both focal adhesion and stress fibers (Figure 3f-g). Together, these results suggest that, in addition to inhibiting FAK activation, *P. taxodiifolius* extract also disrupts paxillin signaling and consequently interferes with the formation of focal adhesions and actin stress fibers, which can explain its effects on invasive properties of glioblastoma.

Discussion

Therapies that target glioma invasiveness and metastasis are effective strategies for preventing tumor recurrence and increasing patient survival rates. We previously showed that *Phyllanthus taxodiifolius* Beille extract suppressed glioblastoma cell invasiveness by reducing cell adhesion, migration, and invasion. Disruption of microtubule dynamics and focal adhesions were also observed in the presence of the extract. In this study, we provide novel findings showing that the *P. taxodiifolius* extract also interferes with several key molecules known to be the hallmarks of the glioblastoma. Specifically, we show that the extract interferes with N-cadherin localization and expression, disrupts vimentin IFs, and reduces focal adhesion and actin stress fibers via dysregulated FAK and paxillin phosphorylation.

The expression of N-cadherin is associated with glioma metastatic behavior (Chen et al., 2018). Several lines of evidence suggest that microtubules are involved in a kinesin-dependent transport of N-cadherin-containing vesicles to the cell surface; whereby, N-cadherin-dependent adhesion regulates directional migration. This is evidenced by the reduction of directional movement of N-cadherin vesicles upon microtubule depolymerization (Mary et al., 2002; Teng et al., 2005). Consistent with this role in directional migration, here, we observed that cadherin-fingers, which indicate polarized cadherin in migrating cells, were diminished in the presence of the extract. Extract-dependent modulation of microtubule dynamics and structure likely interferes with N-cadherin transport to the cell surface, thereby disrupting N-cadherin-mediated effects on cell migration and invasion.

The extract also interfered with the structure and function of vimentin, which is another marker for invasive glioblastoma (Nowicki et al., 2019). In C6 glioblastoma cells, we found a dramatic collapse of the vimentin IF network in the presence of the extract. We previously observed that the extract diminished microtubule polymerization within 15 minutes of exposure (Kwanthongdee et al., 2019), suggesting that disruption of vimentin IFs may result from the microtubule depolymerization effects of the extract. This would be consistent with a previous report showing that microtubule-depolymerizing drug nocodazole disrupted the IF network in astrocytes (Sakamoto et al., 2013). Vimentin has also been shown to link with paxillin, localized to focal adhesion, and regulate cell migration (Ostrowska-Podhorodecka et al., 2021). Here, we showed a decrease in paxillin activation and focal adhesion number in response to the extract. Thus, the extract-mediated interference of microtubules and vimentin IFs may underlie the observed failure of focal adhesion complex formation, which is an initial step for cancer migration.

FAK activity is increased in glioblastoma (Sulzmaier et al., 2014). Importantly, we showed that *P. taxodiifolius* extract reduced the number of FAK-labeled focal adhesions and stress fibers in C6 glioblastoma cells. In addition, activation of paxillin, an important molecule downstream of FAK, was also diminished in the presence of the extract. Paxillin is an important adapter protein that is recruited to nascent focal adhesion at the cell's leading edge. Paxillin plays a key role in focal adhesion turnover during cell migration. Paxillin phosphorylation regulates Rho GTPase signaling, which leads to downstream effects on actin stress fiber formation during migration (Deakin and Turner, 2008). Thus, the disruption of FAK and paxillin phosphorylation by *P. taxodiifolius* extract likely underlies its inhibitory effects on glioblastoma cell adhesion, migration, and invasion.

Activation of membrane-bound integrins by the extracellular matrix initiates signaling pathways critical for focal adhesion formation and stabilization (Nagano et al., 2012). This activation is followed by phosphorylation of FAK and paxillin resulting in the recruitment of other proteins to form the focal adhesion, which is required for the formation of actin stress fiber used to generate the force for cell migration and invasion (Etienne-Manneville, 2013). Microtubules are required for the localization of integrin cell surface receptors to the leading-edge plasma membrane (Gupton and Gertler, 2010). Therefore, microtubule disruption might interfere with the translocation of integrins to the cell surface and consequently reduced FAK and paxillin phosphorylation in glioblastoma cells. However, there are other molecules that are able to regulate FAK activation, including Src-family kinases, EGFR, PDGFR, or G protein-couple receptors (Sulzmaier et al., 2014; Zhou et al., 2019). It is also possible that FAK might be a direct target of some compounds in the extract. Several pure compounds from *P. taxodiifolius* have been identified including Glochidone, Lupenol, Cleistanthin A, Cleistanthin A acetate and Cleistanthin A methyl ether (Tuchinda et al., 2006; Patiwat et al., 2010). Previous studies showed that Cleistanthin A, one of the pure compounds isolated from *P. taxodiifolius* suppressed V-type ATPase activity and FAK phosphorylation (Pan et al., 2017; Jearawuttanakul et al., 2020). However, none of these pure compounds affected microtubule structure and dynamics (data not shown). Identification of active compounds in the extract is necessary in order to prove these hypotheses.

Taken together, our results demonstrate for the first time that *P. taxodiifolius* interferes with adhesion molecules, vimentin intermediate filaments, focal adhesions and actin stress fibers of glioma cells. These multiple modulatory effects of *P. taxodiifolius* on key signaling molecules that regulate glioblastoma metastatic behavior support its potential as a novel therapeutic agent that may be used to inhibit the invasive properties of glioblastoma. Further determination of suitable concentration for further pharmaceutical development and investigation of active compounds in the extract is required to achieve this ultimate goal.

Author Contribution Statement

C.S. performed most of the experimental work. J.K. and K.U. assisted with the experiments. N.C., P.T. and B.M. prepared the *P. taxodiifolius* extract. W.S., C.S. and J.K. conceived the project, designed the experiments and wrote the manuscript. All authors read and approved the final manuscript. All data were generated in-house, and no

paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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