# Combination of the extract from Phellinus linteus with Smilax spp. attenuates colorectal and prostate cancers

**Chiratchaya Chongrak**
Chulalongkorn University
**Peerapat Visitchanakun**
Chulalongkorn University
**Prakaithip Somjit**
Chulalongkorn University
**Dhammika Leshan Wannigama Wannigama**
Yamagata Prefectural Central Hospital
**Suwasin Udomkarnjananun**
Chulalongkorn University
**Panomwat Amornphimoltham**
Mahidol University
**Asada Leelahavanichkul**
Chulalongkorn University
**Pattamawadee Yanatatsaneejit**
pattamawadee.y@chula.ac.th

Chulalongkorn University

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## Abstract

# Background

The present study investigated the effects of traditional Thai herbal remedies, specifically extracts from *Phellinus linteus* (PL) and a combination of PL with *Smilax corbularia* and *Smilax glabra* (PSS), on colorectal and prostate cancers. The use of natural medications for cancer therapy is becoming more widespread. To determine their anti-cancer potential, this work studied PL and PSS and evaluated their anti-cancer qualities both in vitro and in vivo.

# Methods

*In vitro* experiments were conducted using colorectal and prostate cancer cell lines and a kidney cell line. After treatment with PL and PSS, cytotoxicity was assessed using the MTT assay, while flow cytometry with propidium iodide and Annexin V staining was utilized to evaluate cell cycle arrest and apoptosis. RT-qPCR was performed to analyze the expression of cancer proliferation-associated genes, including *MDM2*, *KRAS*, and *MKI67*. The extracts' immune modulation effects were tested on TAMs induced by cancer-conditioned medium, measuring cytokine levels and macrophage polarization markers. Finally, an in vivo mouse xenograft model subcutaneously injected with HCT116 cells was used to determine the specific effects of the extracts on tumor volume.

# Results

*In vitro* analyses revealed that both PL and PSS exhibited cytotoxicity against the tested colorectal and prostate cancer cell lines, with PSS showing a stronger anticancer impact than PL. PSS effectively induced G2 cell cycle arrest in all colorectal cell lines and caused a low rate of apoptosis across the cancer cells. Both extracts reduced the expression of the *MDM2* across all cancer cell types and modulated TAMs by downregulating M2 polarization-associated genes while upregulating M1 polarization-associated genes. In the *in vivo* model, administration of PSS significantly attenuated tumor volume in mice injected with colorectal cancer cells.

# Conclusion

The PSS mixture successfully inhibited the proliferation of cancer cells in vitro through direct cytotoxicity and the modulation of TAMs. Only PSS demonstrated the ability to inhibit the growth of colon malignancies in a live mouse model. These findings suggest that the PSS holds potential clinical value as cancer therapy, though further investigation into its underlying mechanisms and pharmacokinetics is required.

# Introduction

Cancer is a leading cause of death worldwide, especially colorectal and prostate cancer (1), and there are a spectrum of interventions, including surgery, chemotherapy, hormonal treatment and targeted therapies, which are tailored to the stage and type of cancer (2). Since mortality rates and the treatment side effects of cancer are still high, several natural compounds have gained attention in the adjuvant therapy of cancer, with the aim of improving survival and reducing the side effects of the standard treatments (3). Herbal therapy is becoming increasingly popular as a complement to conventional cancer treatments. The information for the proposed approach is still preliminary, but overall, it is promising (4). Various phytochemicals are derived from medicinal plants such as curcumin, ellagic acid, EGCG, berberine, artemisinins, ginseng, gallic acid artesunate and beta-glucan, have been shown to have anticancer properties, including antiproliferative, antiangiogenic, proapoptotic, and antimetastatic effects. They also control autophagy, reverse drug resistance, improve immunity, and aid in chemotherapy development in both vitro and in vivo studies (5).

In Thailand, several herbs have traditionally been used in cancer treatment; for example, *Smilax* spp., especially *Smilax corbularia* (SC) and *S. glabra* (SG) (also referred to as catbriers, greenbriers, prickly ivy and smilaxes), are climbing flowering plants in tropical and subtropical regions that possess anticancer properties, mostly from phenolic compounds (6–9). Additionally, *Smilax* spp. are well-known herbs for the treatment of rheumatism, syphilis, diabetes mellitus, and cancer (9–11). On the other hand, *Phellinus linteus* (PL), a medicinal mushroom, is well-known in alternative medicine due to its anticancer effects, partly through the inhibition of cell proliferation and the induction of apoptosis of malignant cells (12, 13). Accordingly, (1→3)/(1→6)-β-glucan, the major cell wall component of mushrooms, has been demonstrated to exert an antitumor effect on several animal models (14, 15), possibly through the immune modulation of glucan on numerous immune cells (16, 17). While tumor-associated macrophages (TAMs) facilitate the proliferation of malignant cells (18), the administration of glucan reduces the beneficial effects of TAMs on cancer and enhances the tumoricidal activities of TAMs, partly through an alteration in cell energy status (14). Notably, the anticancer effect of herbal medicine might not only have a direct impact on the proliferation of malignant cells, but also on immune activation. Nevertheless, the *in vitro* assessment of herbal extracts on cancer mostly relies on the co-incubation of the substances with malignant cell lines (14, 19–22), which might not cover all possible mechanisms. *In vitro* experiments on immune cells, as well as *in vivo* studies, are necessary to determine the translational use of herbal extracts in cancer therapy.

Since *Phellinus*, an edible mushroom, is commonly found in China, Japan, South Korea and Thailand, and *Smilax* spp. plants are distributed throughout the eastern and south-eastern regions of Asia, the mixtures of PL, SC and SG (denoted as PSS) appear in several traditional Thai medicines. Recently, we demonstrated the direct anti-proliferative activity of PSS on several breast cancer cell lines, which might be useful as an additional treatment (12). Despite the obvious molecular differences between breast cancer, and colorectal and prostate cancer, the effect of PSS on cancer, at least in part, might not be specific to the cancer cell type. Due to the high incidence of colon and prostate cancer cases in the Thai

population (23, 24), PSS has been proposed as a potential herbal therapy for colon, colorectal and prostate cancer in some Thai patients with cancer. Given the potential advantages of PSS against these tumors, as well as the absence of *in vitro* and *in vivo* studies for PL and PSS in these cancer cell types, the present study investigated the impact of PSS in both *in vitro* and *in vivo* experiments. We also addressed the mechanisms involved in cytotoxicity against these cancer cells, such as anti-proliferation through cell cycle arrest, apoptosis and gene-related cell proliferation. In addition, it was hypothesized that the combination of PL and PSS may be effective in the attenuation of malignancy, partly through an impact on TAMs.

# Material and methods

*Cell culture*. Three colorectal cancer cell lines (HCT116, SW620, and HT-29), one prostate cancer cell line (PC3), and one kidney cell line (HEK 293) were purchased from the American Type Culture Collection (ATCC, VA, USA), and short tandem repeat profiling was performed for authentication. These cell lines were cultured in Gibco® Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% Gibco fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% Gibco antibiotic-antimycotic (Thermo Fisher Scientific), and were maintained in a humidified atmosphere with 5% $CO_2$ at 37˚C.

*Preparation of PL and PSS extracts.* All *in vitro* procedures were carried out as previously reported (10). In brief, for PSS, crude powders from the fruiting body of PL, and the rhizomes of SC and SG (Herb for You and Nature Herbs International Holding Company, Bangkok, Thailand) at a ratio of 3:1:1 (PL:SC:SG) were extracted using distilled water with a solid-to-liquid ratio of 1:50 in a Soxhlet apparatus at 80˚C for 5 h, after which they were filtered (Whatman No. 1 paper) and oven-dried at 70˚C overnight. The ratio of PL:SC:SG in the PSS combination employed in the present study was based on a traditional Thai recipe, and the concentrations of PL and PSS were determined by a recent publication indicating the advantages of both drugs in breast cancer (10). The extraction yield was calculated using the following formula: Yield (g/g) = extract obtained after evaporation (g)/dry weight (g).

*MTT assay.* The MTT method was used to assess the cytotoxicity of the extracted samples against each cell line. In brief, all cell lines ($5x10^3$ cells) were plated in 96-well plates and incubated at 37˚C for 24 h. The cells were then treated with PSS and PL at various dosages (75–650 µg/ml and 250-3,750 µg/ml, respectively) for 48 h in colorectal cancer cell lines and a kidney cell line, and for 72 h in the prostate cancer cell line. Untreated cells were used as a negative control. Cisplatin (Sigma-Aldrich) was supplied as a positive control at doses ranging between 2.12 and 4.08 µg/ml. After 48 h or 72 h of treatment, the MTT assay was employed to determine the half-maximal inhibitory concentration ($IC_{50}$). Briefly, after removing the extract medium, each well was incubated with 5 mg/ml MTT dissolved in fresh medium at 37˚C for 3 h. After discarding the solution, DMSO was added and incubated at 27˚C for 5 min. Absorbance was determined using a spectrophotometer at 492 nm and a reference wavelength of 630 nm for all samples. The inhibition percentages for untreated, PSS-, PL- and cisplatin-treated groups were

compared, and presented as percentages of the untreated control. The results of the MTT assay ($IC_{50}$-dependent) were applied to conduct further cell proliferation and apoptosis assays.

*Cell cycle analysis.* Flow cytometric analysis with propidium iodide (PI; DNA staining) was performed using colon and prostate cancer cells ($5x10^5$ cells/well) treated with PSS, PL and cisplatin ($IC_{50}$ values from the MTT experiment) for 48 and 72 h, respectively. Cells were trypsinized, fixed with cold 70% ethanol at 4˚C overnight and centrifuged at 14,000 rpm for 15 min, after which, 50 µl of a 100 µg/ml stock of RNase (Sigma-Aldrich) was added and the cells were stained with PI solution (BD Pharmingen, CA, USA) before analysis using a flow cytometer. Cell cycle arrest at $G_0/G_1$, S and $G_2$ phases was evaluated.

*Apoptosis assay.* The Annexin V-DY-634/PI apoptosis detection kit (Abcam, Cambridge, UK) was utilized to assess apoptosis. After seeding and treating the cells with the $IC_{50}$ values of PSS, PL and cisplatin as aforementioned, the cells were collected and washed twice with PBS. The cells were then stained with DY-634 and PI, as per the manufacturer's instructions. Then, a flow cytometric analysis was performed. Early and late apoptosis, and necrosis were determined by the low and high levels of Annexin V and PI; high levels of Annexin V and low levels of PI indicated early apoptosis, high levels of both Annexin V and PI indicated late apoptosis, and low levels of Annexin V and high levels of PI indicated necrosis.

*Gene expression analysis.* The colon, colorectal and prostate cancer cells that had been treated for 48 or 72 h were trypsinized, and RNA extraction was performed using Invitrogen® TRIzol™ (Thermo Fisher Scientific). Subsequently, 750-1,000 ng total RNA from each sample was used to synthesize cDNA using a cDNA synthesis kit (Biotechrabbit, Hennigsdorf, Germany), as instructed. The cDNA underwent reverse transcription-quantitative PCR (RT-qPCR) using 4X CAPITAL™ qPCR Green Master Mix (Biotechrabbit) with forward and reverse candidate gene primers, as shown in Table I. *GAPDH* served as an internal control (25–28). qPCR amplification was performed using a QuanStudio™ 5 Real-time PCR System (Thermo Fisher Scientific) with the following thermocycling conditions: 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 45 sec. The $2^{-\Delta\Delta Cq}$ technique (29) was used to compare gene expression changes between mixture- or PL extract-treated and untreated cells.

*Macrophage experiments.* Macrophages were derived from human monocytoid THP-1 cells (TIB-202; ATCC) in Roswell Park Memorial Institute medium (RPMI; Thermo Fisher Scientific) supplemented with 10% FBS, 1% Gibco antibiotic-antimycotic and 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich). The induction of TAMs was performed as previously described (14, 30, 31). Because of the effects of secreted chemicals from cancer cells on macrophages, such as succinate, osteopontin and other metabolic byproducts, macrophages recruited to cancer microenvironments are transformed into TAMs (18). As a result, the use of tumor-conditioned medium containing a number of excreted compounds to convert macrophages into TAMs *in vitro* is well established (14, 32). To prepare the conditioned medium for cancer cells (HCT116), $3x10^6$ cells were incubated in 9 ml modified DMEM for 72 h, centrifuged at 1,500 rpm for 10 min at 4˚C and filtered through 0.22-µm filters. Macrophages ($1x10^6$ cells/well) were

treated with 500 µl/well HCT116 conditioned medium for 24 h before being removed with a pipette, rinsed with PBS and cultured in standard DMEM.

TAMs were incorporated into extracts (PL or PSS) or DMEM (control) for 24 h. Cytokines (TNF-α, IL-6 and IL-10) in the supernatants were measured using ELISAs (Invitrogen; Thermo Fisher Scientific) and gene expression was measured using RT-qPCR. The RT-qPCR protocol was the same as that described in earlier publications (33–36) using the primers listed in Table I. Additionally, the tumoricidal activity of macrophages was evaluated using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labeled cancer cells, according to a previously established procedure (14). CFDA-SE (20 µM) in PBS was used to treat $1x10^5$ HCT116 cells for 30 min at 37˚C, according to the manufacturer's protocol. Passively diffused CFDA-SE in the cytoplasm was cleaved by intracellular esterase, resulting in fluorescence activity. CFDA-SE-labeled HCT116 cells ($1x10^5$ cells/well) were then incubated with TAMs ($1x10^5$ cells/well) with and without extracts (PL or PSS at $IC_{50}$ values) for 72 h before being fixed with 2% paraformaldehyde and the nuclei stained with 4', 6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). The fluorescence intensity was then calculated using ImageJ [National Institutes of Health (NIH), Maryland, USA].

*Animal and tumor xenograft model.* The study protocol was approved by the Faculty of Medicine's Institutional Animal Care and Use Committee at Chulalongkorn University (Bangkok, Thailand; ASP SST 018/2563), which followed the NIH in the United States. Male nude BALB/cAJcl-nu mice aged 8 weeks were obtained from Nomura Siam International Co., Ltd. (Bangkok, Thailand). The mice were housed at a specific pathogen-free mouse facility in a controlled environment, with a temperature of 24 ± 2˚C, 50% relative humidity and a 12-h light-dark cycle, with light provided from 7:00 a.m. to 7:00 p.m. Additionally, 5 mice were housed in cages with corncob bedding, which was autoclaved before use. The mice had continuous access to a standard sterile diet and were provided autoclaved tap water from a bottle placed within the cage. To assess the *in vivo* effects of PL and PSS, a human colorectal cancer cell line (HCT116) was used as a representative tumor. HCT116 cells ($1x10^5$) in 100 µl DMEM supplemented with 10% FBS were subcutaneously injected into the mice, with or without daily intralesional injections of extracts (PL or PSS at 2 mg in 200 µl) or normal saline (control group), which started 2 weeks after cancer inoculation. Total of 15 mice were used for subcutaneous cancer injection, with 5 mice for control and 5 mice per group for PL and PSS. The human endpoints include i) any one of severe tumor characteristics (higher than 4,000 $mm^3$ of tumor volume, ulcerated tumor, interference with eating or ambulation, and metastasis), ii) weight loss more than 20%, and iii) moribund appearance (labored breathing, loss of righting reflex, lethargy, ascites, and severe diarrhea). However, no mice reached the human endpoint, and all mice were observed for 30 days. For anesthesia during the subcutaneous injection of tumor cells, 3% isoflurane was adjusted to 0.5–0.75% v/v with an oxygen-nitrous oxide mixture (VetFlo™ Isoflurane Vaporizers) (Kent Scientific, Torrington, CT, USA) during the injection procedure. Notably, 3% isoflurane was released into the induction box to initiate the anesthetic process (anesthesia induction) and maintain by diluting isoflurane into 0.5–0.75% using the machine during the injection process (anesthesia maintenance). During the short anesthesia for cancer injection, the mice

were placed on a temperature control bed (37°C) and checked for the depth of anesthesia (righting and palpebral reflexes, muscular tone, and respiratory rate) before the injection. The tumor volume ($mm^3$) was determined using the following equation: (length x width x width)/2 (37). The control groups received subcutaneous injection of the same volume of autoclaved $dH_2O$. Tumor measurement, as well as routine health examinations of the mice, including assessment of body weight, was performed every 3 days. All of the mice were sacrificed through cardiac puncture under isoflurane anesthesia (the induction and maintenance process) at 3 weeks after intralesional administration. At sacrifice, mice were placed in the induction chamber connected to an isoflurane vaporizer (Kent Scientific, Torrington, CT, USA) before maintenance by 3–5% isoflurane in oxygen (flow rate 1–3 L/min). The mice were unconscious without a righting reflex with relaxing muscle tone before cardiac puncture followed by cervical dislocation to ensure the process. Then, mouse samples were collected post-mortem. Moribund mice, as indicated by the presentation of severe pain, severe distress or suffering conditions, including infection, chronic inflammation, loss of mobility, abnormal resting posture, losing > 20% of their weight or a tumor diameter of > 2 cm, were considered humane endpoints, after which the mice were sacrificed by cardiac puncture under isoflurane anesthesia.

*Statistical analysis.* All data were subjected to statistical analysis using the Statistical Package for Social Sciences software (SPSS 22.0; SPSS Inc., Chicago, IL, USA) and Graph Pad Prism version 10.0 software (Dotmatics, La Jolla, CA, USA). The results are presented as the mean ± standard error. Statistical significance between groups was assessed using unpaired t-test for two-group comparisons, or one-way analysis of variance (ANOVA) with Tukey's test for multiple-group comparisons. The time-point experiments were analyzed using repeated measures ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

# Results

*Impacts of PL and PSS extracts against cancer cell lines.* The effects of PSS and PL extracts were compared to cisplatin (a conventional treatment) on colorectal cancer (HCT116 and SW620, and HT-29), prostate cancer (PC3), and HEK 293 cell lines, particularly regarding cytotoxicity, cell cycle arrest, and cell death. The MTT assay was used to assess cytotoxicity by measuring cell viability following treatment with the extracts and cisplatin in a dose-dependent manner, in order to ascertain the most suitable conditions for subsequent investigations. Table II shows the estimated $IC_{50}$ values for the extracts and cisplatin against all cell lines, as calculated using a linear equation of inhibitory concentrations. As a result, cisplatin revealed cytotoxicity against all tumor cell lines and kidney cell lines at the lowest concentration, followed by PSS, whereas PL showed cytotoxicity only at high dosages (Fig. 1A-E). In terms of PSS, the results showed that it was the most dangerous to SW620 cells, and the least detrimental to HEK 293 cells (Fig. 1E).

To evaluate cancer cell cycle arrest following treatment with $IC_{50}$ doses of PSS, PL and cisplatin, PI staining and flow cytometry were used to determine the DNA content of each cell line. Cell cycle arrest is a halting point in the cell cycle that signifies that the cell is unable to continue dividing. The results

revealed that cisplatin caused an accumulation of all cancer cells in the $G_2$ phase, as well as a decrease in the accumulation of all cancer cell lines in the $G_0/G_1$ and S phases when compared to the untreated control group. Meanwhile, PSS induced $G_2$ cell cycle arrest in all colorectal cell lines, but not in PC3 prostate cancer cells, whereas PL induced $G_2$ cell cycle arrest in just HT-29 and SW620 cells. All agents (cisplatin, PSS and PL) resulted in no accumulation of cells in the $G_0/G_1$ and S phases (Fig. 2). Cisplatin was shown to be the most efficient therapy in producing early apoptosis in all cancer cell lines (10–20%), whereas PL and PSS resulted in ~ 5% HT-29 cell apoptosis. Meanwhile, PL, but not PSS, resulted in 1–2% early PC3 cell apoptosis. Regarding the late apoptosis in all cancer cell lines, with cisplatin being the most powerful inducer in HCT116 and SW620 cells. Cisplatin and PL both triggered late apoptosis in HT-29 cells at equal rates. Only cisplatin and PL induced late apoptosis in the PC3 cell line, with cisplatin being more effective than PL. Across all groups, < 5% of HCT116 cells treated with cisplatin displayed necrosis (Fig. 3). As a result, PL and PSS had less cytotoxic action on cancer cells than cisplatin.

Unlike cisplatin, which has well-known cytotoxic mechanisms through nuclear DNA binding and subsequent interference with transcription and DNA replication (38), the mechanisms of PL and PSS against malignant cells remain unknown and warrant further exploration. Accordingly, to explore the possible mechanisms of PL and PSS against these cancer cells, the expression of several genes associated with cancer cell death and cell proliferation, including murine double minute 2 (*MDM2*), Kirsten rat sarcoma virus (*KRAS*), and marker of proliferation Ki-67 (*MKI67*), was measured by RT-qPCR. Both PL and PSS lowered *MDM2* expression across all cancer cell types, *MKI67* expression in HCT116, HT29, and PC3, and *KRAS* expression in HT-29 and PC3. Only PSS decreased *MKI 67* expression, but PL marginally enhanced it in SW620. Unexpectedly, both PL and PSS boosted *KRAS* expression in HCT116. In SW620, only PL increased *KRAS*, but PSS had no effect on its expression (Fig. 4).

*Influences of PL and PSS extracts on TAMs.* The impacts of the extracts on cancer are possibly not only caused by cell cytotoxicity, but may also be due to the regulation of TAMs, which are immune cells facilitating tumor growth. In the present study, supernatants from a colorectal cancer cell line (HCT116) were used to induce TAMs, whereas macrophages in control culture medium were used as naïve macrophages (M0). Regarding cytokines, TAMs upregulated IL-6 and IL-10 levels, and downregulated TNF-α when compared with M0 macrophages (Fig. 5A-C), supporting the role of IL-6 and IL-10 elevation, and TNF-α downregulation in cancer promotion (39, 40). Treatment with both extracts downregulated the levels of all of these cytokines (TNF-α, IL-6 and IL-10) (Fig. 5A-C), which might have an anticancer effect (reduced IL-6 and IL-10) or could induce cancer promotion (decreased TNF-α). Regarding macrophage polarization, treatment with the cancer cell line supernatant downregulated genes associated with M1 pro-inflammatory polarization (IL-1β and iNOS) and upregulated genes associated with M2 polarization (Fizz-1, TGF-β and Arg-1) (Fig. 5F-H), thus supporting the M2-like condition of TAMs (41). In comparison with TAMs, both extracts altered the characteristics of TAMs, as indicated by elevated IL-1β and iNOS levels, and reduced Fizz-1 and TGF-β expression (Fig. 5D-G). However, only PSS, but not PL, downregulated Arg-1 expression (Fig. 5H) and effectively reduced the abundance of cancer cells after co-incubation with CSFE-stained cancer cells, as determined by the ratio of CFSE/DAPI fluorescence

intensity (Fig. 5I and J). Notably, incubation of CSFE-stained cancer cells with M0 macrophages showed no difference compared with TAMs (Fig. 5I and J), despite the theoretical enhancing effect of TAMs on cancer growth (42).

*Impacts of PL and PSS extracts on a mouse colorectal cancer (HCT116) xenograft model*. Because the extracts demonstrated some beneficial effects on cancer cell lines, especially colorectal cancer, the HCT116 colorectal cancer cell line was selected as a representative cell line for use in a mouse model of subcutaneous cancer injection (Fig. 6A). Notably, subcutaneous injection of HCT116 cells caused the generation of visible tumors in all mice, without alterations in body weight, as indicated in the positive control group (HCT116 cell injection with subcutaneous injection of sterile water) (Fig. 6B-E). Subsequently, the same dose of PL and PSS was administered in mice, with the maximum concentration that can be prepared into an injectable solution used (2 mg in 200 µl). Due to limitations of the solubility of the extracts, the prepared concentration of PL was 4-fold lower than the $IC_{50}$ value of PL, whereas the concentration of the injectable PSS was equal to the $IC_{50}$ value of PSS (Table II). Although there was no alteration in mouse body weights (Fig. 6B), the administration of PSS, but not PL, attenuated tumor volume, despite the fact that injection was performed 2 weeks after cancer injection (Fig. 6C-E).

# Discussion

In the present study, three colorectal cancer cell lines and one prostate cancer cell line were tested. Among the three colorectal cancer cell lines, HCT116 is a highly aggressive cell line without the capacity to differentiate into enterocytes, whereas SW620 cells show resistance to glutamine-based targeting treatment, and HT-29 has an intermediate capacity to differentiate into enterocytes, is sensitive to glutamine-based treatment, and demonstrates AMP-activated protein kinase activation (43, 44). Hence, these three colorectal cancer cell lines represent different characteristics of colon cancer. In parallel, the most commonly used cell lines for prostate cancer research are derived from different metastatic sites; for example, lymph nodes (LNCaP cells), vertebrae (PC3), and brain (DU145) (45). Because androgen receptor-positive prostate cancer can be partially treated with hormone therapies, novel additional therapies may be useful for the treatment of androgen receptor-negative cancer (PC3 and DU145) (46). Subsequently, PC3 was selected as a representative androgen receptor-negative prostate cancer cell line in this study. The impacts of herbal extracts, including PL and PSS, were assessed on both cancer cell lines and mice, as a proof of concept of using the *in vitro* test to select the use of adjuvant cancer therapy *in vivo*.

Both PL and PSS are Thai remedies used for the treatment of several diseases, which might have value in modern medicine, as previously mentioned in breast cancer (12). While SC and SG are climbing plants, PL is a mushroom that predominantly contains polysaccharides, especially β-glucan, with anti-proliferation and immune modulation properties (14). However, PL not only contains β-glucan, but also consists of several phenolic compounds that possibly synergistically attenuate cancer (47), as demonstrated from LC/QTOF-MS chromatographic peaks (10). As such, most of the possible active compounds in PL and PSS are similar. In PL and PSS, there are several substances with previously

mentioned anticancer properties; for example, iridin, ethyl caffeate, sparteine, matrine, formononetin, rhoifolin, rosmanol and angelol (12). Only four compounds, including ethylcamptothecin, octadecanoic acid, methocyindole acetic acid and brevicarine have been found in PSS but not in PL, representing the substances from SC and SG in the PSS mixture. Camptothecin is a quinolone alkaloid that has been used as a chemotherapeutic agent in the treatment of leukemia (48). Meanwhile, fewer mentions have been made regarding the anticancer effects of octadecanoic acid (18-carbon chain saturated fatty acid, which is referred to as stearic acid) and indole-3-acetic acid (the most common plant hormone of the auxin class that regulates various aspects of plant growth and development) (49, 50). In the present study, the PSS and PL extracts exhibited cytotoxicity against colorectal and prostate cancer cells. Extracts are harmful to various cancer cell lines. This finding is consistent with the research conducted by Florento et al., which discovered that there were varying concentrations of anticancer drugs in cancer cell lines (51). Cancer cells have different genetic characteristics from normal cells, and they also vary among cancer cell types. Different cell lines have diverse histological and genetic properties, which result in a response to varying extract doses. Many studies have demonstrated that the drug sensitivity of cancer cells is dependent on alterations to the genome, such as point mutations, copy number changes, and epigenetics (52, 53). Herbal medicines exert distinct anti-cancer effects on different cell types due to the influence of mutations on aberrant signaling pathways that can contribute to cancer. Our findings revealed that PSS was more toxic in HCT116 ($IC_{50}$=159 ug/mL) and SW620 ($IC_{50}$=150 ug/mL) than in HT29 ($IC_{50}$=517 ug/mL) and PC3 ($IC_{50}$=259 ug/mL). However, PSS's cytotoxic effect on each cell line did not correlate with molecular levels detected for *MDM2*, *KRAS*, and *MKI67*. *MDM2* expression was the most promising gene associated with cytotoxicity. *MDM2* expression in cells treated with PSS, compared to untreated cells, was reduced in HCT116 and SW620. Considering cytotoxicity, PSS exhibited toxicity toward HCT116 and SW620. This may suggest a possible correlation between the cytotoxicity of PSS and the downregulation of *MDM2* expression in both cell types. At the molecular level, HCT116 cells do not harbor a p53 mutation, whereas SW620 cells do. Despite this difference, both cell lines yielded comparable cytotoxicity results and demonstrated a similar reduction in *MDM2* gene expression. This reflects that PSS possesses a versatile, and highly efficient mechanism of action that is not restricted solely to the conventional p53-dependent pathway. In SW620 with a p53 mutation, a reduction in *MDM2* in this specific cell line points directly toward p53-independent mechanisms of tumor suppression. The capacity of MDM2 to directly bind to the RB protein, resulting in driving cell cycle progression, is one example of a p53-independent mechanism. In addition, MDM2 can activate NF-κB, increasing the production of anti-apoptotic genes. In contrast, *MDM2* expression in PC3 following PSS treatment was reduced less than in HT29, whereas PSS is more cytotoxic in PC3 than in HT29. The contrasting results observed between PC3 and HT29 cell lines highlight the multi-target nature of the PSS extract and the complexity of cell-type-specific responses. Although PSS treatment resulted in a more pronounced downregulation of *MDM2* expression in HT29 compared to PC3, the cytotoxicity was paradoxically lower in HT29. This suggests that while *MDM2* suppression is a prominent molecular event in HT29, it is not solely sufficient to induce massive cell death, possibly due to intrinsic compensatory survival pathways robustly expressed in this specific cell line. Conversely, the higher cytotoxicity observed in PC3, despite a modest reduction in *MDM2*, implies that the phytochemical constituents of

PSS likely activate alternative, highly lethal p53/MDM2-independent apoptotic pathways in these cells. Although SW620 and HT29 share the same p53 mutation, their response to PSS is different. This might be because both cells possess distinctly different driver mutations, which directly impact their survival potential under cellular stress. Therefore, the anti-cancer efficacy of PSS is not uniformly dependent on *MDM2* downregulation but rather hinges on the unique genetic background and molecular vulnerabilities of each cancer cell type. *KRAS* expression was the most unexpected finding. Its expression was reduced in HT29 and PC3 following PSS treatment compared to untreated cells; however, it increased in HCT116 and remained unchanged in SW620 after PSS treatment, in contrast to PSS's cytotoxicity against these cell lines. The unexpected effects of *KRAS* in HCT116 and SW620 may be due to the fact that both cell lines are *KRAS* mutants. HCT116 harbors a mutation at codon 13 (G13D), while SW620 has a mutation at codon 12 (G12V). These mutations result in the KRAS protein being in a constitutively active state. Mutated genes often possess impaired feedback regulation mechanisms, making it difficult for natural extracts or various drugs to easily suppress their transcription or reduce their levels. Even if the PSS extract cannot down-regulate the expression of *KRAS*, this does not imply that the substance is non-toxic to the cells. Phytochemicals within the extract can attack cancer cells through alternative pathways that operate independently of *KRAS* regulation. The increased expression of *KRAS* in HCT116 cells upon treatment with PSS is likely a compensatory survival response. When the cell detects toxicity or stress induced by the extract, it attempts to stimulate survival signals through its primary oncogene (which is *KRAS*), forcing it to overexpress in order to resist death. Ultimately, however, if the biochemical damage caused by PSS is severe enough, the signals from KRAS will be unable to withstand it, and the cell will inevitably undergo cell death. *MKI67* expression was lower in all cancer cell lines following PSS treatment compared to untreated. Its expression was the strongest decrease in HT29 cells, contradicting PSS's cytotoxicity towards the cells, since PSS had the lowest cytotoxicity against HT29. PSS may cause cellular senescence, in which cells irreversibly lose their proliferative potential (causing MKI67 to drop to very low levels), although these cells can survive and remain resistant to cytotoxic medicines. The findings differed from our prior breast cancer study, which found reduced expression of *MDM2*, *KRAS*, and *MKI67* in breast cancer cell lines treated with PSS compared to untreated cells (12). These data suggest that PSS affects distinct cells with diverse genetic backgrounds. However, the molecular-level effect of PSS in this study remains uncertain; only three genes were identified, which may be insufficient. To identify the genes affected by PSS, it is preferable to examine the transcriptome.

In this study, HEK293 represents normal kidney cells. Although HEK 293 is not a good representation of normal cells because it is an immortal human embryonic kidney cell, it is not cancerous. The findings suggested that PSS was more deleterious to HEK293 than HT29. It is possible to conclude that PSS is not appropriate for the treatment of HT29. Taken together, it is crucial to recognize that this herbal medicine is not suitable for all individuals if it is to be used in the clinic.

PSS had a stronger anticancer impact than PL due to its lower $IC_{50}$ value. PSS was the least harmful to HEK 293 cells, indicating that it has little effect on kidney cells. However, cisplatin proved to be the most potent anticancer agent. Cisplatin is more successful than PSS in cancer treatment, although it has

greater side effects. It would be interesting to investigate the combination of PSS with cisplatin to reduce the use of cisplatin and its adverse effects while maintaining anticancer activity.

Following exploration of the cell cycle, the present study showed that cisplatin was the most effective agent in inducing cancer cell toxicity through $G_2$ arrest, followed by PSS and PL, respectively. Notably, none of the tested agents induced the accumulation of cells in $G_0/G_1$ and S phases, indicating that $G_2$ phase interference may be an underlying mechanism. Because of the well-known association between cell cycle arrest and apoptosis (54), it is not surprising that cisplatin strongly induced cell death (early apoptosis, late apoptosis and necrosis). The present study demonstrated that cisplatin was more effective in inhibiting cancer cell proliferation than PSS and PL. PSS could induce apoptosis in cancer cells at a low rate (< 5%), indicating that the potential of the extracted samples to induce apoptosis in these cancer cell lines was low.

The anticancer effect is not only dependent on direct cancer cytotoxicity, but may also be due to an impact on immunity. As such, the effects of both PSS and PL on TAMs, the M2-like polarization of macrophages that can enhance tumor growth, and cytokine responses were also tested. Because TNF-α from macrophages might induce death processes in malignant cells (55), the downregulation of TNF-α in naive M0 macrophages in response to cancer cell conditioned medium might enhance tumor growth, thus supporting the role of TAMs in cancer (18). Likewise, the upregulated expression of IL-6 and IL-10 genes in TAMs might be beneficial for cancer, as IL-6 and IL-10 promote cancer cell proliferation and inhibit cancer cell apoptosis (40, 56). Notably, PL and PSS downregulated the expression levels of IL-6 and IL-10, which might have a beneficial anticancer effect; however, the extracts also demonstrated a possible role in cancer promotion through the downregulation of TNF-α. On the other hand, the HCT116 colorectal cancer supernatant induced M2-like characteristics, as indicated by the upregulation of several genes associated with M2 polarization (Fizz-1, TGF-β and Arg-1) and the reduced characteristics of M1 polarization (IL-1β and iNOS levels). Meanwhile, both PL and PSS similarly downregulated these M2 polarization-associated genes and upregulated the M1 polarization-associated genes (IL-1β and iNOS), which might be beneficial in patients with numerous types of cancer (42). Hence, PL and PSS demonstrated several antitumor effects, as indicated by downregulation of IL-6 and IL-10, and the reduction in the direction of M2 polarization; however, they may also have had a tumor-promoting impact through the downregulation of TNF-α. Nevertheless, as determined in the CFSE-stained tumor cell experiments, only PSS, not PL, demonstrated an antitumor effect and reduced the abundance of the tumor cells, implying that the downregulation of TNF-α had a negligible effect on tumor promotion in this situation. Notably, PL did not exert an antitumor effect in the CFSE-stained tumor cell experiments, despite the similar impacts of PL and PSS on TAMs. Since Arg-1 expression was higher in TAMs after activation by PL compared with PSS, the reduced Arg-1 expression might be responsible for the greater antitumor effect of PSS over PL. Notably, the incubation of TAMs with the CFSE-stained tumor cells did not demonstrate any beneficial effects on the cancer cells different from the well-known benefits of TAMs in cancer (18), suggesting a limitation of this experimental system. More mechanistic tests using other *in vitro* experiments may be interesting. The results of the *in vitro* macrophage experiments

indicated that both PL and PSS might be candidates for additional anticancer therapies, and PSS demonstrated a more potent beneficial impact on some parameters. Hence, the different potencies between PSS and PL underscore the importance of doses and the intrinsic characteristics of the extracts. Furthermore, the higher $IC_{50}$ value of PL, which may result in difficulties in the preparation of the extract in a soluble form, implies a possible limitation of the *in vivo* use of PL. In the present study, the HCT116 colorectal cancer cell line was selected as a representative cell line for the *in vivo* experiment and only PSS, not PL, attenuated the tumors in mice. Indeed, the lack of anticancer properties of PL in mice might partly be due to the use of too low a concentration of PL *in vivo* due to the limited solubility of the high concentration of PL. Additionally, the benefits of PSS (PL + *Smilax* spp.) over PL alone might partly be due to the major compound of *Smilax* spp., especially camptothecin substances, which are not a component of PL. More studies on camptothecin against colon cancer may be interesting.

Several limitations also need to be mentioned. First, different parts of the herbs might provide different bioactive agents (57), and the various solvents used in herbal preparation may be associated with the active ingredients (58). For example, the ethanol extracts of PL indicate strong antioxidant activity (59), whereas the aqueous PL extracts demonstrate more potent inhibitory effects on angiogenesis (60). The use of other solvents might demonstrate different effects on cancer. Second, the *in vivo* test (subcutaneous cancer cell injection) was performed only with HCT116 cells, and the combination of these extracts with standard chemotherapy was not tested. Also, the lack of animal experiments with PC3 cells is a limitation of the present study. Because of the metastatic characteristics of PC3 (a prostate cancer cell line) (61), the use of intralesional PL and PSS is not appropriate, and the development of proper drug delivery to different sites of metastasis is needed. More studies are required for the translation of the present findings into patient treatment. Third, the extracts were tested only in THP-1 cells (monocytes isolated from the peripheral blood of a patient with acute monocytic leukemia), which is a cancer cell line, not a normal cell line. In addition, the ratio of tumor cells to macrophages used was ~ 1:3 followed our previous protocol (14); the results might be different with the different concentrations of the extract with various ratios between the malignant cells and TAM. Tests on primary normal noncancerous macrophages directly extracted from volunteers and the non-cancerous from organs (colons and prostate glands) might also have different results. Fourth, the mechanistic exploration was performed only regarding RNA expression, not at the protein level, and the extracts might affect cancer cells through direct cytotoxicity and indirect TAM inhibition. More studies aiming to explore the mechanisms, not only the proof of concept, will be interesting. Thus, the present results were only a proof of concept to use the PSS extract, adapted from a Thai remedy, in cancer. The concentrations of PSS and PL employed in the current study are lower than those that could be obtained through herbal therapies.

Administration of the extract through other routes, including oral and intravenous administration, a concentration higher than the $IC_{50}$ value reported from this in vitro research is necessary. This is dependent on the pharmacokinetics of both extracts, which require more research to provide information for future therapy applications. Although the use of these extracts might not be ready to use as herbal

oral therapy or tea-liked supplement, our proof-of-concept results supported a possible tumoricidal effect from these herbs derived from the Thai traditional medicine.

# Conclusion

In summary, the cytotoxicity of PSS and PL has been proven against a variety of cancer cell lines. Notably, in response to PSS, the inhibition of cell proliferation was more significant than the effects on cell apoptosis. The immune modulation effects on TAMs were also demonstrated, and the effects of PSS were more pronounced than those of PL. In addition, PSS inhibited the growth of colon malignancies in rodents as proof of concept. Future assessment of the underlying mechanisms, and the use of PSS and PL in other malignancies may be intriguing.

# Abbreviations

PSS Mixture extract of *P.linteus*, *S. corbularia* and *S. glabra*

PL *P.linteus* extract

SC *S. corbularia*

SG *S. glabra*

TAMs tumor-associated macrophages

# Declarations

### Ethics approval and consent to participate

The study protocol was approved by the Faculty of Medicine's Institutional Animal Care and Use Committee at Chulalongkorn University (Bangkok, Thailand; ASP SST 018/2563).

### Consent for publication

Not applicable.

### Availability of data and materials

The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

### Competing Interests

All authors declare that they have no conflicts of interest.

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**Authors' contributions**

CC conducted the experiments, analyzed the data and drafted the original manuscript. PV, PS, DL, SU, and PA conducted the experiments and analyzed the data. PY and AL designed the study, supervised the study, wrote the proposal for grants, and revised the manuscript. All authors have read and approved the final manuscript.

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# Tables

Tables 1 and 2 are available in the supplementary files section

# Figures

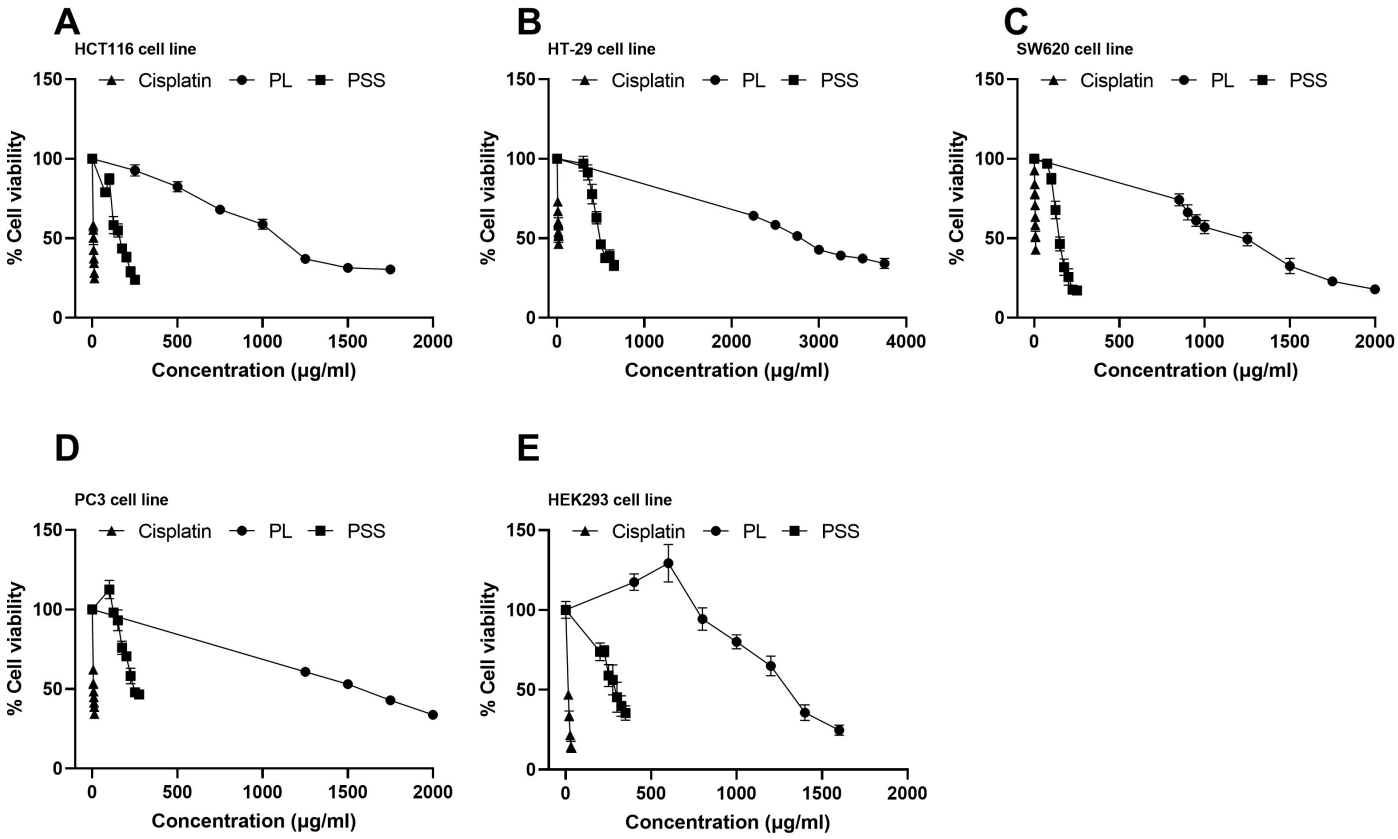
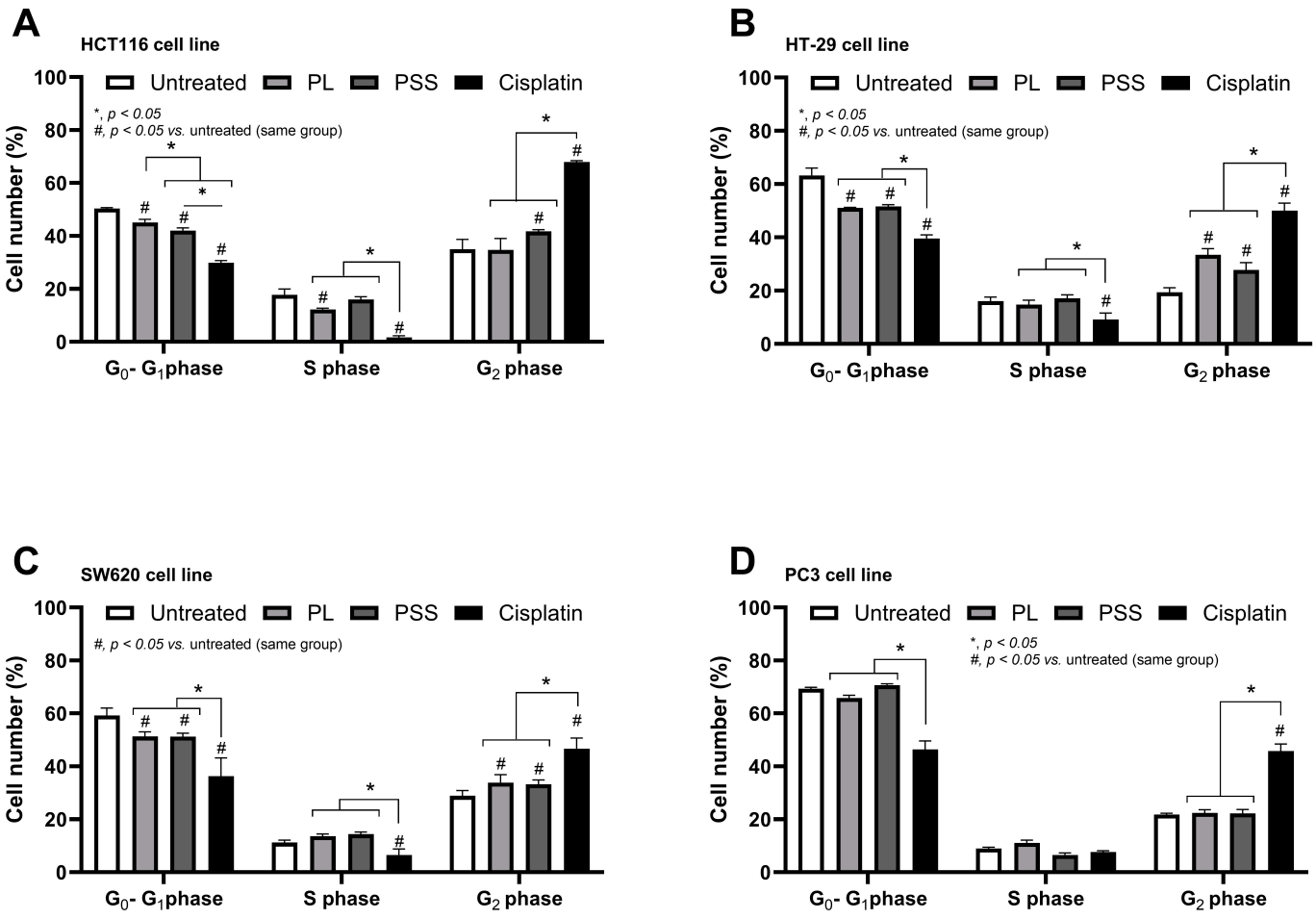


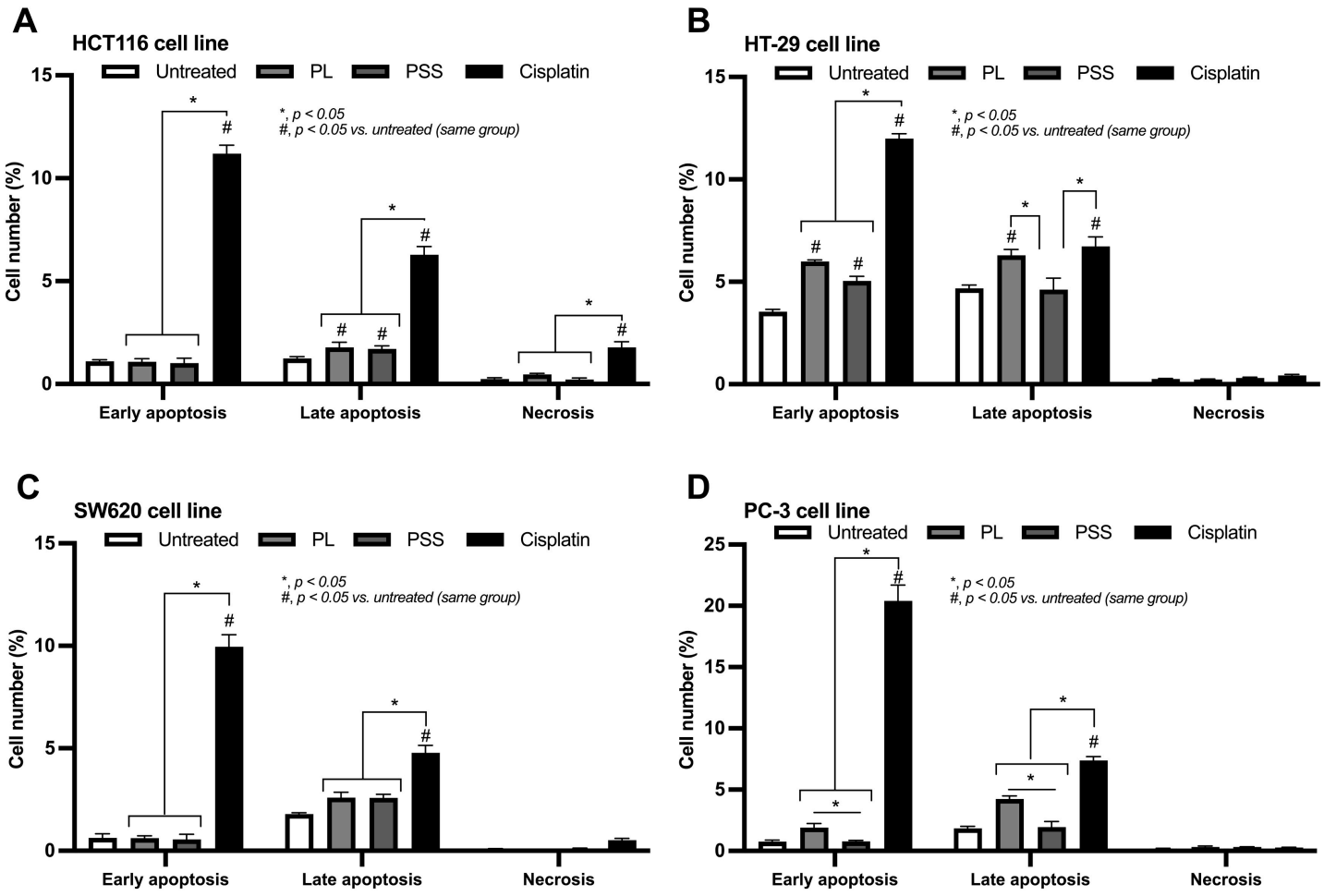

**Figure 1**

Characteristics of the impacts of compounds from PL and PSS mixture and Cisplatin against colorectal cancer cell lines, including HCT116 (A), HT-29 (B), and SW620 (C) prostate cancer cell line PC3 (D), and PSS against kidney cell line HEK293 (E) as indicated by the dose-dependent cytotoxicity. *, $p < 0.05$ between the indicated groups; #, $p < 0.05$ vs. the untreated.




## Figure 2

Characteristics of the impact of compounds from PL and PSS mixture (PL+ Smilax corbularia + Smilax glabra at the ratio 3:1:1) and Cisplatin against colorectal cancer cell lines, including HCT116 (A), HT-29 (B), and SW620 (C), and prostate cancer cell line PC3 (D) as indicated by cell cycle arrest. *, $p < 0.05$ between the indicated groups; #, $p < 0.05$ vs. the untreated




## Figure 3

Characteristics of the impact of compounds from PL and PSS mixture (PL+ Smilax corbularia + Smilax glabra at the ratio 3:1:1) and Cisplatin (a standard cancer treatment) against colorectal cancer cell lines, including HCT116 (A), HT-29 (B), and SW620 (C), prostate cancer cell line PC3 (D), as indicated by cell death. *, $p < 0.05$ between the indicated groups; #, $p < 0.05$ vs. the untreated

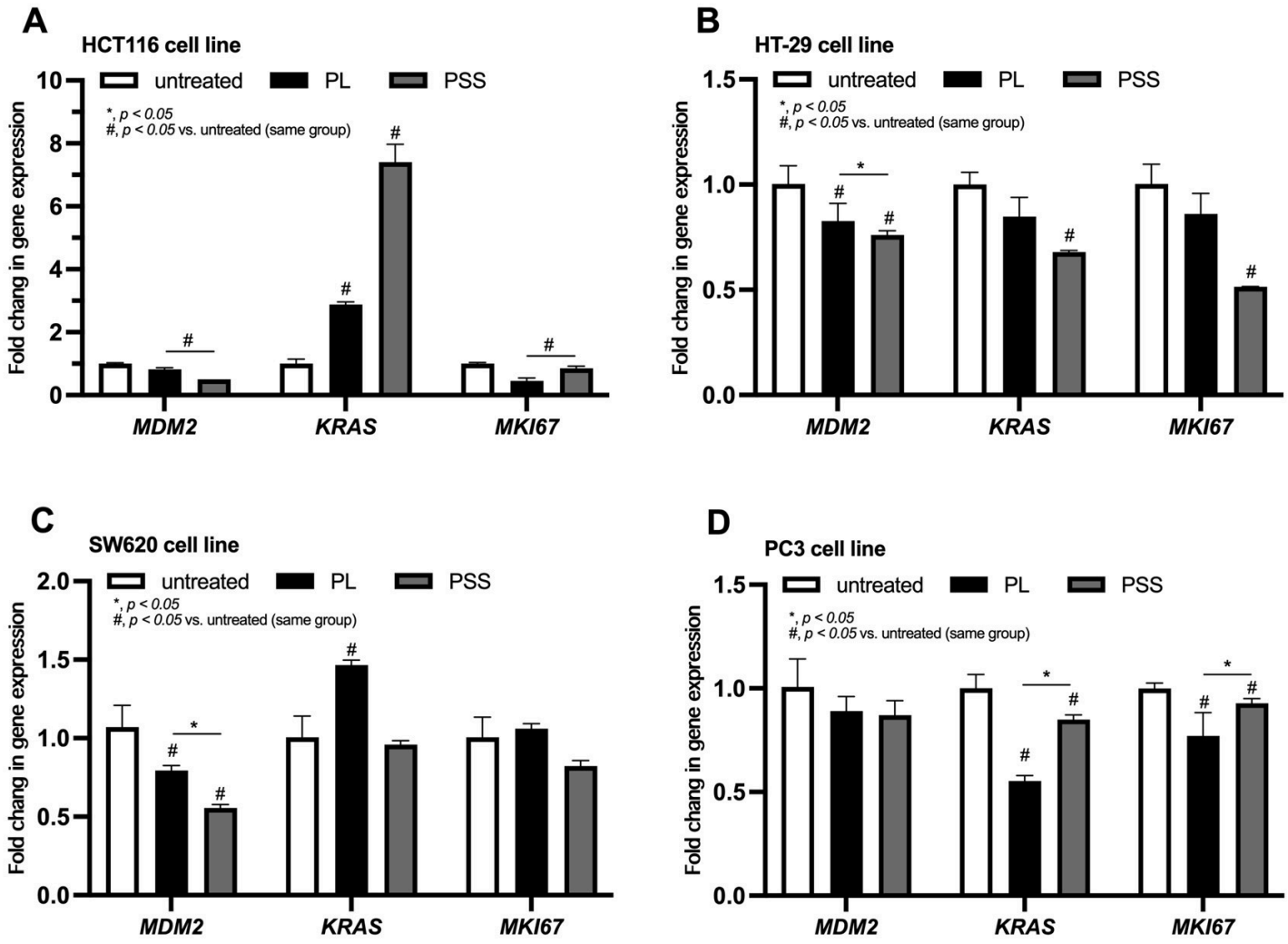
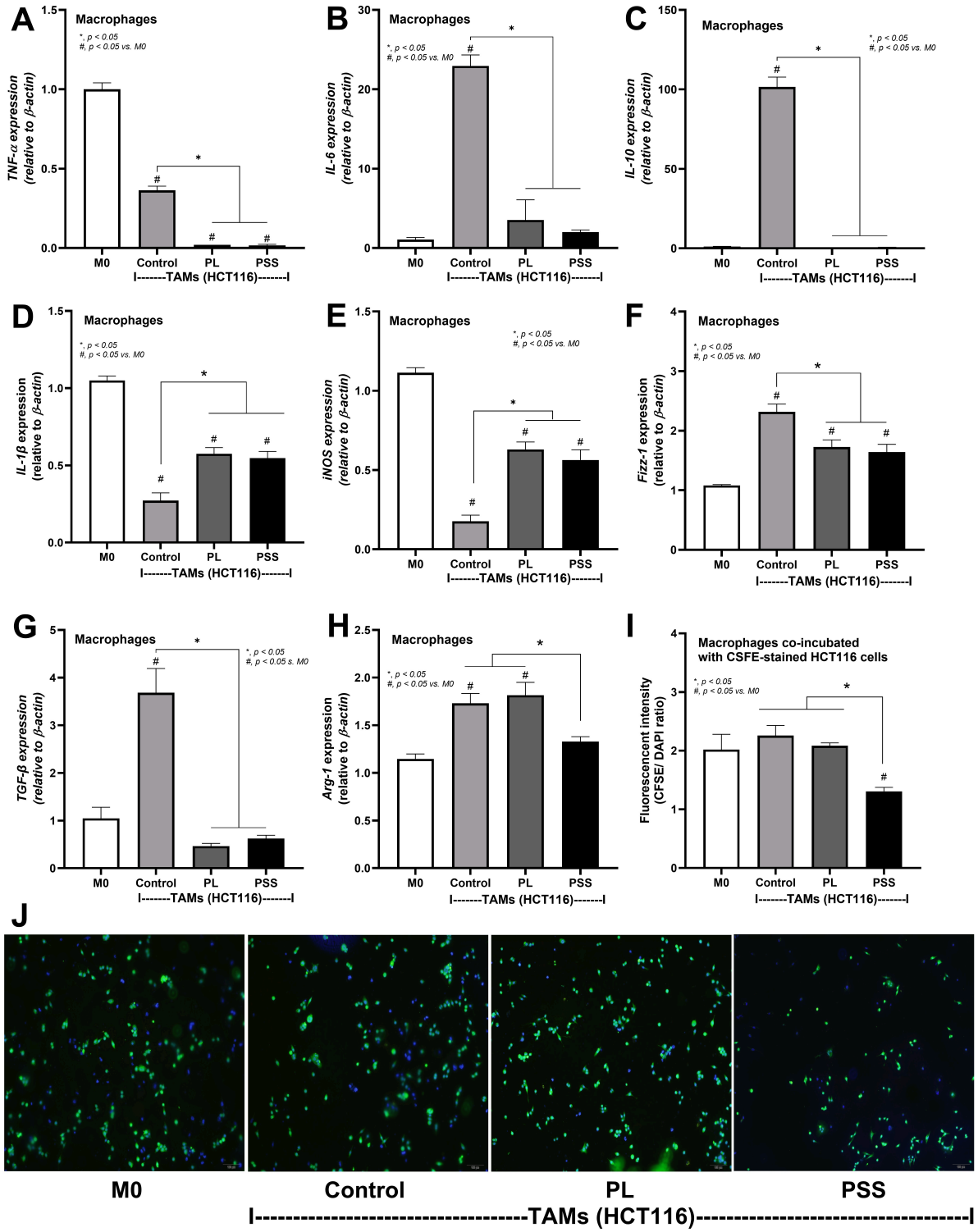



**Figure 4**

Characteristics of the impacts of compounds from PL and PSS mixture (PL+ Smilax corbularia + Smilax glabra at the ratio 3:1:1) and untreated control (untreated) against colorectal cancer cell lines, including HCT116 (A), HT-29 (B), and SW620 (C) and prostate cancer cells (PC3) (D) as indicated by the expression of several genes, including murine double minute 2 (*MDM2*), Kirsten rat sarcoma virus (*KRAS*), and marker of proliferation Ki-67 (*MKI67*), of the malignant cells are demonstrated. Data derived from isolated triplicate experiments. *, $p < 0.05$ between the indicated groups; #, $p < 0.05$ vs. the untreated (within the same gene)




## Figure 5

Characteristics of the impacts of compounds from PL and PSS mixture (PL+ Smilax corbularia + Smilax glabra at the ratio 3:1:1) and untreated control (untreated) against tumor-associated macrophages (TAMs) using the supernatant from a colorectal cancer cell lines (HCT116) (see method) as indicated by the expression of genes for cytokines (TNF-α, IL-6, and IL-10) (A-C), M1 macrophage polarization (IL-1β and iNOS) (D, E) and M2 macrophage polarization (Fizz-1, TGF-β and Arg-1) (F-H) with the cytotoxicity

against carboxyfluorescein succinimidyl ester (CSFE)-stained malignant cells as indicated by fluorescent intensity and the representative pictures (I and J) are demonstrated. Data derived from isolated triplicate experi-ments. *, $p < 0.05$ between the indicated groups; #, $p < 0.05$ vs. M0

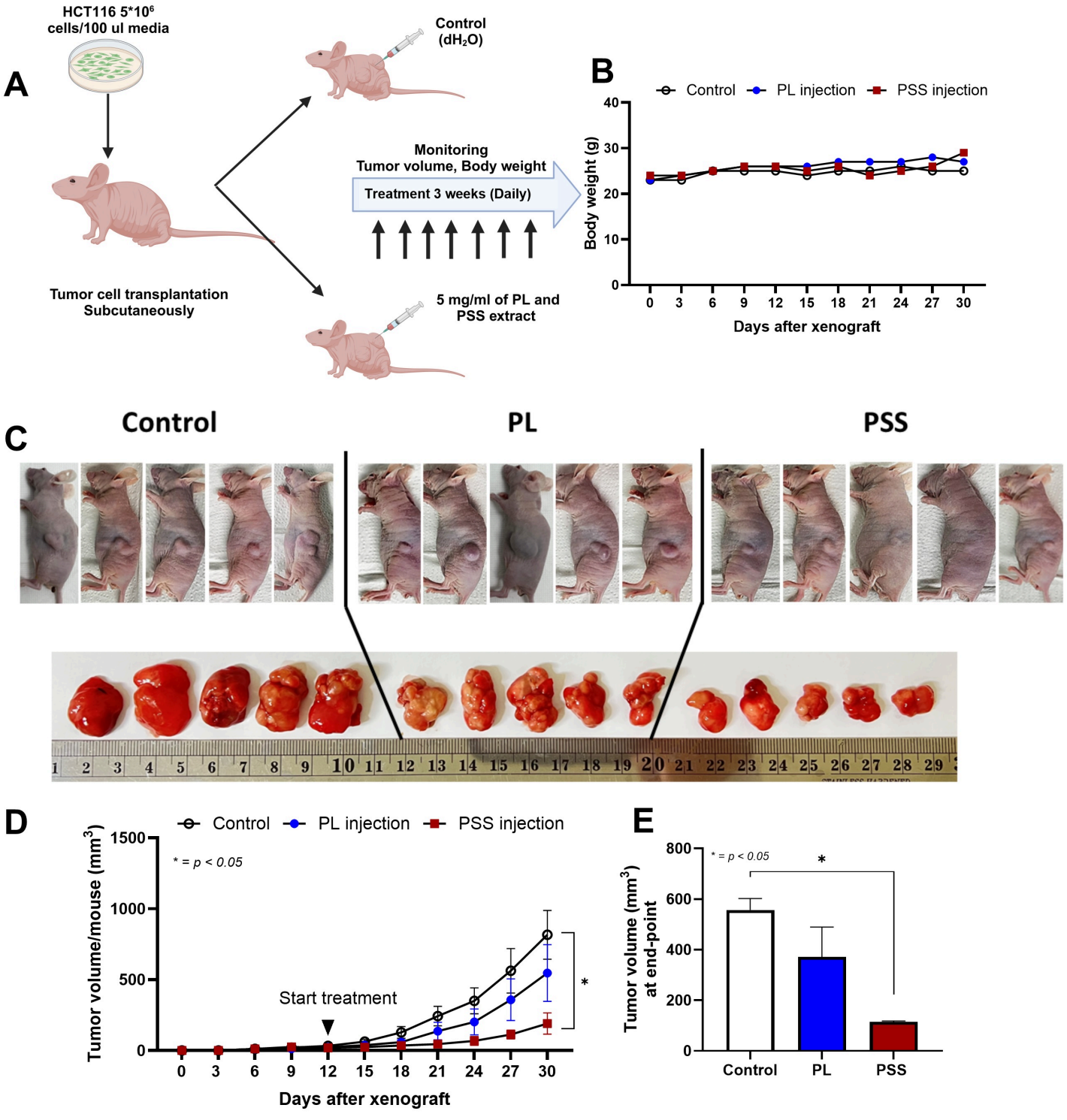



**Figure 6**

Characteristics of the impacts of compounds from PL or PSS mixture (PL+ Smilax corbularia + Smilax glabra at the ratio 3:1:1) and control (water injection) against sub-cutaneous colorectal cancer cell line (HCT116) as indicated by the schema of experiments (A), body weight (B), the tumor volume in time-point data and at the end-point (30 days post-cancer injection) with representative pictures of the tumors in mice and the pictures of tumors after excision (C-E) are demonstrated (n = 5 per time-point for B and D) (n = 5 per group for E). *, $p < 0.05$ between the indicated groups

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Table2.docx