IN VIVO *39*: 778-794 (2025)
doi: 10.21873/invivo.13882

Standardized *Pluchea Indica* Leaf Extract Exhibited Antiproliferative Activity Against TGF-β-induced Prostate Stromal Cells (WPMY-1) Through G_0/G_1 Phase Cell Cycle Arrest *via* SMAD2/3 and ERK1/2 Signaling Pathways

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Abstract

Background/Aim: Benign prostatic hyperplasia (BPH) is characterized by the abnormal proliferation of prostate stromal cells, resulting in the enlargement of the prostate gland and the manifestation of troublesome symptoms, such as nocturia, urinary retention, and urinary incontinence. Dihydrotestosterone (DHT) and interleukin-17 (IL-17) are known to be key factors in promoting the overproduction of transforming growth factor-beta (TGF-β) in prostate stromal cells, contributing to their excessive proliferation, leading to BPH.

Materials and Methods: In this study, selected plant extracts traditionally used to alleviate urinary symptoms were subjected to primary screening for their anti-proliferative activity by evaluating DHT- and IL-17-induced proliferation in WPMY-1 prostate stromal cells. This was followed by a secondary screening using TGF-β induction.

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Received October 29, 2024 | Revised November 6, 2024 | Accepted November 7, 2024

Results: The extract that significantly inhibited cell proliferation was standardized and further investigated for its anti-proliferative effects through the TGF-β signaling pathway. Results showed that the leaf extract of *P. indica* significantly inhibited cell proliferation induced by DHT, IL-17, and TGF-β. It was demonstrated that *P. indica* has anti-proliferative properties *via* the TGF-β signaling pathways by inhibiting PCNA protein expression and inducing cell accumulation at the G_0/G_1 phase, while reducing the cell population at the S phase. Additionally, it down-regulated the expression of both canonical (p-SMAD2/3) and non-canonical (p-ERK1/2) proteins in TGF-β-induced WPMY-1 cells.

Conclusion: The standardized leaf extract of *P. indica* showed notable anti-proliferative activity against TGF-β-induced WPMY-1 cells by arresting the cell cycle at the G_0/G_1 phase through the SMAD2/3 and ERK1/2 signaling pathways.

Keywords: Transforming growth factor-beta, benign prostatic hyperplasia, *Pluchea indica*, WPMY-1, antiproliferation, chlorogenic acid.

Introduction

Benign prostatic hyperplasia (BPH) refers to the abnormal growth of cells in the prostate, leading to its enlargement. This condition is prevalent among males, particularly those aged 40 to 80 (1). In BPH, there is an abnormal overgrowth of stromal and epithelial cells in the prostate, particularly in the transitional zone near the urethra (2, 3). Prostate stromal cells exert a more significant influence on BPH compared to epithelial cells by releasing substances that stimulate cell proliferation, alter tissue structure, and induce inflammation (4, 5). This leads to an enlarged prostate, which compresses the urethra and causes various urinary symptoms (6).

BPH, which is linked to aging and hormonal changes (7, 8), involves the binding of testosterone and dihydrotestosterone (DHT) to prostate cell androgen receptors, leading to cell proliferation by disrupting the balance between growth and death *via* increased transforming growth factor-beta (TGF-β) levels (9). Metabolic syndrome, oxidative stress, and infections contribute to prostate inflammation, tissue injury, and IL-17 secretion, further elevating TGF-β levels (10). Elevated TGF-β levels stimulate excessive prostate cell proliferation through both canonical (SMAD2/3 signaling) and non-canonical pathways including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and the c-Jun N-terminal kinase (JNK) pathways (10, 11). Extracellular signal-regulated kinases 1 and 2 (Erk1/2) in the MAPK pathway regulate cell proliferation (12). Proliferating cell nuclear antigen (PCNA) is essential for DNA replication, which controls the cells entering to the S phase of the cell cycle, facilitating cell proliferation (13).

Finasteride, tadalafil, and alfuzosin are typically prescribed as first-line medications for BPH management. However, they lead to adverse effects, such as bleeding, gynecomastia, and sexual dysfunction (3, 14). Due to side effects, some BPH patients use herbal remedies to relieve their symptoms (15). The medicinal plants including *Curcuma wanenlueanga* Saensouk, Thomudtha & Boonma, *Paederia linearis* Hook f., *Derris scandens* (Roxb.) Benth., *Pluchea indica* (L.) Less., and *Andrographis paniculata* (Burm.f.) Wall ex Nees, are commonly found in tropical areas of Southeast Asia and have been traditionally used for relieving uncomfortable urinary symptoms (16-19). Additionally, a previous study has demonstrated the anti-proliferative effects of *Crinum latifolium* L., a plant belonging to the Amaryllidaceae family, on TGF-β-induced prostate stromal cells (3). The other plants in the Amaryllidaceae family including *C. thaianum* J. Schulze, *C. amabile* Donn., and *C. amoenum* Roxb. may possess anti-proliferation properties as well. Therefore, this study aimed to assess the inhibitory potential of selected medicinal plants, as listed above, by initially examining their effects on DHT and IL-17-induced proliferation of WPMY-1 prostate stromal cells as the primary screening,

Table I. *Compilation of selected medicinal plants from various sources in Thailand used in this study.*

No.	Scientific name	Part used	Source (provinces in Thailand)	Voucher no.	Traditional used	Ref.
1	*Curcuma wanenlueanga* Saensouk, Thomudtha & Boonma	Rhizome	Uttaradit	SS-894	Diuretic, Antiinflammation	(44)
2	*Paederia linearis* Hook f.	Root	Prachinburi	SS-889	Diuretic, Bladder stone	(45)
3	*Paederia linearis* Hook f.	Leaf	Prachinburi	SS-888	Diuretic, Bladder stone	(45)
4	*Derris scandens* (Roxb.) Benth.	Stem	Prachinburi	SS-890	Diuretic, Relieve aches and pains	(46)
5	*Pluchea indica* (L.) Less.	Leaf	Prachinburi	SS-891	Diuretic	(47)
6	*Andrographis paniculata* (Burm.f.) Wall ex Nees.	Leaf	Bangkok	SS-892	Diuretic, Antiinflammation	(48)
7	*Crinum latifolium* L.	Leaf	Ratchaburi	SS-893/1	Antiproliferation in BPH	(3)
8	*Crinum latifolium* L.	Bulb	Ratchaburi	SS-893/2	Antiproliferation in BPH	(3)
9	*Crinum thaianum* J. Schulze	Leaf	Phang Nga	SS-895/1	Antiinflammation	(49)
10	*Crinum thaianum* J. Schulze	Bulb	Phang Nga	SS-895/2	Antiinflammation	(49)
11	*Crinum amabile* Donn.	Leaf	Prachinburi	SS-896/1	Antiinflammation	(50)
12	*Crinum amabile* Donn.	Bulb	Prachinburi	SS-896/2	Antiinflammation	(50)
13	*Crinum amoenum* Roxb.	Leaf	Loei	SS-897/1	Antiinflammation	(51)
14	*Crinum amoenum* Roxb.	Bulb	Loei	SS-897/2	Antiinflammation	(51)

followed by a secondary screening using TGF-β induction. Extract(s) demonstrating significant inhibition of cell proliferation were standardized, and their antiproliferative effects were investigated regarding protein expression involved in proliferation and cell cycle distribution through the TGF-β signaling pathway.

Materials and Methods

Plant materials and preparation of plant extracts. Medicinal plants were selected for antiproliferation screening based on prevailing wisdom, traditional knowledge, and folk medicine regarding their use in relieving related urinary symptoms (Table I). Plants were gathered, cleaned, chopped, and dried for 24 h at 50˚C in a hot oven. The ground materials were macerated in 70% ethanol (1:40 w/v) until exhausted. The extract was then filtered using No. 1 filter paper, and the combined filtrates were concentrated using a rotary evaporator under lower-than-normal pressure. The residue was dried to give the final extract. A selection of medicinal plants' yields is shown in Supplementary Table S1.

Standardization of P. indica leaf extract. The ethanolic extract of *P. indica* was standardized by using chlorogenic acid, its major compound. The chemical quantity was determined using validated high performance liquid chromatography (HPLC) in the same manner as in a previous study (20). A standard stock solution of chlorogenic acid was produced at a concentration of 1,000 ppm in methanol. Each standard solution was diluted to five different concentrations (100, 50, 25, 12.5, and 6.25 ppm) to generate a calibration curve. A modified HPLC method was utilized to quantitatively analyze the chlorogenic acid by a Shimadzu HPLC (Kyoto, Japan). The system included a thermostatted column compartment (CTO-40C), diode array detector (SPD-M40), and autosampler (SIL-40C). Data were obtained using Shimadzu LabSolutions software. The chromatographic separation was performed on a C18 guard column and an ACE® C18 column (150×4.6 mm, *i.d.*, 5 μm; Phenomenex, Torrance, CA, USA). The elution process involved the use of solvent A (0.5% acetic acid in water) and solvent B (methanol) as the mobile phase. A gradient was applied, starting from 10% B and increasing to 50% B over 40 min,

followed by a period of 100% B for 10 min. The flow rate was maintained at 1.0 ml/min, with a constant temperature of 25˚C. Prior to analysis, the extract was diluted in methanol (1,000 ppm) and filtered through a 0.2 mm membrane filter. Detection was performed using a DAD detector set at 326 nm, and a 5 μl volume of injection was used for all samples and standards.

Cell culture and cell viability. Human normal prostate stromal cells (WPMY-1) were obtained from the American Type Culture Collection (ATCC #CRL-2854TM). The cells were maintained in Dulbecco Modified Eagle Medium (DMEM) with an addition of 10% fetal bovine serum (FBS), 1% GlutaMax, and 1% penicillin–streptomycin. The cells were kept in a standard cell culture incubator at 37˚C with 5% CO_2. To evaluate cell viability, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay (MTT) was conducted. Initially, WPMY-1 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated at 37˚C with 5% CO_2 for 24 h. Following this, the cells were treated with various concentrations of the extracts for an additional 24 h. Subsequently, serum-free medium containing MTT reagent (Sigma-Aldrich, St. Louis, MO, USA, cat #298-93-1) was added and incubated for 4 h, after which DMSO was added to dissolve the formazan crystals. The absorbance was measured at OD 570 nm using a microplate reader (CLARIOstar Plus, Ortenberg, Germany).

Primary and secondary screening. The maximum non-toxic dose of each extract was screened for their anti-proliferation effect using the MTT assay with the same procedure as described above. For the initial screening, after seeding the WPMY-1 cells for 24 h, the culture medium was substituted with serum media containing either 10 ng/ml of DHT (TCI, cat #521-18-6) or IL-17 (Biolegend, San Diego, CA, USA, cat #570504), supplemented without or with the extracts for 24 h. For the secondary screening, the cells were tested for their anti-proliferation property using 5 ng/ml of TGF-β (Biolegend, cat #781804) without or with the extracts after 24 h of treatment. A positive control of Mitomycin C at a concentration of 5 ng/ml was utilized. The experiments were performed in triplicate. Then, the extracts demonstrating significant inhibitory effect were subsequently screened for TGF-β-induced WPMY-1 cell proliferation as a secondary assessment. This screening aimed to select extracts for further investigation of their anti-proliferative effects at the molecular level *via* the TGF-β signaling pathway.

Cell cycle analysis. The WPMY-1 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and cultured at 37˚C for 24 h. Following this, the cells were exposed to either 5 ng/ml of TGF-β alone or in conjunction with 1 or 5 μg/ml of *P. indica* leaf extract. After 24 h of treatment, the cells were detached using trypsin and then fixed with 70% ethanol at 4˚C overnight. Next, the cells were rinsed with cold phosphate-buffered saline (PBS) and stained with RNase (0.1 mg/ml) and propidium iodide (40 μg/ml) for 30 min at 37˚C in the dark. Cell cycle distribution was then analyzed using flow cytometry (Guava® easyCyte HT System, Luminex Corporation, Austin, TX, USA).

Protein localization. The protein localization and intensity were assessed using an indirect immunofluorescence assay. Initially, WPMY-1 cells were seeded at a density of 1×10^3 cells/well in 96-well plates and incubated at 37˚C for 24 h. Following this, the cells were treated with 5 ng/ml of TGF-β, either alone or in combination with 1 or 5 μg/ml of *P. indica* leaf extract, for an additional 24 h. Subsequently, the cells were fixed with cold absolute methanol for 45 min, washed with PBS, and permeabilized with 0.25% Triton X-100 for 10 min. The cells were then incubated with either anti-PCNA antibody (Biolegend, cat #307902, 1:500 dilution), anti-p-SMAD2/3 antibody (Cusabio, Houston, TX, USA, cat #CSB-PA000650, 1:500 dilution), or anti-p-ERK1/2 antibody (Cusabio, cat #675502, 1:500 dilution) at 4˚C for 2 h. After PBS washing, FITC-labeled goat anti-rabbit or mouse IgG antibodies were added and incubated for 1 h. The nucleus was counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 15 min. Fluorescent

images were captured in three randomly selected areas using a fluorescence microscope (Eclipse Ni model with Digital Sight 10 camera, Nikon Corporation, Tokyo, Japan).

Protein expression. WPMY-1 cells were cultured in 6-well plates at an initial density of 5×10^5 cells/well. The cells were exposed to TGF-β at a concentration of 5 ng/ml, either alone or in combination with 1 or 5 μg/ml of *P. indica* leaf extract, and then incubated for 24 h. Following the incubation period, the cells were lysed using a lysis buffer containing RIPA buffer (Sigma-Aldrich, cat #SLCG2240) and protease inhibitor (Sigma-Aldrich, cat #25535-16-4) at a volume of 100 μl/well for 5 min on ice. Subsequently, the cells were scraped and then centrifuged at 12,000 rpm, 4˚C, for 20 min to collect the protein present in the supernatant. The protein concentration was quantified using Bradford protein reagent (TCI, cat #B5702). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA, cat #1620115). The membranes were then incubated with 5% skim milk at room temperature for 1 h to block nonspecific protein binding. Following that, the membranes were exposed to primary antibodies, including anti-PCNA antibody (1:1,000 dilution), anti-SMAD2/3 (Cusabio, cat #Q15796/P84022 and 1:1,000 dilution), p-SMAD2/3 antibody (1:1,000 dilution), anti-ERK1/2 (Biolegend, cat #686902 and 1:1,000 dilution), and p-ERK1/2 antibody (1:1,000 dilution) at 4˚C overnight. Next, the membranes were washed three times with Tween 20/Tris-buffered saline (T/TBS) buffer before being incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. Following the washing process, protein expression was visualized using a chemiluminescence imaging system (Chemi Doc Touch Image System, Bio-Rad Laboratories).

Statistical analysis. All data were represented as mean values accompanied by their respective standard deviations (mean±SD). We employed GraphPad Prism 9 (GraphPad Software, LLC, San Diego, CA, USA) software to perform a one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. A significance level of $p<0.05$ was deemed statistically significant.

Results

P. indica extract inhibited DHT-, IL-17-, and TGF-β-induced WPMY-1 cell proliferation. The maximum non-toxic dose of each extract was determined to screen for anti-proliferation effects on WPMY-1 cells (Supplementary Figure S1). Concerning the screening process (Figure 1), primary screening involved testing cells with 10 ng/ml of DHT or IL-17, either alone or in combination with extracts from nine plants. Extracts that effectively inhibited proliferation in both DHT- and IL-17-induced cells were then subjected to secondary screening with TGF-β induction (Figure 1A). The results showed a significant increase in WPMY-1 cell proliferation following treatment with either DHT or IL-17 alone. Compared to the control group, DHT treatment led to a maximum increase of 150.39±3.99% ($p<0.001$), while IL-17 treatment resulted in a maximum increase of 128.13±4.52% ($p<0.001$). The cell proliferation induced by DHT significantly decreased after treatment with extracts from *P. linearis*, *D. scandens*, *P. indica*, *A. paniculata*, *C. thaianum*, *C. thaianum*, *C. amabile*, *C. latifolium*, and *C. latifolium*. The proliferation induced by IL-17 was significantly inhibited by treatment with extracts from *P. linearis*, *D. scandens*, *P. indica*, *C. thaianum*, *C. amabile*, *C. latifolium*, and *C. latifolium*. Based on the primary screening results, extracts with significantly inhibited cell proliferation induced by both DHT and IL-17 were identified as *P. linearis*, *D. scandens*, *P. indica*, *C. thaianum*, *C. thaianum*, *C. amabile*, *C. latifolium*, and *C. latifolium* extracts (Figure 1B). These extracts were then subjected to secondary screening to evaluate their antiproliferative effects caused by TGF-β induction. In the secondary screening, WPMY-1 cells treated with 5 ng/ml of TGF-β alone exhibited a significantly increased cell proliferation, reaching 146.29±0.35% ($p<0.001$) compared to 100% in untreated cells. However, cell proliferation was

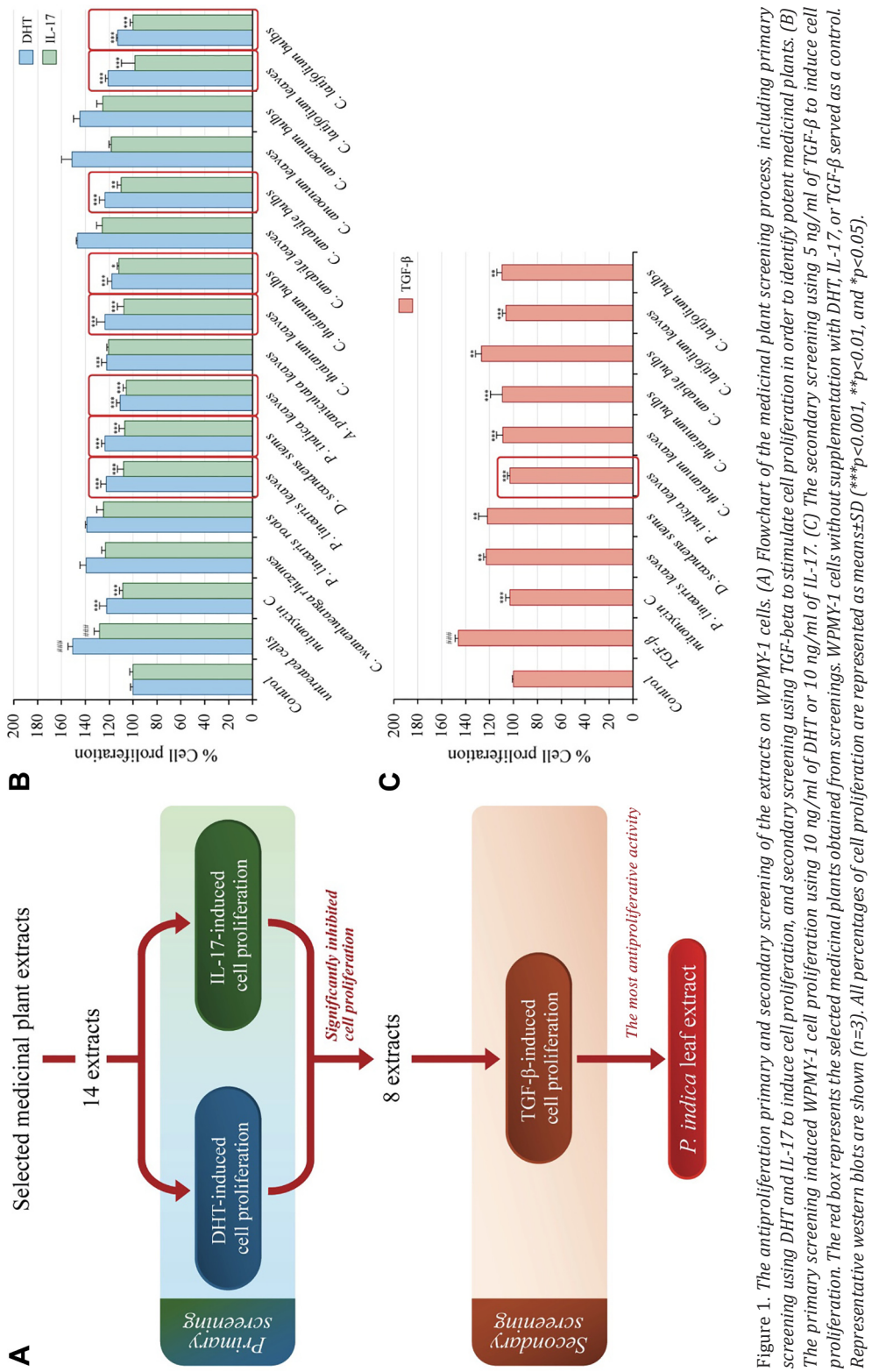

Figure 1. *The antiproliferation primary and secondary screening of the extracts on WPMY-1 cells. (A) Flowchart of the medicinal plant screening process, including primary screening using DHT and IL-17 to induce cell proliferation, and secondary screening using TGF-beta to stimulate cell proliferation in order to identify potent medicinal plants. (B) The primary screening induced WPMY-1 cell proliferation using 10 ng/ml of DHT or 10 ng/ml of IL-17. (C) The secondary screening using 5 ng/ml of TGF-β to induce cell proliferation. The red box represents the selected medicinal plants obtained from screenings. WPMY-1 cells without supplementation with DHT, IL-17, or TGF-β served as a control. Representative western blots are shown (n=3). All percentages of cell proliferation are represented as means±SD (***p<0.001, **p<0.01, and *p<0.05).*

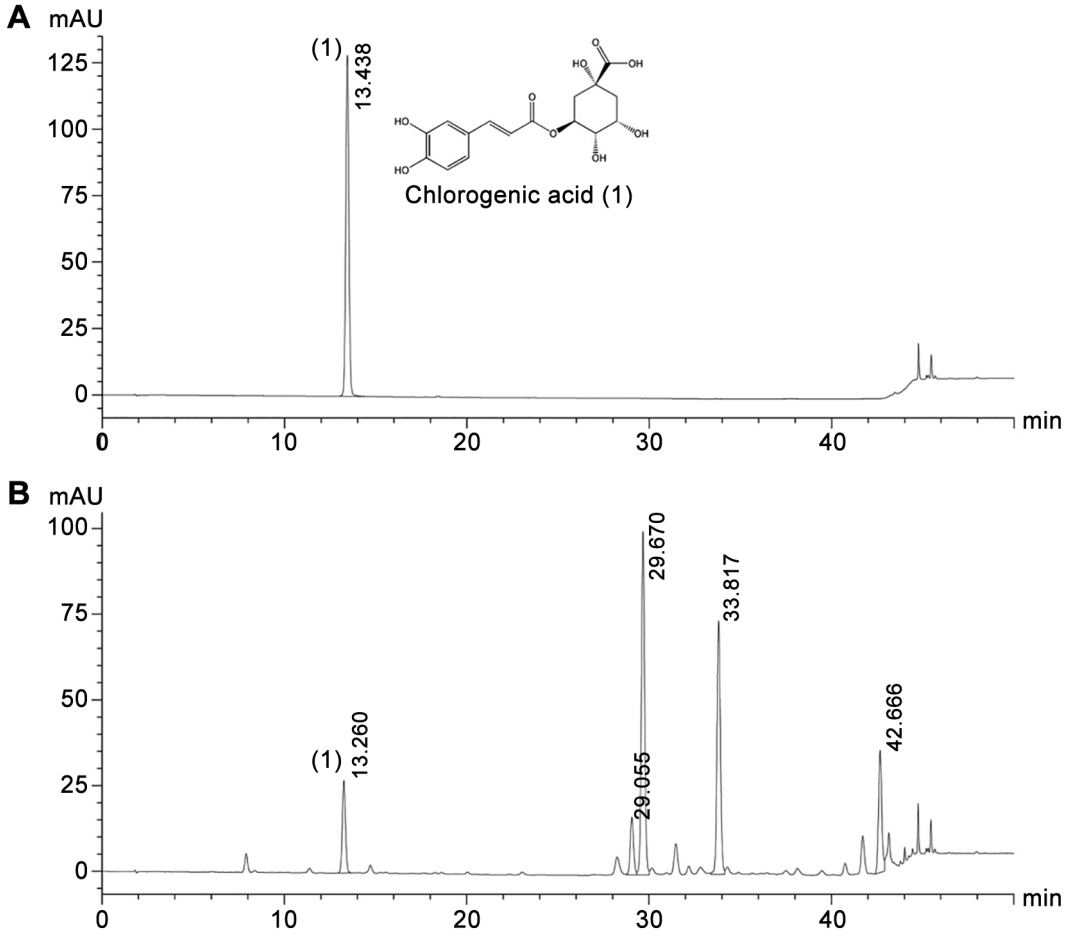

Figure 2. *HPLC chromatograms of (A) standard chlorogenic acid (1) and (B) ethanolic extract of P. indica.*

significantly reduced after treatment with all the extracts (Figure 1C). In the second screening, the leaf extract of *P. indica* exhibited the most potent effect on TGF-β-induced antiproliferation of WPMY-1 cells, as assessed by the percentage of inhibition in cell proliferation.

Standardization of the P. indica leaf extract by HPLC. The HPLC chromatograms depicted both the standard chlorogenic acid and the ethanolic extract of *P. indica* (Figure 2). Sharp and symmetric peak shapes were achieved with a rapid analysis time and high resolution, using a two-solvent mobile phase system: solvent A, a weak acid solution of 0.5% acetic acid in water, and solvent B, pure methanol. The reference standard chromatogram of chlorogenic acid appeared at 13.438 min, while in the extract, it had a retention time of 13.260 min (Figure 2A and B). The calibration curve for the chlorogenic acid standard exhibited a high level of reliability, with a correlation value (R^2) of 0.9997 (Supplementary Figure S2). In this study, the average concentration of chlorogenic acid was determined to be 1.18±0.03% w/w.

P. indica extract suppressed TGF-β-induced WPMY-1 cell proliferation via PCNA protein inhibition. To assess the effect of *P. indica* extract on PCNA protein expression, we utilized indirect immunofluorescence to examine the localization of PCNA and western blot assays to evaluate

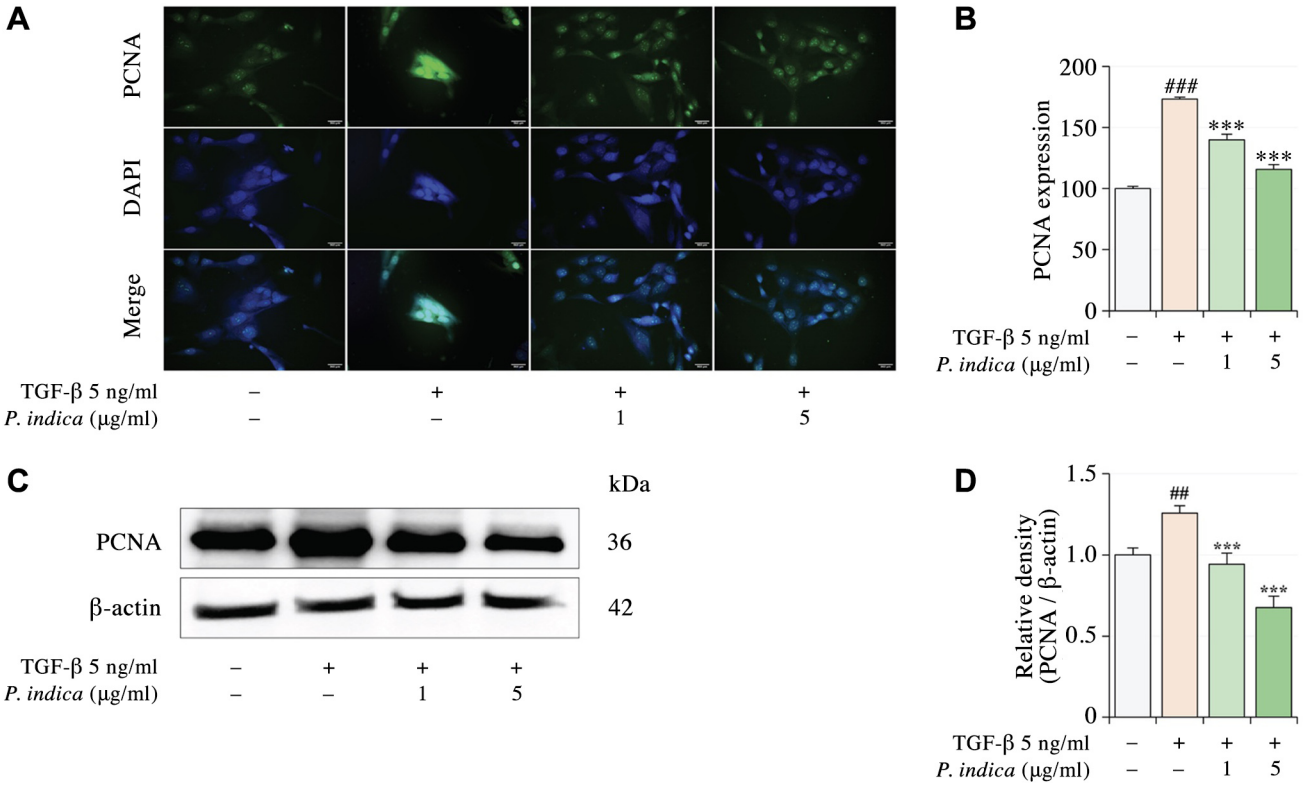

Figure 3. *Effects of P. indica extract on TGF-β-induced PCNA expression. WPMY-1 cells were treated with 5 ng/ml of TGF-β alone or in combination with P. indica extract at concentrations of 1 μg/ml and 5 μg/ml. (A) Immunofluorescence images depict the population of WPMY-1 cells, with green signals indicating PCNA protein expression in the nucleus, while blue represents nuclear staining with DAPI. Scale bar: 960 μm, magnification 200X. (B) Bar graph represents PCNA intensity quantification using ImageJ software. (C) Protein bands of PCNA and (D) expression of PCNA is normalized to β-actin protein and reveal as relative density compared to the control, presented as means±SD ($^{###}p<0.001$ and $^{##}p<0.01$ compared with control and $^{***}p<0.001$ compared with TGF-β).*

its expression levels (Figure 3). WPMY-1 cells were treated with 5 ng/ml of TGF-β alone or in combination with the extract of *P. indica* at concentrations of 1 μg/ml and 5 μg/ml. The cells stained with an antibody against the PCNA protein appeared green, while nuclei stained with DAPI appeared blue. Observations revealed that cells treated with TGF-β exhibited higher green signal expression in the nucleus compared to cells treated with the extract (Figure 3A and B). In the western blot assay, the results showed that WPMY-1 cells treated with TGF-β exhibited a significant increase in PCNA protein expression ($p<0.01$) compared to the cells without TGF-β treatment. In contrast, treatment with the extract significantly reduced the amount of PCNA protein in a dose-dependent way ($p<0.001$). Lower doses of the extract resulted in reduced levels of PCNA protein (Figure 3C and D). These findings show that the anti-proliferation activity of *P. indica* extract was due to the suppression of PCNA protein expression in the nucleus of WPMY-1 cells.

The extract of P. indica efficiently halted the proliferation of WPMY-1 cells triggered by TGF-β-induced cell cycle arrest specifically at the G_0/G_1 phase. The effect of *P. indica* extract on cell cycle distribution in TGF-β-induced WPMY-1 cell proliferation was evaluated using flow cytometry (Figure 4). WPMY-1 cells were treated with 5 ng/ml of TGF-β, without or with the extract at concentrations of 1 μg/ml and 5 μg/ml for 24 h. The histogram depicted the distribution

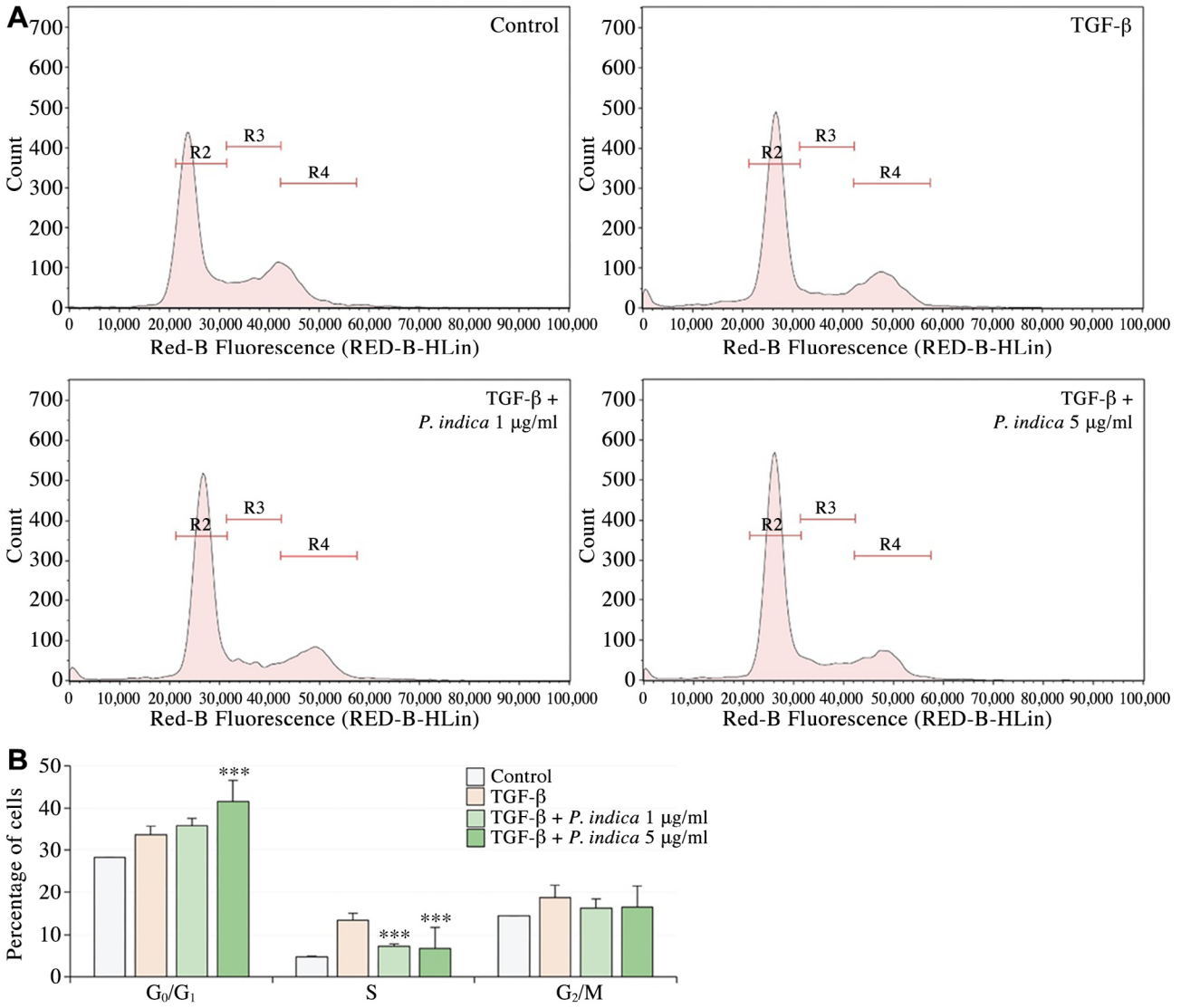

Figure 4. *The P. indica extract on cell cycle distribution in TGF-β-induced WPMY-1 cells. Cells were induced without TGF-β (control) or with 5 ng/ml of TGF-β alone, or in combination with the extract at concentrations of 1 μg/ml and 5 μg/ml, and then analyzed for cell cycle distribution using flow cytometry. (A) The histogram illustrates cell cycle distributions in G_0/G_1 (R2), S (R3), and G_2/M (R4) phases. (B) The percentage of cells in each phase is depicted in the bar graph as mean±SD (*** $p<0.001$ compared with TGF-β alone).*

of cell cycle phases, with R2, R3, and R4 representing the G_0/G_1, S, and G_2/M phases, respectively. The results showed that cells accumulated at the S phase in the TGF-β treatment group compared to the untreated group. Additionally, we observed a higher peak at the G_0/G_1 phase and a reduced peak at the S phase when the cells were exposed to the extract in a dose-dependent manner (Figure 4A). Furthermore, the extract at a concentration of 5 μg/ml significantly induced cell cycle arrest at the G_0/G_1 phase ($p<0.001$), while inhibition of cell proliferation at the S phase occurred starting from a concentration of 1 μg/ml ($p<0.001$), compared to the TGF-β treatment group (Figure 4B). This discovery highlighted the anti-proliferation effect of *P. indica* extract, which not only suppressed proliferating

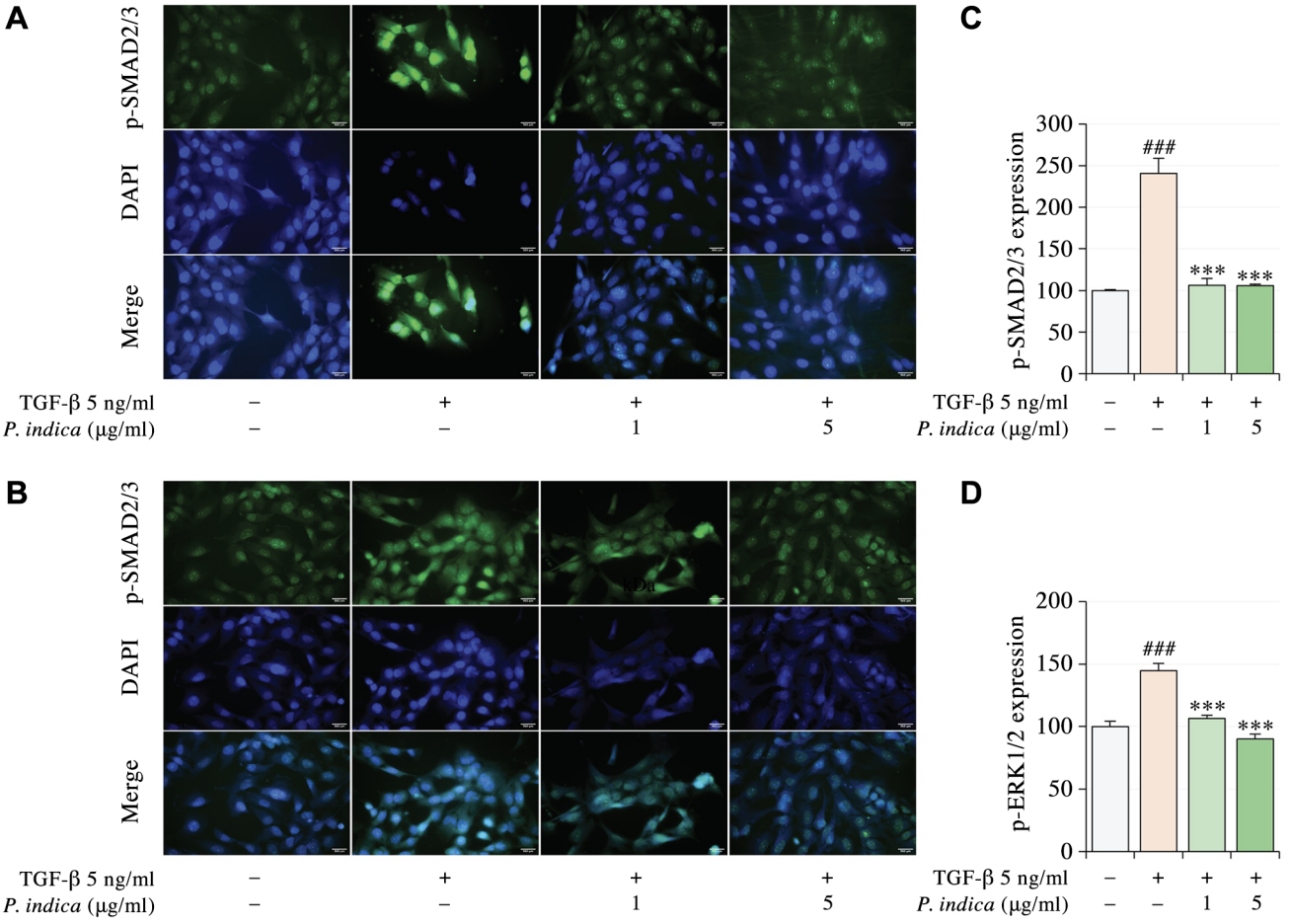

Figure 5. *Effects of the P. indica extract on protein localization and intensity in the TGF-β signaling pathway. WPMY-1 cells were treated without (control) or with 5 ng/ml of TGF-β alone or in combination with the extract at concentrations of 1 μg/ml and 5 μg/ml, respectively. The immunofluorescence images illustrate the WPMY-1 cell population, with green signals indicating (A) p-SMAD2/3 and (B) p-ERK1/2 protein expression in the nucleus, while blue represents nuclear staining with DAPI. The scale bar is 960 μm, with a magnification of 400´. Bar graphs represent (C) p-SMAD2/3 and (D) p-ERK1/2 intensity quantification using ImageJ software (n=3) and presented as means±SD (###p<0.001 compared with control, ***p<0.001 compared with TGF-β).*

proteins but also promoted cell cycle arrest at the G_0/G_1 phase in TGF-β-induced WPMY-1 cells.

The extract from P. indica effectively down-regulated the expression of p-SMAD2/3 and p-ERK1/2 proteins in the TGF-β signaling pathway. To validate the mechanisms of action of the *P. indica* extract on the TGF-β signaling pathways in WPMY-1 cells, we analyzed the localization and levels of expression of proteins involved in the SMAD and ERK signaling pathways, which represent the canonical and non-canonical signaling pathways of TGF-β, respectively. An indirect immunofluorescence assay was used to assess the phosphorylation of SMAD2/3 (p-SMAD2/3) and ERK1/2 (p-ERK1/2) proteins localization (Figure 5). The immunofluorescence images illustrated strong green signals of p-SMAD2/3 and p-ERK1/2 in the nucleus of WPMY-1 cells after treatment with 5 ng/ml of TGF-β alone, compared to the group without TGF-β treatment. *P. indica* extract inhibited nuclear translocation of the proteins, as indicated by slightly green signals in

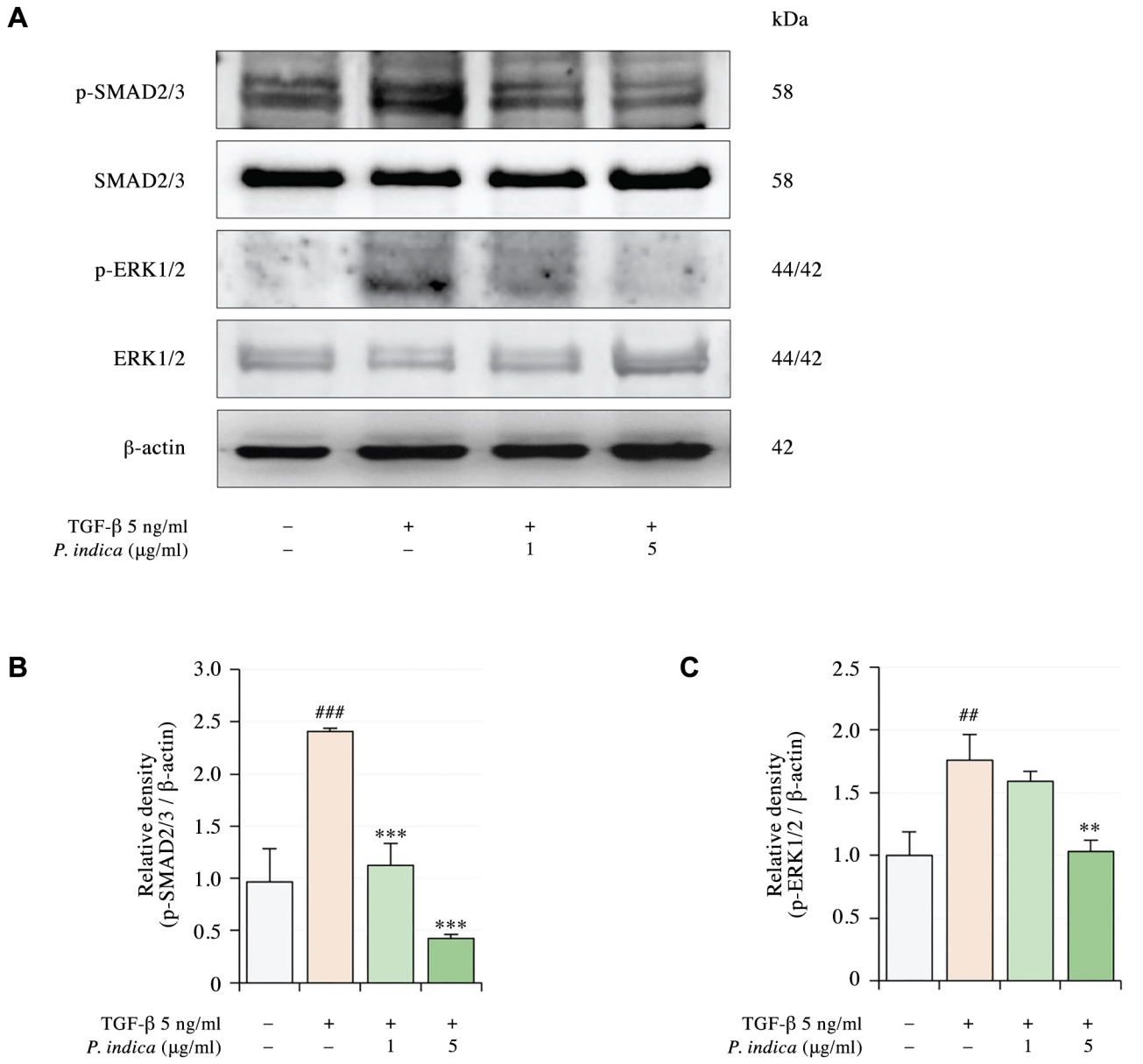

Figure 6. *Effects of the extract of P. indica on the expression of proteins involved in the TGF-β signaling pathway. WPMY-1 cells were treated either without (control) or with 5 ng/ml of TGF-β alone or in combination with the extract at concentrations of 1 μg/ml and 5 μg/ml, respectively. (A) Protein bands of p-SMAD2/3, SMAD2/3, p-ERK1/2, and ERK1/2 expression. (B) Expression levels of p-SMAD2/3 and (C) p-ERK1/2 are normalized to β-actin protein levels, demonstrated as relative density compared to the control. Representative western blots are shown (n=3). The data are presented as means±SD ($^{###}p<0.001$ and $^{##}p<0.01$ compared with the control, and $^{***}p<0.001$ and $^{**}p<0.01$ compared with TGF-β).*

the nucleus (Figure 5A and B). The extract significantly inhibited the intensity of p-SMAD2/3 and p-ERK1/2 proteins at concentrations starting from 1 μg/ml. The extract demonstrated a stronger effect on the inhibition of p-SMAD2/3 compared to p-ERK1/2 protein (Figure 5C and D). To confirm the inhibitory effect on the TGF-β

signaling pathway, we investigated the expression levels of p-SMAD2/3 and p-ERK1/2 proteins through western blot analysis (Figure 6). Our findings revealed that TGF-β-treated WPMY-1 cells exhibited an increase in the intensity of p-SMAD2/3 and p-ERK1/2 protein bands. Conversely, the protein bands decreased after treatment with the extract (Figure 6A). Additionally, TGF-β significantly up-regulated the expression of p-SMAD2/3 ($p<0.001$) and p-ERK1/2 ($p<0.01$) proteins compared to untreated cells. Treatment with the extract significantly decreased the levels of p-SMAD2/3 ($p<0.001$) in a dose-dependent manner, while only the highest concentration of the extract (5 μg/ml) significantly inhibited the expression of p-ERK1/2 protein ($p<0.01$), as compared to TGF-β treatment alone (Figure 6B and C). These results show that *P. indica* extract inhibited prostate stromal cell proliferation through the TGF-β signaling pathway.

Discussion

The exact cause of BPH remains unclear (21). However, the primary factor is associated with changes in testosterone hormone levels (22). Within the prostate gland, the enzyme 5-alpha reductase plays a crucial role in converting testosterone to its more potent form, DHT, which then binds to androgen receptors on prostate cells. This binding triggers the stimulation of prostate cell proliferation under the control of growth factors, such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), and notably TGF-β (23). In addition to hormone alteration, metabolic syndrome, oxidative stress, and infections also contribute to BPH by inducing prostate inflammation, causing tissue injury, and increasing the secretion of inflammatory cytokines, including members of the IL-17, IL-1β, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (24). These pro-inflammatory agents, especially L-17, trigger inflammation by promoting the production of signaling molecules like IL-6, IL-8, and chemokines, such as CCL2, CCL5, CXCL12, and CXCL10, which can further promote prostate cell proliferation (25). A study has reported that IL-17 can also disrupt the balance between prostate cell proliferation and death by increasing the amount of TGF-β (26). As elevated level of TGF-β is associated with numerous diseases, we aimed to evaluate the anti-proliferation activity of extracts by initially screening their response to DHT and IL-17 induction, which served as the primary screening method before subjecting them to TGF-β in the secondary screening to assess their specific anti-proliferation effects on BPH.

Interestingly, in the primary screening, eight out of fourteen selected extracts demonstrated significant inhibition of cell proliferation, indicating their potential to suppress cell growth at a high rate. This finding can be supported by their history of use in traditional medicine for alleviating diuretic and inflammation symptoms, leading to their strong anti-proliferative action on WPMY-1 cells induced by DHT and IL-17. Notably, all extracts that met the primary screening exhibited inhibitory effects on TGF-β-induced WPMY-1 cell proliferation in the secondary screening, with most of them being plants from the Amaryllidaceae family (Figure 1). These findings align with previous studies that reported the inhibitory effects of *Crinum latifolium* on TGF-β-induced prostate cell proliferation, attributing it to alkaloids, lycorine and 6α-hydroxybuphanidrine, as the bioactive compounds responsible for this effect (3). However, Amaryllidaceae plants including *C. amabile*, *C. thaianum* (27), and *C. latifolium* (28) have limitations in their utilization, as they are rare to find, grow slowly, and some are considered conservation plants. Based on the screening results, the ethanolic extract of *P. indica* demonstrated the highest inhibitory effect on cell proliferation induced by all three factors, decreasing cell proliferation by 92%, 87%, and 93% in DHT-, IL-17-, and TGF-β-induced WPMY-1 cells, respectively (Supplementary Table S2 and S3). *P. indica*, a plant in the Asteraceae family, is commonly found along Thailand's coastline and in warm climate locations across the world, including Malaysia, Indonesia, Australia, Taiwan, India, and Mexico (29). Studies on the phytochemical fractionation of *P. indica* extract have shown the presence of flavonoids, anthocyanins, phenolic acids, and several

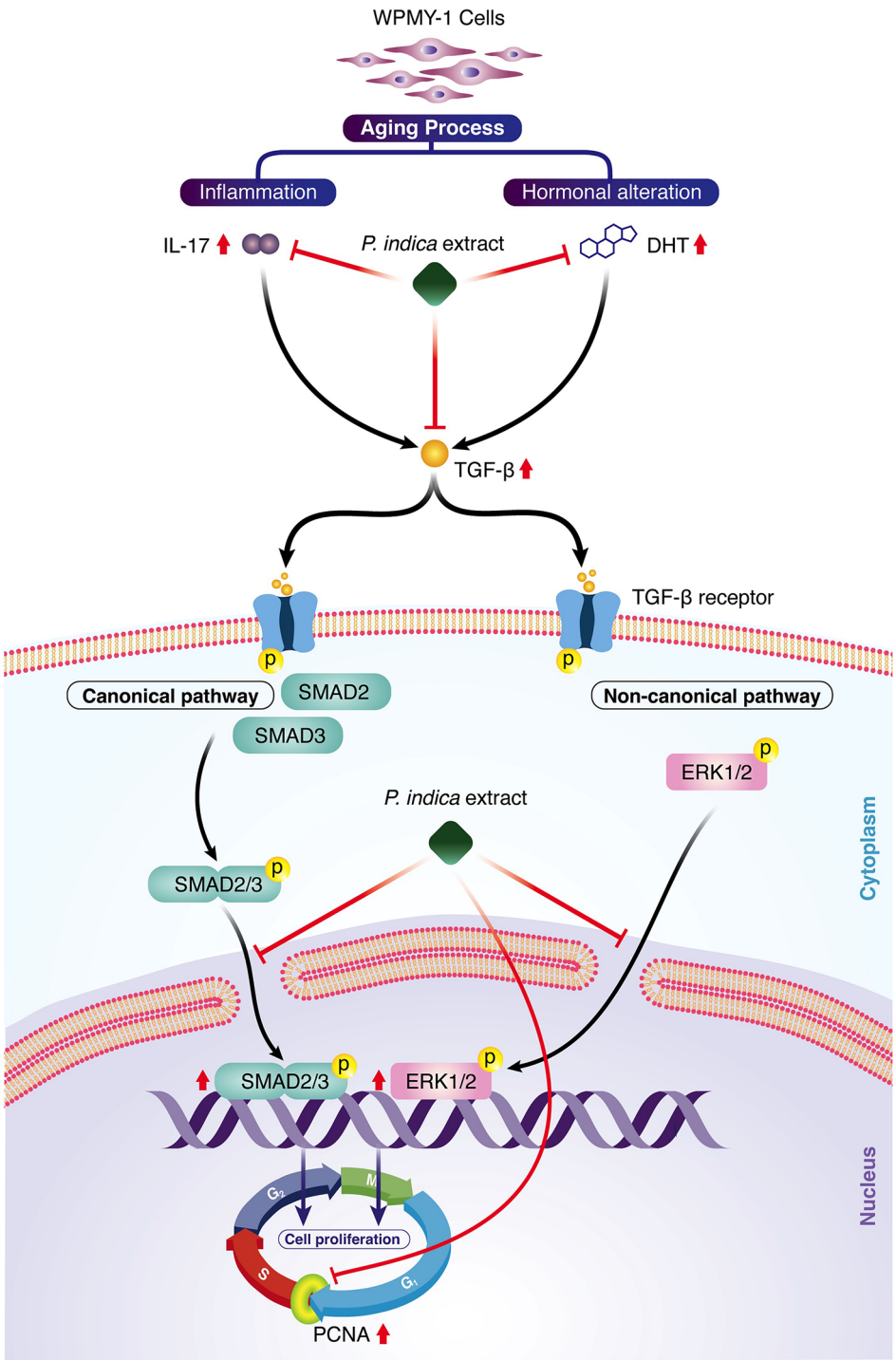

Figure 7. *The schematic illustrates the antiproliferative effect of P. indica extract and the proposed mechanism through the TGF-β mediated SMAD2/3 and ERK1/2 pathways. The extract of P. indica exhibits inhibition of WPMY-1 cell proliferation in both primary and secondary screenings. It also inhibits cell proliferation via the TGF-β signaling pathway by reducing the expression of both p-SMAD2/3 and p-ERK1/2 proteins. It diminishes the expression of PCNA protein, a marker indicative of cell proliferation, and impedes cell proliferation.*

kinds of carotenoids (30, 31). The *P. indica* extract was standardized using chlorogenic acid; however, despite dicaffeoylquinic being present in the highest amounts (Figure 2), its anti-BPH effect remains unclear. However, a previous study demonstrated the anti-BPH activity of chlorogenic acid by decreasing type II 5-alpha-reductase blood levels (32). Additionally, chlorogenic acid in *P. indica* extract has shown pharmacological properties in reducing colon and liver carcinogenesis in hamsters induced by methylazoxymethanol acetate, as well as anti-proliferative effects on cervical cancer cell lines (31). Moreover, chlorogenic acid represents the same biosynthetic pathway as dicaffeoylquinic (20), suggesting its potential as a representative for dicaffeoylquinic. Nevertheless, more research is necessary to investigate the inhibitory effects of chlorogenic acid on the cell proliferation of prostate stromal cells.

This study investigated the inhibition mechanism of cell proliferation, focusing on the expression of the protein PCNA, which plays a crucial role in promoting cell cycle progression and increasing cell numbers (33). It was noted that the expression of the PCNA protein is controlled by a wide variety of transcription factors, including TGF-β, TNF, YY1, E2F, CREB, ATF, RFX, GRH, and AP1 (34). Previous studies have indicated that PCNA is highly expressed in BPH tissue compared to normal prostate tissue (35, 36). This finding aligns with previous research indicating elevated expression of the PCNA protein induced by TGF-β in WPMY-1 cells. In this study, it is highlighted that the extract from *P. indica* significantly reduced the expression of the PCNA protein (Figure 3). In addition to being a protein linked to the cell cycle, PCNA is essential for managing DNA synthesis and the progression of the cell cycle (35, 37). Our findings indicate that the *P. indica* extract inhibits the increase in cell numbers in the S phase and induced the cell cycle arrest in the G_1 phase before entering the S phase (Figure 5), which is the stage where DNA replication begins, and cell division initiates. The results of these experiments confirmed the anti-proliferative effects of the extract through suppressing PCNA protein expression and promoting the retention of proliferated cells in the G_0/G_1 phase, leading to cell cycle arrest. Additionally, the investigation of the expression of PCNA protein and cell cycle distribution confirmed that the inhibitory effect directly targeted cell proliferation without inducing cell death.

The TGF-β signaling pathway consists of both canonical and non-canonical pathways. The canonical pathway involves the activation of receptor-regulated SMADs (R-SMADs) proteins, particularly SMAD2 and SMAD3 (38). TGF-β binds to the TGF-β receptor, leading to the phosphorylation of both the TGF-β receptor and specific SMAD proteins, namely SMAD2/3. p-SMAD2/3 then translocate into the nucleus, resulting in the expression of proteins that promote cell proliferation (39). On the other hand, the non-canonical pathway involves signaling through mechanisms other than SMAD. The non-canonical pathways of TGF-β comprise various signaling pathways that elicit different cellular processes responses (40) such as the MAPK pathway, which is associated with increased cell proliferation. In the MAPK pathway, TGF-β binding to its receptor triggers signal transduction through Ras, leading to the phosphorylation of proteins, such as Raf, MEK1/2, and ERK1/2, ultimately resulting in the formation of p-ERK1/2 (41). This phosphorylated form then translocates into the nucleus, inducing the expression of proteins that contribute to cell proliferation (12, 42). Previous studies have also supported the role of TGF-β in inducing cell proliferation in WPMY-1cells (3, 43). From the study of the TGF-β signaling pathway, specifically by examining the expression of proteins p-SMAD2/3 and p-ERK1/2, it is evident that the *P. indica* extract inhibits cell proliferation through the TGF-β signaling pathway. The *P. indica* extract significantly reduced the expression of proteins p-SMAD2/3 and p-ERK1/2 (Figure 5 and Figure 6). These results suggested that *P. indica* leaf extract inhibited prostate stromal cell proliferation through both canonical (SMAD2/3) and non-canonical (ERK1/2) pathways within the TGF-β signaling pathway, by strongly down-regulating protein expression in the TGF-β-mediated SMAD pathway more effectively than in the ERK signaling pathway.

According to the findings, the results of this experiment indicate that the *P. indica* extract is effective in inhibiting the enlargement of the prostate by suppressing cell proliferation through the TGF-β signaling pathway (Figure 7). Furthermore, the screening process reveals the potential of the extract to inhibit cell proliferation induced by DHT, IL-17, and TGF-β, all significant factors in BPH. This implies that *P. indica* extract might target multiple mechanisms involved in BPH. This serves as a confirmation of the effects of the *P. indica* extract in inhibiting prostate enlargement. This discovery provides crucial in-depth information supporting the significant inhibitory effects of *P. indica* extract on the proliferation of prostate stromal cells. However, our findings have only been confirmed in *in vitro* cell experiments, necessitating further *in vivo* studies for clinical application. Additionally, there is a need to identify and study the specific active compounds responsible for the anti-proliferative effects of the *P. indica* leaf extract.

Conclusion

The evaluation of various medicinal plant extracts revealed that the leaf extract of *P. indica* displayed significant inhibition of cell proliferation induced by DHT, IL-17, and TGF-β, surpassing other extracts in terms of inhibitory activity. This study reaffirms the effect of *P. indica* extract in curtailing prostate proliferation by suppressing the PCNA protein and inducing cell cycle arrest at the G_0/G_1 phase through both canonical (SMAD2/3) and non-canonical (ERK1/2) pathways within the TGF-β signaling pathway. The standardization of the *P. indica* extract was achieved using chlorogenic acid. These findings offer crucial insights at the molecular level, indicating the promising potential of *P. indica* extract for the management of BPH.

Supplementary Material

The supplementary material can be downloaded using the DOI: 10.6084/m9.figshare.27627282

Funding

Not applicable.

Conflicts of Interest

Suchada Sukrong and Pasarapa Towiwat are the co-founders of Herb Guardian Co., Ltd. Yanisa Kanphet reports a relationship with Herb Guardian Co., Ltd., which includes funding grants. The remaining Authors declare no competing interests in relation to this study.

Authors' Contributions

Y.K.: Investigation, Methodology, Validation, Formal analysis, Writing original draft manuscript. TU: Methodology, Writing-review and editing. BV: Investigation, Methodology. TB: Writing Review and Editing. NN: Writing Review and Editing. SJ: Writing Review and Editing. PT: Resources, Data Curation, Supervision. SS: Conceptualization, Supervision, Methodology, Writing Review and Editing, Funding acquisition. The final manuscript was read and approved by all Authors.

Acknowledgements

The Authors would like to extend their gratitude to the Pharmaceutical Sciences and Technology Program (PST) for their support. Additionally, the Authors would like to thank the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, and the Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, for providing access to laboratory instruments. This research project is supported by the 90th Anniversary of Chulalongkorn University Scholarship and Herb Guardian Co., Ltd.

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