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Original Article

Complementary effects of *Orthosiphon stamineus* standardized ethanolic extract and rosmarinic acid in combination with gemcitabine on pancreatic cancer

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ABSTRACT

Background: Pancreatic cancer is one of the most notorious cancers and is known for its highly invasive characteristics, drug resistance, and metastatic progression. Unfortunately, many patients with advanced pancreatic cancer become insensitive towards gemcitabine treatment. *Orthosiphon stamineus* (O.s) is used widely as a traditional medicine for the treatment of multiple ailments, including cancer in South East Asia. The present in vitro study was designed to investigate the complementary effects of an ethanolic extract of O.s (Et. O.s) or rosmarinic acid in combination with gemcitabine on Panc-1 pancreatic cancer cells.

Method: Cell viability and colony formation assays were used to determine the 50% inhibitory concentration (IC₅₀) of Et. O.s, rosmarinic acid, and gemcitabine. Different doses of gemcitabine in combination with Et. O.s or rosmarinic acid were tested against Panc-1 to select the best concentrations which possessed synergistic effects. Elucidation of molecular mechanisms responsible for mediating chemo-sensitivity in Panc-1 was performed using Quantitative Real-time PCR (QPCR), flow cytometry and immunohistochemistry.

Results: Et. O.s was found to significantly sensitise Panc-1 towards gemcitabine by reducing the gene expression of multidrug-resistant protein family (MDR) (MDR-1, MRP-4, and MRP-5) and molecules related to epithelial-mesenchymal transition (ZEB-1 and Snail-1). An induction of the human equilibrate nucleoside transporter-1 (hENT-1) gene was also found in cells treated with Et. O.s-gemcitabine. The Et. O.s-gemcitabine combination induced cellular senescence, cell death and cell cycle arrest in Panc-1. In addition, the inhibition of Notch signalling was demonstrated through the downregulation of Notch 1 intracellular

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domain in this treatment group. In contrast, rosmarinic acid-gemcitabine combination showed no additional effects on cellular senescence, apoptosis, epithelial mesenchymal transition (EMT) markers, the MRP-4 and MRP-5 multi-drug resistance protein family, hENT-1, and the Notch pathway through Notch 1 intracellular domain.

Conclusion: This study provides valuable insights on the use of Et. O.s to complement gemcitabine in targeting pancreatic cancer *in vitro*, suggesting its potential use as a novel complementary treatment in pancreatic cancer patients.

At a glance commentary

Scientific background on the subject

Chemoresistance poses a big challenge in the treatment of pancreatic cancer. Ethanolic extract of *O.s* (Et *O.s*) suppressed vascularisation and the growth of colon xenograft tumours through the down-regulation of VEGF protein expression. However, little is known about Et *O.s* in complementary to gemcitabine in pancreatic cancer.

What this study adds to the field

Et *O.s* sensitised pancreatic cancer cells to gemcitabine by down-regulating multidrug resistance and epithelial-mesenchymal transition genes but induced senescence and cell death through Notch 1 inhibition *in vitro*. This study provides the fundamentals for developing Et *O.s* as a botanical drug for complementary therapy in pancreatic cancer.

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related deaths globally. Despite advances in combination chemotherapy regimens, the survival rate in pancreatic cancer patients still stands at less than 5%. Multiple reasons, including late diagnosis as well as resistance to conventional chemo- and radiation therapy, have been suggested to be responsible for such a low survival rate. Gemcitabine, (2', 2'-difluorodeoxycytidine), is a first-line therapy approved by the US Food and Drug Administration for pancreatic cancer [1]. However, overall success rates are poor [2]. Multiple adverse effects, mainly unforeseen toxicity observed in a large number of patients when treated with a combination of gemcitabine with other cancer drugs, often reduce its clinical relevance. The multi-drug resistance (MDR) or P-glycoprotein is mainly responsible for the development of insensitivity to chemodrugs, including gemcitabine [3]. MDR-1 is abundantly expressed in the liver, pancreas, colon, kidney, and jejunum [4]. MDR-associated proteins (MRP) play a key role in the emergence of drug insensitivity in pancreatic tumours towards chemotherapeutic drugs such as gemcitabine and 5-fluorouracil [5]. The human equilibrative nucleoside transporter-1 (hENT-1) is a nucleoside transporter that brings gemcitabine into the cells [6].

A growing body of evidence has revealed that increased expression of cellular hENT-1 protein can elevate the intracellular uptake of gemcitabine [6]. Epithelial-mesenchymal transition (EMT) has been implicated as a potential mechanism for drug insensitivity [7]. However, the molecular mechanisms involved in the EMT process are not well understood. In addition, studies have also shown that the involvement of multiple oncogenic signalling pathways, including the Notch pathway is essential for the induction of EMT [8,9]. A study by Wang et al. has illustrated that Notch-2 and its ligand, Jagged-1, were highly activated in gemcitabine-resistant pancreatic cancer cells, and the suppression of Notch signaling was associated with decreased invasion of gemcitabine-resistant cells [10].

Orthosiphon stamineus (*O.s*) of the lamiaceae family is traditionally used for the treatment of a variety of chronic ailments, including cancer in Asia [11]. Multiple studies utilising *in vitro*, *ex vivo* and *in vivo* techniques have demonstrated the antitumor potential of this medicinally important herb in colorectal and breast cancers [12]. *O.s* has been shown to arrest angiogenesis through the downregulation of vascular endothelial growth factor (VEGF), and the promotion of VEGF receptor (VEGFR) phosphorylation [13,14]. Plant-derived potent anti-cancer drugs such as vincristine, camptothecin, and paclitaxel have been shown to serve as a primary treatment protocol for cancer as reviewed in Yehya et al. [15]. The potent anti-angiogenic activity of *O.s* and its preventative activity against human breast tumours in a xenograft model has also been established [15]. *O.s* suppressed vascularisation and inhibited the growth of implanted human colon tumours [11]. Phytochemical studies have reported that leaves of *O.s* contain more than 20 phenolic bioactive compounds, including rosmarinic acid, eupatorin, sinesitin, pentacyclic triterpenes, betulinic acid, oleanolic acid, ursolic acid, and β -sitosterol [11,15].

This study is a continuation of our previous work, and aims to investigate *in vitro* efficacy of ethanolic extract of *O.s* (Et. *O.s*) in combination with gemcitabine compared to its major active compound (rosmarinic acid) as a complementary therapy in pancreatic cancer.

Materials and methods

Cell lines

The Panc-1 pancreatic cancer cell line was purchased from American Type Culture Collection. Cells were maintained in

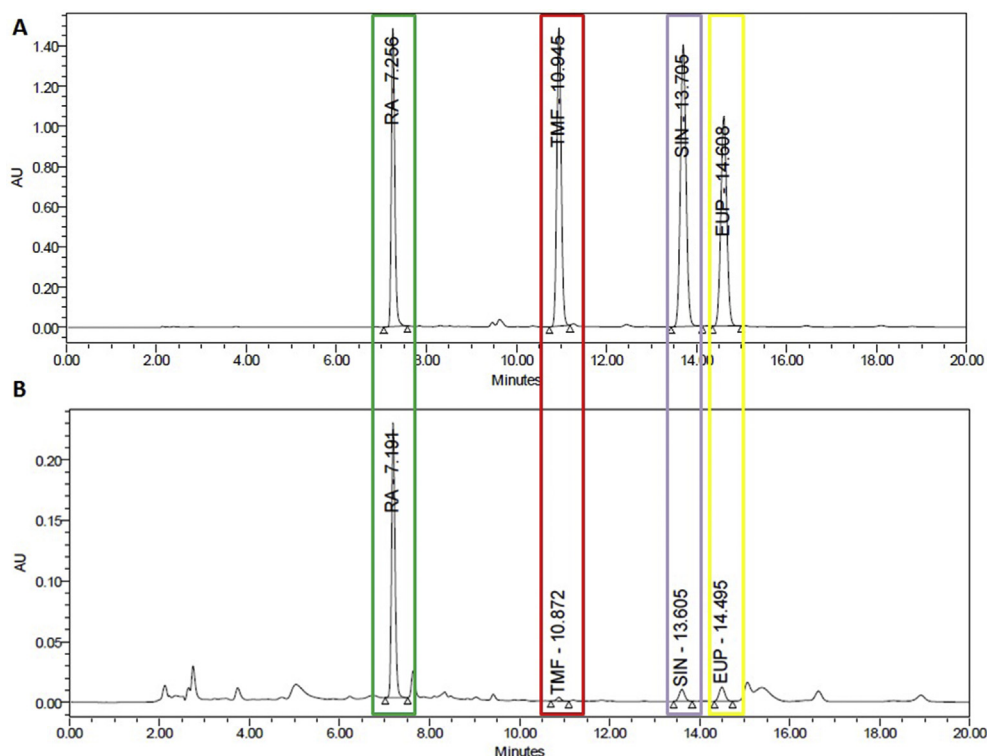


Fig. 1 HPLC chromatograms. (A) HPLC chromatogram of standard markers, rosmarinic acid (RA), 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), sinesitin (SIN), and eupatorin (EUP). (B) HPLC chromatogram of Et. O.s showing different proportions of respective markers.

Dulbecco's Modified Eagle Medium (DMEM) (Thermo Scientific, USA) supplemented with 10% fetal bovine serum (Biowest, USA) and 100 units/ml penicillin-streptomycin (Nacalai Tesque, USA). Cells were kept at 37 °C in a humidified incubator with 5% CO₂ atmosphere.

Plant materials and compounds

O.s as 50% standardised ethanol extract (Catalogue No: 931886-P) was purchased from Nature Ceuticals Sendirian Berhad Malaysia. Gemcitabine (Catalogue No: S1149) was purchased from Selleckchem, USA. Rosmarinic acid (Catalogue No: 536,954) and eupatorin (Catalogue No: E4660) were purchased from Sigma–Aldrich, USA. Sinesitin (Catalogue No: P201) was purchased from Indofine/USA and 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (Catalogue No: CDS007106-10 MG) was procured from Sigma–Aldrich, USA.

High-performance liquid chromatography (HPLC)

Instrumentation

The HPLC was performed using the Agilent Technologies series 1260 infinity (Waldronn, Germany) system equipped with a quaternary pump (G1311C), autosampler (G1329B), column oven (G1316A) and ultraviolet (UV) detector (G1314F).

Preparation of standard compounds and samples

For the preparation of standard compounds stock solution, 5 mg of each standard was dissolved in 5 ml of methanol and then filtered through a 0.45 m filter (Whatman). A series of

working standard solutions were prepared by diluting the above solution with methanol. OS-E and OS-EL (100 mg) were dissolved in 10 ml of HPLC grade methanol. Working sample solutions of a concentration of 5 mg/ml were prepared by diluting the stock solutions with HPLC grade methanol. The samples were filtered through a 0.45 m filter (Whatman).

Cell viability assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide) cell viability assay was performed in a 96-well plate format to determine the IC₅₀ (50% inhibitory concentration) of Et. O.s, rosmarinic acid, and gemcitabine. Different doses of gemcitabine (12.5, 25, 50, 100, and 200 nM), Et. O.s (0, 15, 30, 60, and 120 µg/ml), and rosmarinic acid (0, 10, 20, 40, and 80 µM) were tested on Panc-1 (3 × 10³ cells/0.1 ml) for 72 h to select the best cytotoxic concentrations for further detailed studies.

Combination index (CI): assessment of CI for Et. O.s/Rosmarinic acid and gemcitabine combination treatments

Different concentrations of Et. O.s (0, 15, 30, 60, and 120 µg/ml) or rosmarinic acid (0, 10, 20, 40, and 80 µM) and gemcitabine (12.5, 25, 50, 100, and 200 nM) were used for combination treatment [Fig. 1]. MTT was used to measure the cell viability of Panc-1 cells at 72 h post-treatment. The CI was determined using CompuSyn software (CompuSyn, Inc, Paramus, NJ. 07652 USA).

Colony formation assay

The effect of different treatments on the clonogenic potential of Panc1 cells as previously reported [16]. Briefly, Panc-1

(1×10^3 cells/ml) were suspended in DMEM medium and seeded into each well of a 6-well plate. Cells were then incubated at 37 °C in a humidified incubator with an atmosphere of 5% CO₂ (v/v) for 24 h to allow cell attachment. Subsequently, the medium was gently aspirated and replaced with fresh medium containing different concentrations of treatments. After 72 h, the medium was removed; cells were washed twice with PBS, and replaced with fresh medium without treatments. After incubation for ten days, colonies were rinsed twice in cold PBS, and then fixed with 4% paraformaldehyde prior to staining with 0.2% crystal violet. Colonies containing more than 50 cells were counted and analysed under a microscope, using the following equation:

$$\text{PE (\% plating efficiency (PE))} = \left(\frac{\text{number of colonies formed}}{\text{number of cells seeded}} \right) \times 100\%$$

Survival fraction (SF) in each group was calculated using the following formula;

$$\text{SF\%} = \left[\frac{\text{number of colonies after treatment}}{\text{number of cells seeded} \times \text{PE}} \right] \times 100\%$$

The results were presented as the mean percentage of SF \pm SD of three independent experiments (n = 3).

Cell migration assay

Cell migration assay was performed following a previously described method [17]. Briefly, medium containing 5×10^4 cells/ml was transferred into each well of a 6-well plate. After 24 h, an even scratch was created using a 20 μ l micropipette tip on the confluent monolayer. Different concentrations of treatment were prepared and added to each well. Subsequently, images of the wounds were taken at different time-points (0, 12, and 24 h), followed by the measurement of wound width using the Image J software (LOCI, University of Wisconsin).

Cellular senescence assay

Approximately 500 cells/well were seeded into 6-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 24 h to allow cell attachment. The medium was gently removed and replaced with fresh medium containing different concentrations of treatment. After 72 h, the senescence β -Galactosidase staining kit (Cell Signaling Technology, USA) was used to detect β -galactosidase activity, according to the manufacturer's instructions. Cells were incubated at 37 °C in an overnight dry incubator, in the absence of CO₂. Cells were later observed under a microscope (200 \times magnifications) and analysed for cellular senescence.

Detection of cell cycle arrest

The Panc-1 cell suspension containing 5×10^4 cells/ml in different treatment concentrations were incubated for 72 h in a 6-well plate. Cells were trypsinised and washed in cold 1 \times PBS with gentle shaking, followed by fixation in 1 ml of 70% ethanol (molecular grade) for 30 min. Cells were subsequently centrifuged and washed with cold PBS multiple times, and

centrifuged at 45 \times g for 10 min. The resultant cell pellet was stained with 500 μ l of propidium iodide (PI) solution containing 50 μ l of RNase A stock solution (Life Technologies, USA). Stained cells were incubated for 30 min on ice. Stained cells were kept on ice in the dark until scheduled for Flow cytometry analysis using a BD FACSCalibur (BD Biosciences, USA).

Apoptosis detection

Flow cytometry using the Annexin V-FITC apoptosis detection kit (eBioscience, Austria) was employed to detect apoptosis in treated cells. Panc-1 (5×10^4 cells/ml) were seeded in growth medium and incubated for 24 h at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ to allow cell attachment. Cells were then treated with Et. O.s, rosmarinic acid and gemcitabine, either alone or in combination for another 72 h. Cells were trypsinised and centrifuged at 10 \times g for 5 min. The resultant cell pellets were then washed with PBS and stained with propidium iodide and annexin V for 10–15 min according to the manufacturer's instructions. Cells were then subjected to analysis using the BD FACSCalibur flow cytometer (BD Biosciences, USA).

Quantitative real-time PCR (QPCR)

Panc-1 cells (5×10^4 cells/ml) were exposed to different treatment conditions for 72 h. The RNA from each treatment group was extracted using T GENEzol™ reagent (Geneaid, Taiwan) following the manufacturer's protocol. RNA was then converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington). QPCR was performed using the Applied Biosystem 7500 Fast Real-time PCR System (Applied Biosystem, US). The cDNA samples were then programmed through 40 cycles of amplification at 95 °C for 15 s followed by 60 °C for 1 min. All the genes were normalized to the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. The following primer sets were used in this study:

- GAPDH: Forward: 5- ACCCACTCCTCCACCTTTGA -3.
Reverse: 5- CTGTTGCTGTAGCCAAATTCGT -3.
Snail-1: Forward: 5- TCGGAAGCCTAACTACAGCGA -3.
Reverse: 5- AGATGAGCATTGGCAGCGAG -3.
ZEB-1: Forward: 5- TTACACCTTTGCATACAGAACCC -3.
Reverse: 5- TTTACGATTACAGCCAGACTGC -3.
MDR-1: Forward: 5- CCCATCATTGCAATAGCAGG -3.
Reverse: 5- GTTCAAACCTTCTGCTCCTGA -3.
MRP-4: Forward: 5- GGATCCAAGAAGCTGATGAGTTAAT -3.
Reverse: 5- TCACAGTGCTGTCTCGAAAATAG -3.
MRP-5: Forward: 5- GCTGTTTCTGAGTGGCAGTGTGAG -3.
Reverse: 5- TCAGCCCTTGACAGCGACCTT -3.
hENT1: Forward: 5- CACCAGCCTCAGGACAGATACAA -3.
Reverse: 5- GTGAAATACTGAGTGGCCGTCAT -3.
Notch-1: Forward: 5- GACAACGCCTACCTCTGCTT -3.
Reverse: 5- ACTTGTACCCGTTGAGGCTG -3.
HEY-2: Forward: 5- GTTGCGGGCTGGGAAAGAG -3.
Reverse: 5- GTGTGGTCAAAGTAGCCTTTA -3.

Analyses were carried out on data from three independent experiments (n = 3).

Western blot

Briefly, protein extraction from cultured cells 72 h post-treatment was performed using RIPA lysis buffer (Nacalai

Table 1 Percentage quantification of four marker compounds in 50% Et. O.s extracts.

Component	Et. O.s (%)
Rosmarinic acid	7.421
Sinesitin	0.755
Eupatorin	0.592
3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone	0.175

Tesque, USA) according to the manufacturer's instructions. Protein samples were quantified at 280 nm wavelength using the NanoDrop ND-1000 spectrophotometer (ThermoScientific, USA). Protein samples and rainbow protein markers were loaded at 40 $\mu\text{g}/\text{well}$ into the wells of different percentage bis-acrylamide gel (Nascalai Tesque, Japan). Proteins were then transferred from the gel onto the Immobilon-polyvinylidene fluoride transfer membrane (Millipore, Watford). The

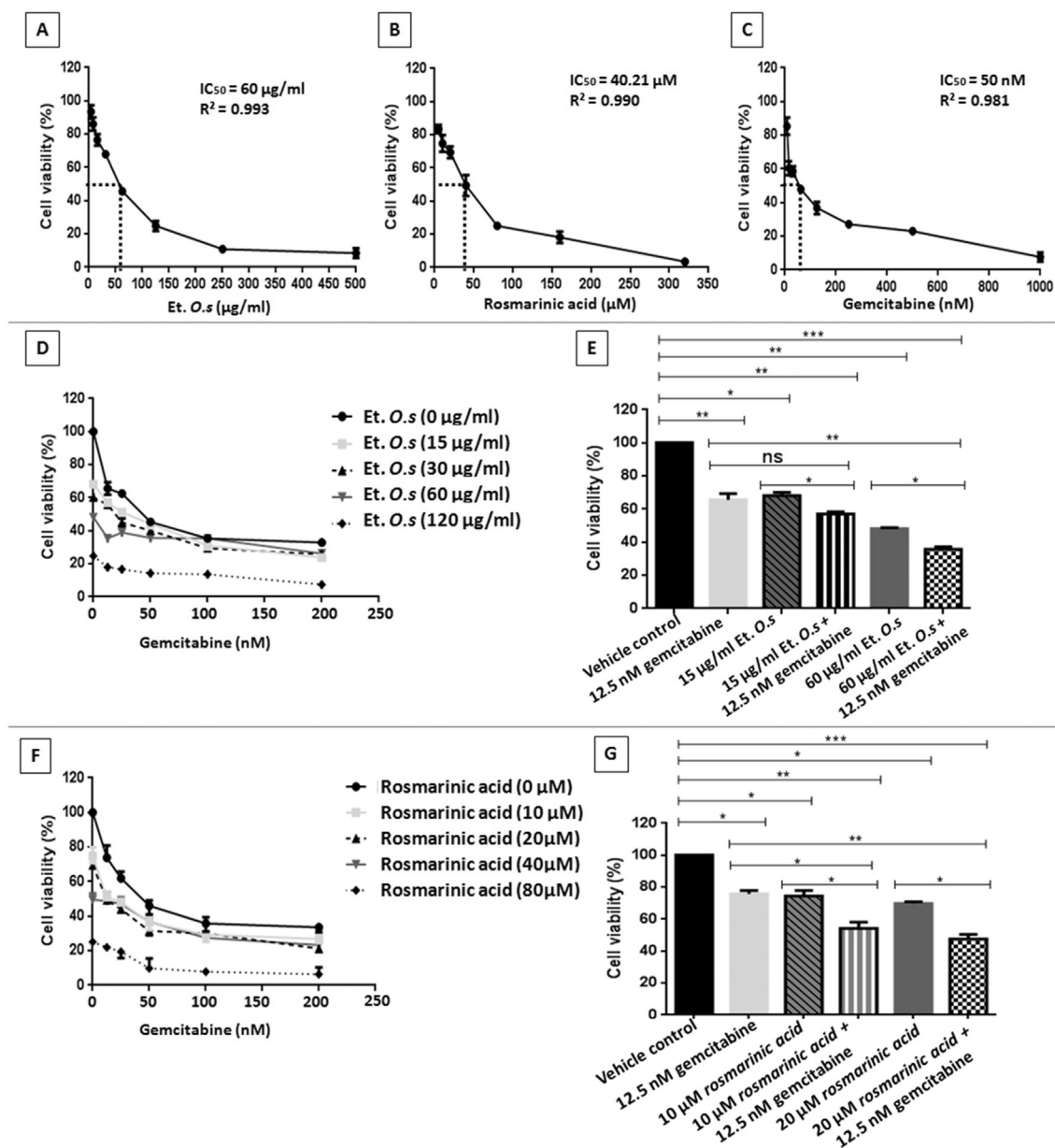


Fig. 2 Effect of Et. O.s, rosmarinic acid, and gemcitabine treatments on cell viability of Panc-1 pancreatic cancer cells 72 h post treatment. (A) Et. O.s, (B) Rosmarinic acid, and (C) Gemcitabine reduced cell viability with increasing doses. (D) Effect of combined treatment of Et. O.s and gemcitabine on Panc-1 cell viability. (E) Panc-1 cells treated with Et. O.s (15 $\mu\text{g}/\text{ml}$ and 60 $\mu\text{g}/\text{ml}$) demonstrated had synergistic effect with gemcitabine (12.5 nM). (F) Effect of combination treatment of rosmarinic acid and gemcitabine on Panc-1 cells. (G) Panc-1 cells were treated with 10 μM and 20 μM of rosmarinic acid had synergistic effect with 12.5 nM gemcitabine. Error bars represent SEM. Statistical analysis (ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, One way ANOVA was performed using GraphPad Prism 6.0 software ($n = 3$). R² = Explained variation/Total variation.

membrane was immersed in a blocking solution consisting of 5% milk powder and 0.1% Tween-20 in PBS, for 1 h at room temperature. Subsequently, the membrane was probed with primary antibodies (Notch 1 ICD, Vimentin, E-Cadherin, and Caspase-3 purchased from Santa Cruz, USA; Cleaved PARP purchased from Cell Signalling Technology, USA; β -Actin purchased from Sigma–Aldrich, USA) overnight at 4 °C. On the next day, the membrane was rinsed in PBST three times for 10 min each and probed with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. The protein bands on the blots were detected using Chemi-Lumi one super detection reagents (Nacalai Tesque, USA) and visualised using the C-Digit blot scanner (Lincoln, Nebraska, USA). Densitometry analysis was performed using Image J by calculating the relative density of each peak which corresponds well with the size and intensity of each band in on the blot, and normalised to the loading control (β -Actin). Analyses were carried out on data from three independent experiments ($n = 3$).

Statistical methods

Prism (GraphPad, USA) and Excel (Microsoft, USA) were used for statistical analysis. Data was presented as mean \pm S.E.M., and statistical analyses were performed using One-Way Analysis of Variance (ANOVA) followed by post-hoc should

be italicized Tukey to compare the mean values among three or more data sets and to assess significance among samples. A p value < 0.05 was considered significant when compared to the respective control group of all groups.

Results

HPLC analysis of Et. O.s

Quantitative determination of flavonoids (rosmarinic acid, sinesitin, eupatorin, and 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone) was performed using the HPLC method. The HPLC chromatograms of Et. O.s (absorbance at 330 nm) along with the mixed standard of bioactive markers are shown in Fig. 1A and B. The HPLC analyses for the four marker compounds in 50% Et. O.s are depicted in Table 1.

Et. O.s and rosmarinic acid reduced Panc-1 cell viability and survival in combination treatment with gemcitabine

The IC₅₀ values of Et. O.s, rosmarinic acid, and gemcitabine towards Panc-1 were evaluated to be 60 μ g/ml, 40.21 μ M, and 50 nM, respectively [Fig. 2A, B, and C]. According to the combination index (CI) analysis [Tables S1 and S2], Et. O.s at 15 μ g/

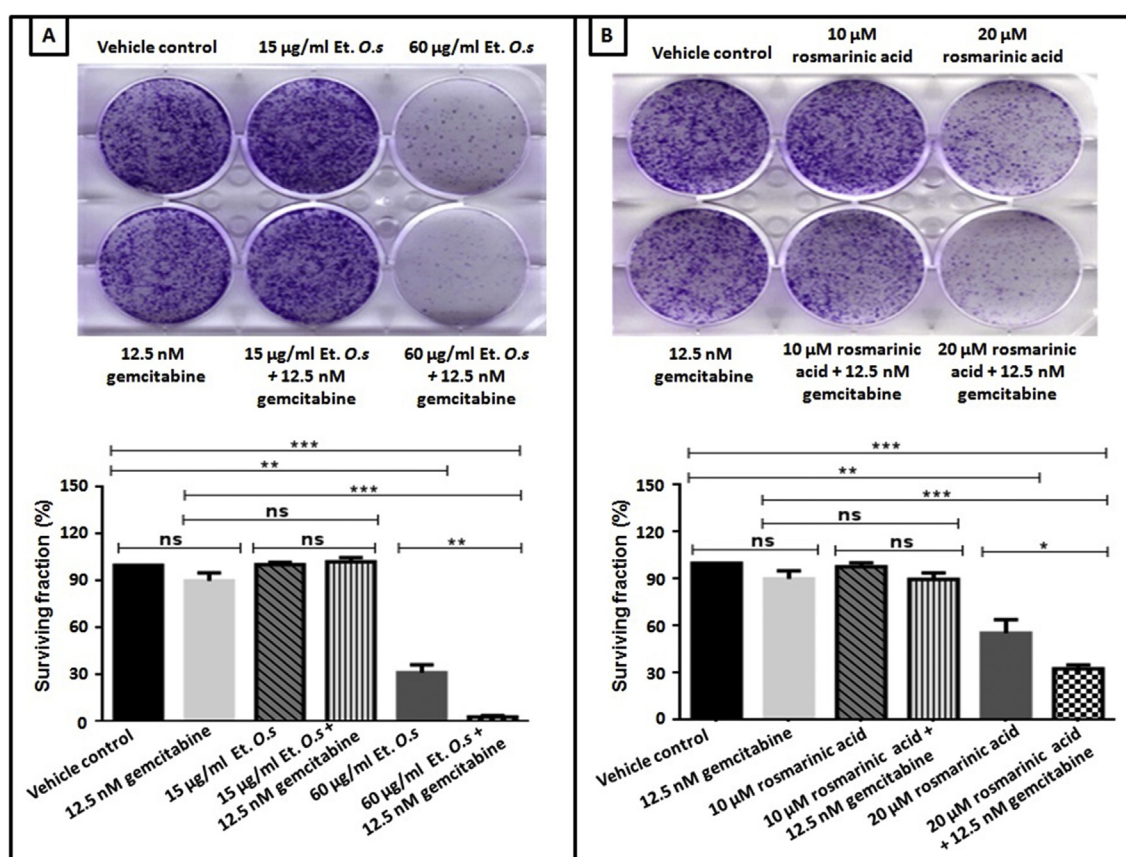


Fig. 3 The effect of Et. O.s or rosmarinic acid and gemcitabine combination treatments on colony formation of Panc-1 cells 72 h post treatment. (A) Colony formation of Panc-1 cells post treatment with Et. O.s (15 and 60 μ g/ml) with/without gemcitabine (12.5 nM) compared to vehicle control. (B) Colony formation of Panc-1 cells treated with rosmarinic acid (10 μ M and 20 μ M) with/without gemcitabine (12.5 nM) compared to vehicle control. Error bars represent SEM. Statistical analysis (ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, One way ANOVA, $n = 3$ independent experiments) using GraphPad Prism 6.0 software.

ml and 60 $\mu\text{g/ml}$ exhibited synergistic cytotoxic effects in combination with gemcitabine at 12.5 nM. However, Et. O.s (60 $\mu\text{g/ml}$) and gemcitabine (12.5 nM) displayed prominent cytotoxic effects by reducing Panc-1 viability compared to gemcitabine (12.5 nM) and Et. O.s (60 $\mu\text{g/ml}$) single treatments [Fig. 2D]. At 10 μM and 20 μM , rosmarinic acid together with gemcitabine (12.5 nM) showed synergistic cytotoxic effects towards Panc-1 [Fig. 2E]. Interestingly, the combination of rosmarinic acid (20 μM) and gemcitabine (12.5 nM) was more effective in reducing Panc-1 viability compared to single treatments of either one [Fig. 2E].

The data from the cell survival assay demonstrated that the combination of Et. O.s (15 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$) and gemcitabine (12.5 nM) significantly inhibited colony potential of Panc-1 compared to either single treatment [Fig. 3A]. At 72 h post-treatment with 15 and 60 $\mu\text{g/ml}$ of Et. O.s and 12.5 nM of gemcitabine alone, the percentage survival fraction (%SF) in Panc-1 was found to be 99%, 32%, and 90% respectively compared to 100% in vehicle control group. The %SF in Panc-1 treated with a combination of gemcitabine (12.5 nM) and Et. O.s (15 $\mu\text{g/ml}$ or 60 $\mu\text{g/ml}$), was at 102% and 3% respectively [Fig. 3A]. However, the % SF in Panc-1 treated with the combination of rosmarinic acid (10 μM or 20 μM) and gemcitabine

(12.5 nM) was at 89% and 33% respectively, when compared with vehicle control group. The % SF in Panc-1 treated with 10 μM or 20 μM of rosmarinic acid alone was found to be at 97.5% and 56.5%, respectively [Fig. 3B]. Based on the results of the cell viability and colony formation assays, Et. O.s (60 $\mu\text{g/ml}$), rosmarinic acid (20 μM), and gemcitabine (12.5 nM) were selected for further downstream assays.

Combination treatment of Et. O.s or rosmarinic acid and gemcitabine inhibited Panc-1 migration compared to either single treatment and vehicle control

Et. O.s, rosmarinic acid, and gemcitabine treatments alone displayed no inhibition of the migratory potential in Panc-1, compared to vehicle control group 24 h post-treatment [Fig. 4]. The percentage of gap (cell-free area) in Et. O.s, rosmarinic acid, and gemcitabine single treatment groups were at 32%, 56%, and 90%, respectively compared to the cell-free area of vehicle control group (96%) [Fig. 4]. The percentage of gap closure in combination treatment groups i.e., either Et. O.s or rosmarinic acid with gemcitabine was at 5% and 33%, respectively [Fig. 4], compared to the vehicle control group.

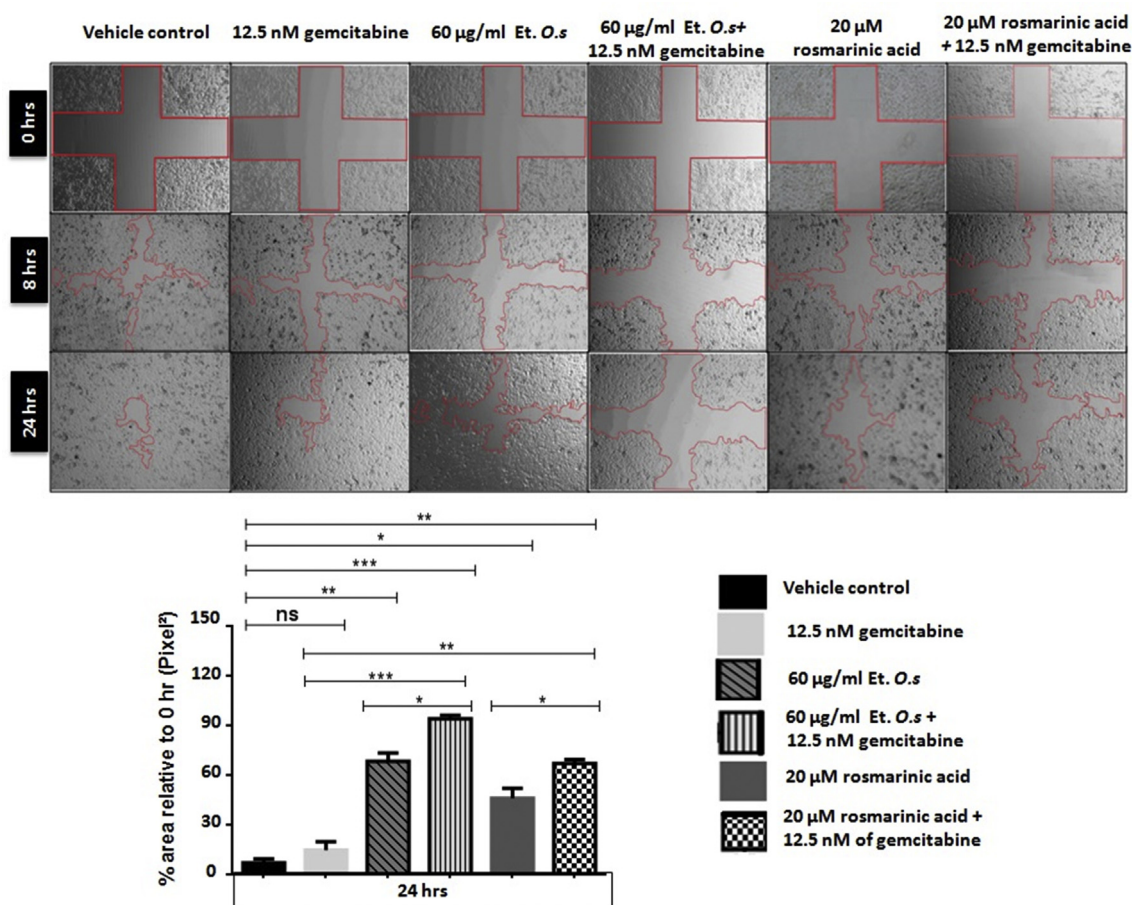


Fig. 4 The effect of Et. O.s or rosmarinic acid and gemcitabine on combination treatments on Panc-1 cells migration 24 h post treatment. Cells were treated with Et. O.s (60 $\mu\text{g/ml}$) or rosmarinic acid (20 μM) with/without gemcitabine (12.5 nM). Images were captured at 0, 8, and 24 h time points (200 \times magnification). Areas of scratch were captured and quantified using Image J software. Error bars represent SEM. Statistical analysis (ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, One way ANOVA, $n = 3$ independent experiments) using GraphPad Prism 6.0 software.

Combination treatment of Et. O.s and gemcitabine promoted cellular senescence in Panc-1 cells

Significant induction of senescence was established in Panc-1 treated with the combination of Et. O.s and gemcitabine, compared to vehicle and single treatment groups [Fig. 5]. However, the combination treatment of rosmarinic acid and gemcitabine did not result in a significant regulation of senescence when compared to vehicle and single treatment groups [Fig. 5].

Combination treatment of Et. O.s and gemcitabine induced cell cycle arrest in Panc-1 cells

At single doses, Et. O.s significantly arrested Panc-1 at the G1 phase, rosmarinic acid at S phase, and gemcitabine at the S

phase [Fig. 6]. Combination treatment of Et. O.s and gemcitabine significantly arrested Panc-1 S phase compared to single treatment and vehicle control [Fig. 6]. There was no additional effect on cell cycle arrest in cells treated with the rosmarinic acid–gemcitabine combination compared to gemcitabine treatment alone [Fig. 6]. 5-FU was used as positive control.

Combination treatment of Et. O.s and gemcitabine induced necrosis and early apoptosis in Panc-1 cells

Combination treatment of Et. O.s and gemcitabine significantly triggered cellular necrosis and early apoptosis, compared to single treatments of either one [Fig. 7]. Further induction of apoptosis through cleaved PARP and caspase-3 proteins was demonstrated when Panc-1 cells were treated with Et. O.s in combination with gemcitabine, compared with

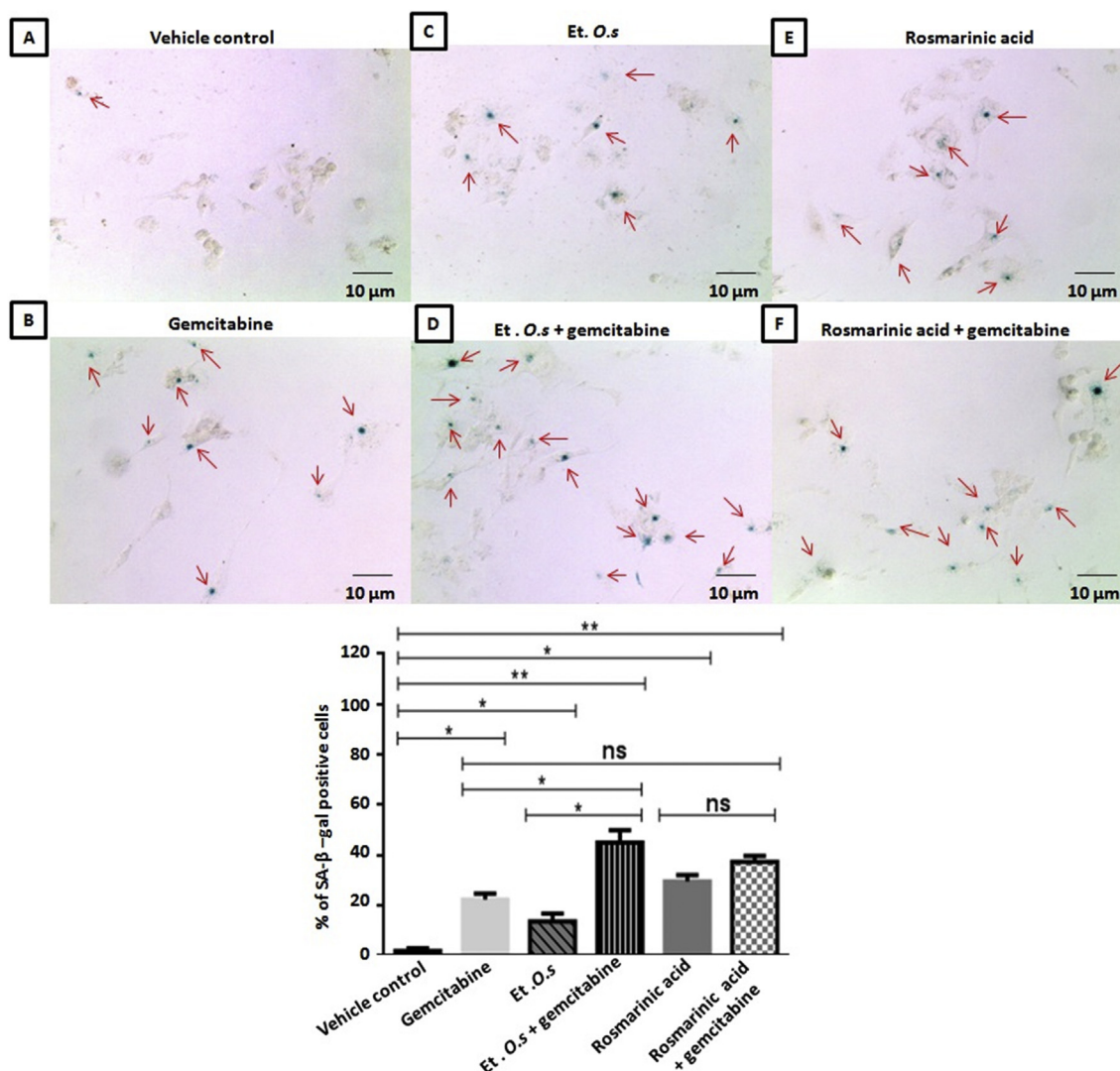


Fig. 5 Combination treatment of Et. O.s and gemcitabine induced cellular senescence in Panc-1 after 72 h treatment compared to rosmarinic acid and vehicle control. Senescence-associated-β-gal activity was measured on day 6 and scored as percentage of senescence-associated-β-gal positive cells (blue) over total cells (200× magnification) and quantified using Image J software. Arrows indicate senescent cells. Error bars represent SEM. Statistical analysis (ns = not significant; *P < 0.05; **P < 0.01; One way ANOVA was performed using GraphPad Prism 6.0 software (n = 3).

vehicle control and single treatments. Rosmarinic acid and gemcitabine stimulated apoptosis by reducing the protein expression of cleaved PARP and caspase-3, compared to vehicle control [Fig. 8]. However, there was no significant effect on apoptosis cell death between the combination treatment of rosmarinic acid and gemcitabine, and the single treatment groups [Fig. 8].

Combination treatment of Et. O.s and gemcitabine inhibited notch signalling pathway in Panc-1 cells

Et. O.s treatment alone significantly downregulated Notch-1 ICD protein in Panc-1 compared to vehicle control. Interestingly, gemcitabine and rosmarinic acid single treatment had no effect on Notch-1 ICD protein expression in Panc-1, compared to vehicle control [Fig. 9]. The combination treatment of Et. O.s or

rosmarinic acid with gemcitabine downregulated Notch-1 ICD protein expression significantly in Panc-1, compared to vehicle control and single treatments [Fig. 9].

An increased expression of the HEY2 and Notch-1 genes was detected in Panc-1 cells treated with gemcitabine alone when compared to the vehicle control group [Fig. 9B and C]. The HEY2 gene was also up-regulated by Et. O.s, whereas Notch-1 was not affected by Et. O.s treatment in Panc-1 cells, when compared to the vehicle control group [Fig. 9B and C]. Combination treatment of Et. O.s and gemcitabine significantly reduced the expression of HEY2 and Notch-1 genes in Panc-1 cells compared to single treatment and vehicle control groups [Fig. 9B and C]. However, the combination treatment of rosmarinic acid and gemcitabine did not significantly reduce the expression of HEY2 and Notch-1 in Panc-1 cells compared to the vehicle control group [Fig. 9B and C].

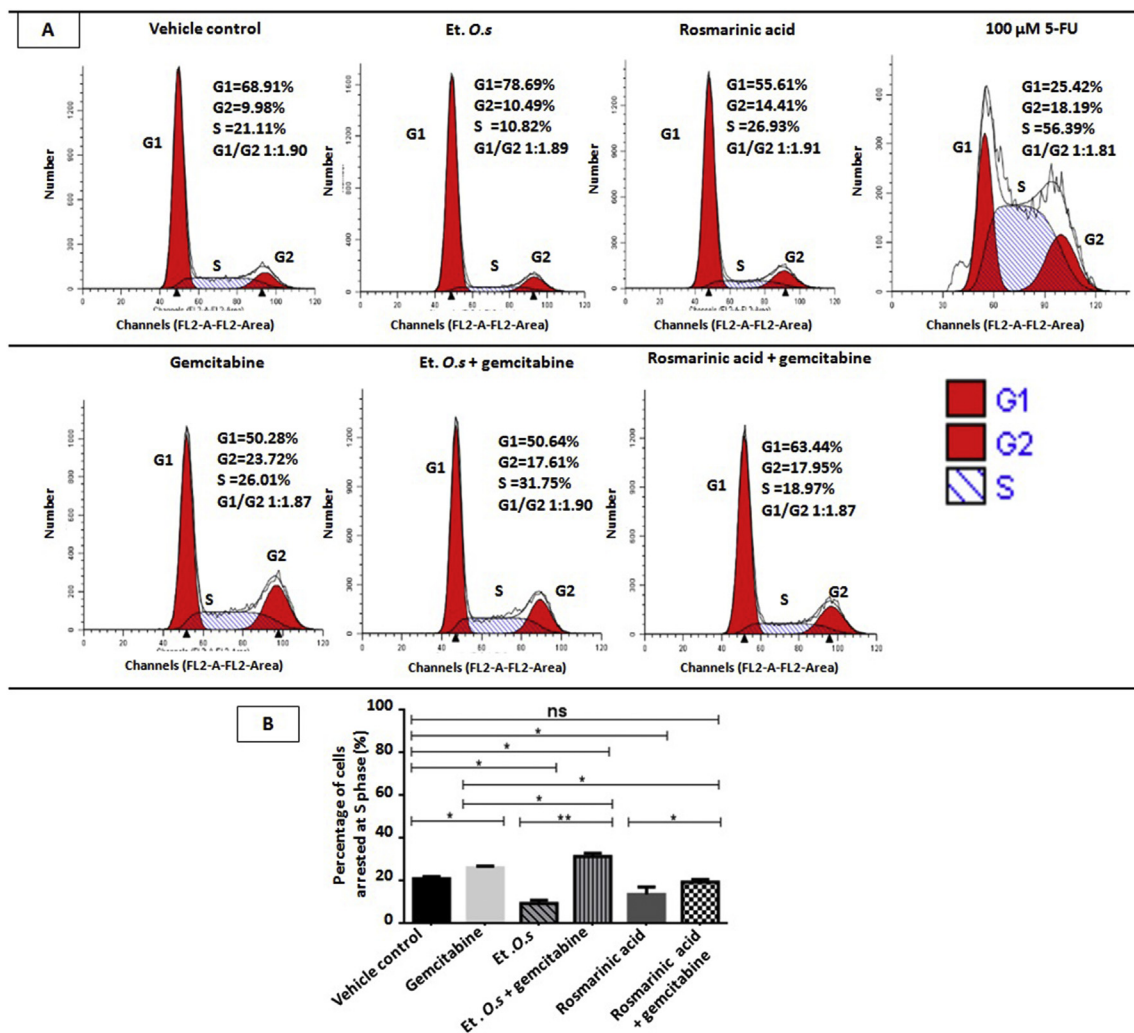


Fig. 6 Combination treatment of Et. O.s and gemcitabine induced cell cycle arrest in Panc-1 cells at 72 h post treatment compared to combination treatment of rosmarinic acid and gemcitabine. At single dose, Et. O.s significantly arrested cells at G1 phase, rosmarinic acid at S phase, and gemcitabine at S phase. (A) FACS pictograms of cells after 72 h incubation with Et. O.s or rosmarinic acid and gemcitabine. (B) The percentage of cells at S phase in different treatment groups. 5-FU was used as positive control. Error bars represent SEM. Statistics analysis (ns = not significant; * $P < 0.05$; ** $P < 0.01$; One way ANOVA, $n = 3$ independent experiments) using GraphPad Prism 6.0 software.

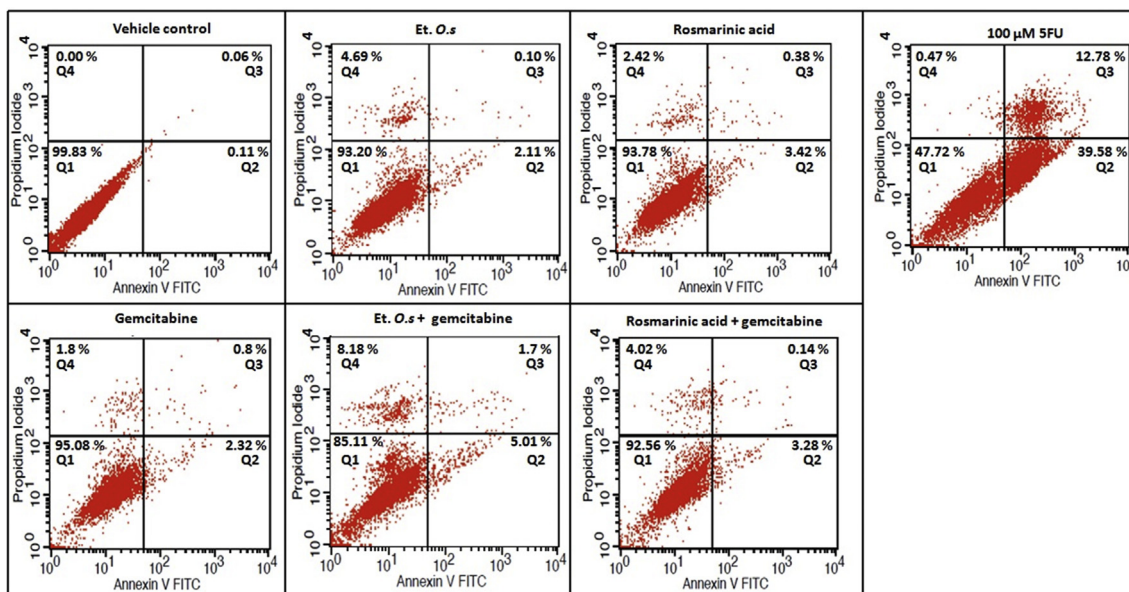


Fig. 7 Flow cytometry cytograms of cells treated with Et. O.s and/or gemcitabine. Combination treatment of Et. O.s and/or gemcitabine induced necrosis (Q4) and early apoptosis (Q2) compared to the combination treatment of rosmarinic acid and gemcitabine and single treatment. The apoptotic fractions of Panc-1 cells were detected by Annexin V-PE and PI double staining. Results were expressed as a percentage of total cells. Cytogram showed Q1 (Live cells), Q2 (early apoptosis), Q3 (late apoptosis), and Q4 (necrotic cells). 5FU was used as positive control.

Combination of Et. O.s and gemcitabine reduced expression of EMT markers, MDR-1, MRP-4, and MRP-5 and induced expression of hENT-1 gene in Panc-1 cells

Et. O.s, rosmarinic acid, and gemcitabine treatments alone induced protein expression of vimentin in Panc-1 72 h post-treatment, compared to vehicle control [Fig. 10A]. Combination treatment of Et. O.s and gemcitabine significantly reduced protein expression of vimentin compared to single treatment [Fig. 10A]. However, the combination treatment of rosmarinic

acid and gemcitabine showed no further reduction in vimentin protein expression compared to either single treatment [Fig. 10A]. Gemcitabine reduced the E-Cadherin cell junction protein expression compared to vehicle control [Fig. 10A]. Combination treatment of Et. O.s and gemcitabine significantly induced the protein expression of E-Cadherin compared to single treatment [Fig. 10A]. However, the combination treatment of rosmarinic acid and gemcitabine did not affect E-Cadherin protein expression, compared to rosmarinic acid single treatment [Fig. 10A].

Et. O.s or gemcitabine single treatment induced the expression of ZEB-1 and Snail-1 genes in Panc-1 72 h post-treatment compared to vehicle control [Fig. 10B and C]. Combination of Et. O.s with gemcitabine downregulated the expression of ZEB-1 and Snail-1 genes in Panc-1 72 h post-treatment, compared to single treatments, respectively [Fig. 10B and C]. However, there was no further reduction in the gene expression of ZEB-1 and Snail-1 when treated with rosmarinic acid and gemcitabine combination, compared to rosmarinic acid alone [Fig. 10A, B, and C].

Gemcitabine treatment induced the expression of the MDR-1, MRP-4, and MRP-5 genes in Panc-1 compared to cells exposed individually to vehicle control, Et. O.s and rosmarinic acid, respectively [Fig. 10D, E, and F]. Et. O.s-gemcitabine combination significantly reduced the expression of MDR-1, MRP-4, and MRP-5 genes and up-regulated the gene expression of the hENT-1 in Panc-1 compared to either single treatments [Fig. 10D, E, F, and G]. In contrast, the rosmarinic acid-gemcitabine combination down-regulated the MDR-1 gene expression when compared to single treatment, but did not further reduce the expression of MRP-4, MRP-5, and hENT-1 genes in Panc-1 [Fig. 10D, E, F, and G].

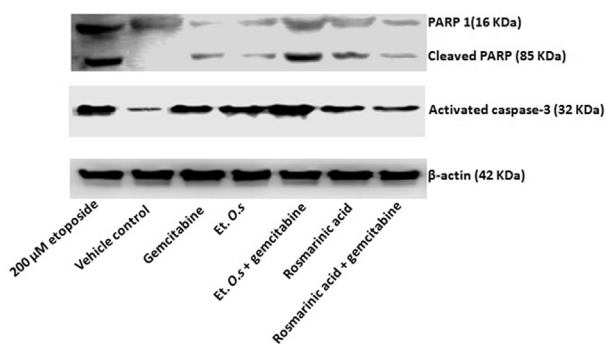


Fig. 8 Protein expression profile of PARP and activated caspase-3 proteins in Panc-1 cells treated with Et. O.s or rosmarinic acid and gemcitabine at 72 h (n = 3). Etoposide used as positive control. Combination treatment of Et. O.s and gemcitabine showed increased protein expressions of cleaved PARP and activated caspase-3 proteins in Panc-1 cells compared to combination treatment of rosmarinic acid and gemcitabine.

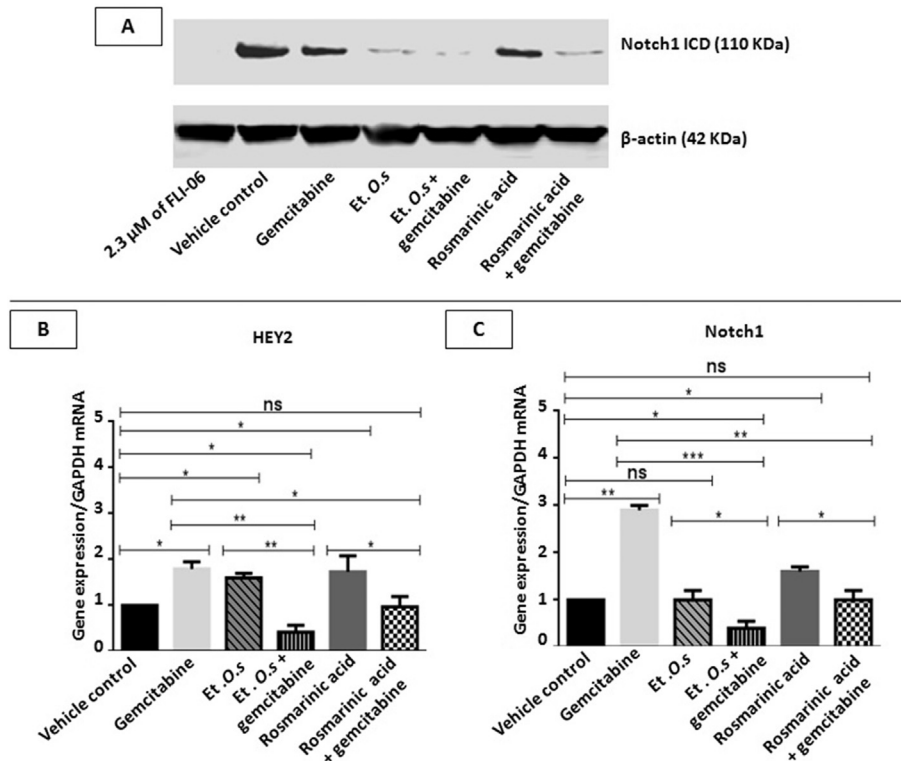


Fig. 9 Protein expression profile of Notch 1 ICD and gene expression profile of HEY2 and Notch1 in Panc-1 cells 72 h post treatment with Et. O.s, rosmarinic acid and gemcitabine. (A) Representative Image of Western blot for Notch1 ICD protein expression investigated in Panc-1 cells. (B) HEY2 gene expression in Panc-1 cells. (C) Notch1 gene expression in Panc-1 cells. Error bars represent SEM. Statistical analysis (ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, One-way ANOVA was performed using GraphPad Prism 6.0 software ($n = 3$ independent experiments).

Discussion

One of the challenges associated with the use of gemcitabine in pancreatic cancer is the loss of chemo-sensitivity. Herbal products containing multiple phytochemicals are known to decrease incidences of chemotherapy-induced side effects. However, the lack of mechanistic insights into herb–drug combinations warrants in-depth research. The proposed Et. O.s-gemcitabine combination has been demonstrated to be non-toxic in our previous *in vivo* study [12]. The current study describes the molecular mechanisms responsible for the anti-cancer effects of ethanol Et. O.s or rosmarinic acid, a marker compound of Et. O.s, in combination with gemcitabine in pancreatic cancer. Two pancreatic cancer cell lines i.e., Panc-1 [Fig. 2D] and MiaPaCa-2 [12] were initially screened and based on the IC_{50} values of combination treatment; Panc-1 cell line was selected for detailed *in vitro* studies. Numerous *in vitro* studies have previously reported the ability of Et. O.s in reducing cell viability in breast (MDA-MB-231), prostate (PC3), and colon (HCT116) cancer cell lines [18,19]. Our work corroborated these findings, as a marked reduction in cell viability [Fig. 2], clonogenic [Fig. 3] and migratory [Fig. 4] potentials of Panc-1 cells were demonstrated when treated with combinations of Et. O.s or rosmarinic acid and gemcitabine. However, these effects were obtained at doses less than IC_{50} values of individual treatments. Other studies have also depicted rosmarinic acid as the principal marker constituent

of Et. O.s [18,20], as also demonstrated in our HPLC analysis [Fig. 1 and Table 1]. Rosmarinic acid has previously been shown to exert anti-lipid peroxidative, anti-cancer, and apoptotic effects in 7,12-dimethylebenz(a)anthracene-induced skin carcinogenesis in Swiss albino mice [21]. These attributes could also possibly explain the diminished cell viability in pancreatic cancer cells in the current study, although this remains to be confirmed experimentally.

Cellular senescence is a protective mechanism to halt the growth of pre-cancerous lesions through indefinite cell cycle arrest. Interestingly, the combination treatment of rosmarinic acid and gemcitabine did not induce cellular senescence in Panc-1 cells, when compared to the combination treatment of Et. O.s and gemcitabine [Fig. 5]. However, an arrest in the S-phase cell cycle was detected in Panc-1 cells treated with gemcitabine in combination with Et. O.s [Fig. 6], suggesting that impediment of cell cycle progression may be one of the mechanisms involved in the reduction of cell viability. In addition, rosmarinic acid significantly triggered cell cycle arrest at the S phase compared to the vehicle control group [Fig. 6]. According to a study conducted by Hashiesh and colleagues, rosmarinic acid also exerted anti-proliferative activity through induction of cell cycle arrest in G1/S and apoptosis in cancer cells [22].

A majority of anti-cancer therapies are known to induce apoptosis. Over the course of treatment, pancreatic cancer cells have evolved to adopt a wide variety of molecular

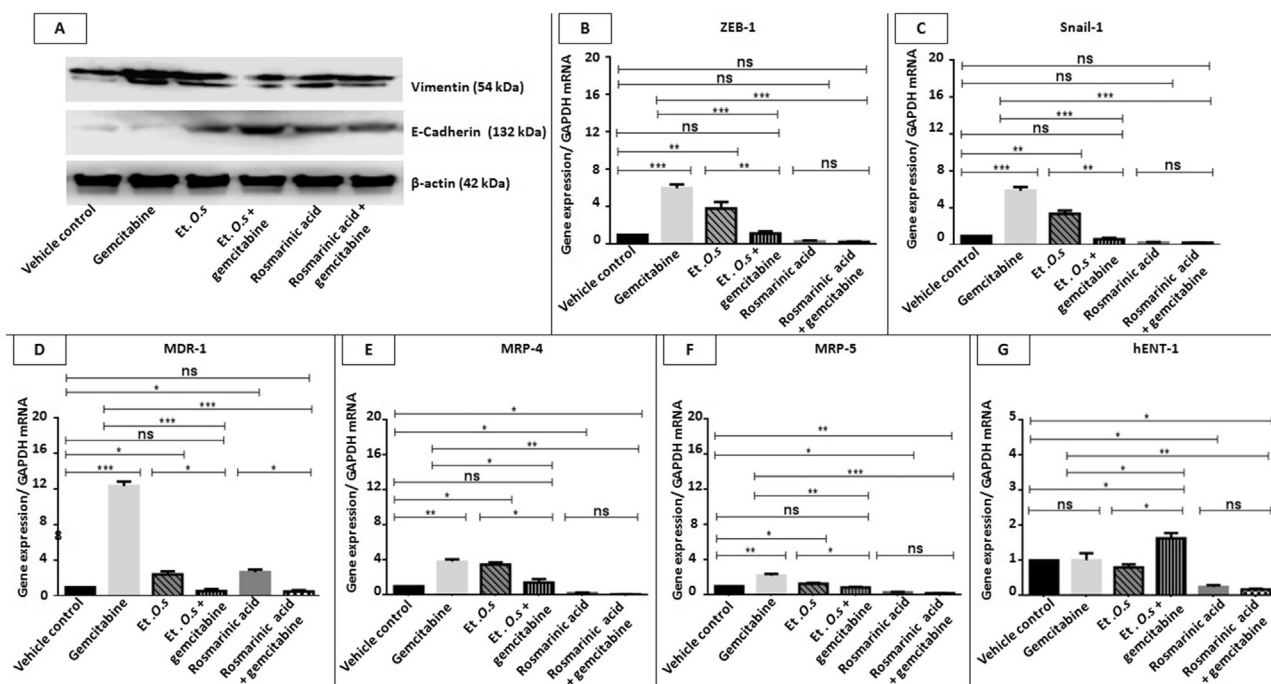


Fig. 10 The effect of Et. O.s or rosmarinic acid with gemcitabine on the EMT markers, multidrug genes (MDR-1, MRP-4, and MRP-5), and hENT-1 genes in Panc-1 cells 72 h post treatment. The effect of Et. O.s/rosmarinic acid and gemcitabine on the expression of (A) vimentin and E-Cadherin proteins, (B) ZEB-1, (C) Snail, (D) MDR-1, (E) MRP-4, (F) MRP-5, and (G) hENT-1 genes. β -actin was used as loading control. Densitometry analysis was performed using Image J software and graphs were plotted using GraphPad Prism 6.0 software. Error bars represent SEM. Statistical analysis (ns = not significant; * P < 0.05; ** P < 0.01; *** P < 0.001, One way ANOVA, n = 3 independent experiments).

mechanisms to bypass the cell death pathways [23]. Therefore, agents which can sensitise the cancer cells to chemotherapies warrant further investigations. Apoptosis can be triggered by the caspase cascade reaction. As a DNA damage repair protein, PARP is degraded by caspase-3 and other cysteine proteinases during apoptosis. The degradation of PARP protein is regarded as an early molecular sign of apoptosis [24]. In the current study, Et. O.s, in combination with gemcitabine activated caspase-3 in Panc-1 to trigger early apoptosis through the cleavage of PARP proteins [Figs. 7 and 8]. Interestingly, rosmarinic acid–gemcitabine combination showed an inferior apoptotic activity compared to the O.s-gemcitabine combination [Fig. 8]. A growing body of evidence has also demonstrated that structural differences in polyphenols could lead to considerable differences in their biological activities [25]. Rosmarinic acid has been suggested to interact with gemcitabine to form new complexes, leading to the alteration of biological activities of rosmarinic acid. Altogether, these suggest that multicomponent chemo-herbal (Et. O.s-gemcitabine) combination may be a more effective option to treat pancreatic cancer compared to the rosmarinic acid–gemcitabine combination, at least *in vitro*.

Given the inhibitory effect on cell migration of this chemo-herbal combination, the molecular pathway responsible for these attributes was further examined. The Notch signalling pathway plays a central role in pancreatic tumorigenesis. This signalling pathway is activated through the binding of Notch ligands to the receptors to produce active fragments, known as the Notch intracellular domain (NICD). The activation of

canonical Notch signalling pathway eventually prompts the up-regulation of Notch target genes, including the HEY family genes [26]. These genes are involved in the regulation of diverse processes including cell cycle progression, cell proliferation, apoptosis, differentiation, migration, invasion, and survival, all of which are related to pancreatic cancer development and progression [27]. Notch 1 is abundantly expressed in invasive pancreatic ductal adenocarcinoma, and has been reported as a key member involved in the pancreatic carcinogenesis [28]. The activation of Notch 1 is also known to induce ZEB1 and Snail-1, EMT markers in pancreatic cancer cells [29]. HEY2 has been reported to play a key function in tumorigenesis and cancer development [30]. Significant downregulation of Notch signalling via the Notch 1 ICD protein and its downstream target genes (Notch 1 and HEY2) was demonstrated in Panc-1 cells treated with gemcitabine in combination with Et. O.s or rosmarinic acid [Fig. 9], indicating that hampered Notch signalling could possibly be responsible for the anti-migratory attribute that involves the regulation of EMT.

The Et. O.s–gemcitabine combination treatment induced the expression of E-Cadherin protein with simultaneous downregulation of vimentin protein, as well as ZEB-1 and Snail-1 genes [Fig. 10A, B, C]. In an elegant study, Gradiz and colleagues have reported that Panc-1 cells expressing low levels of E-Cadherin protein had a higher tendency to undergo EMT [31]. However, the overexpression of mesenchymal marker vimentin in cancer cells, including pancreatic cancer, is positively associated with accelerated tumour growth [32].

ZEB-1, Snail-1 and other regulators of EMT have also been described to confer drug resistance in human pancreatic cancer cells [33], hence the impediment of these molecular targets in the Et. O.s-gemcitabine treatment group may explain the sensitisation of Panc-1 cells to the treatment to reduce cell viability.

Mounting evidence has also indicated EMT markers to be important regulators of ABC transporters [34], which are responsible for the reduction of drug uptake/penetration into the cancer cells to cause drug insensitivity. Hence, the diminished expression of EMT markers and their downstream components, including the ABC transporters are one of the strategies for sensitisation of cancer cells to treatments [35]. In the current study, treatment with gemcitabine alone induced the expression of MDR-1, MRP-4, and MRP-5 genes four times higher compared to vehicle control cells [Fig. 10D, E, and F]. However, the combination treatment of Et. O.s or rosmarinic acid with gemcitabine was found to reduce the expression of MDR-1, MRP-4, and MRP-5 genes, delineating a potential role of these chemo-herbal combinations in the treatment of resistant pancreatic cancers. Rosmarinic acid has been reported to reverse multi-drug resistance in human gastric cancer cells by decreasing the expression of P-glycoprotein gene [36], as also observed in our study [Fig. 10D–F].

Moreover, Et. O.s could reduce the expression of the multi-drug resistant genes through the increased expression of human equilibrative nucleoside transporter type 1 (hENT-1) gene in Panc-1 cells. Recently, mounting evidence has shown that elevated expression of hENT-1 proteins in cells could increase the intracellular uptake of gemcitabine [6]. In the current study, treatment with Et. O.s and gemcitabine significantly induced the expression of hENT-1 gene [Fig. 10G]. It is essential to highlight that relatively low doses of gemcitabine were used in combination treatments, however, due to the ability of Et. O.s to induce hENT-1 expression [Fig. 10G], gemcitabine may be transported into Panc-1 cells to possibly produce the profound effects which otherwise were not possible at such a low dose. In contrast, rosmarinic acid alone or in combination with gemcitabine downregulated hENT-1 gene expression in Panc-1 cells compared to vehicle control cells [Fig. 10G]. The hENT-1 protein has been reported to reverse gemcitabine-induced drug resistance and facilitate gemcitabine entry into cancer cells [37]. Treatment with rosmarinic acid alone reduced the expression of hENT-1 gene in Panc-1, which may eventually hinder the influx of gemcitabine. This may account for the low expression of MRP-4 and MRP-5 genes in rosmarinic acid-treated cells or when gemcitabine was added in combination with rosmarinic acid.

Overall, it is postulated that Et. O.s worked in synergy with gemcitabine to reduce pancreatic cell viability and induced apoptosis compared to single treatments of either Et. O.s or gemcitabine and bioactive compound, rosmarinic acid. Therefore, based on current findings, we propose that 50% Et. O.s has the potential to be used in combination with gemcitabine to treat pancreatic cancer *in vitro*.

Conclusion

For the first time, we have highlighted the beneficial effects of standardised 50% ethanol extract of O.s alone and in

combination with gemcitabine towards pancreatic cancer. The downregulation of the Notch signalling pathway via Notch ICD along with its downstream components is postulated to be responsible for observed anti-migratory, proapoptotic and anti-cancer attributes of current chemo-herbal combination in pancreatic cancer, *in vitro*. This study also showed that rosmarinic acid, being the principal marker compound in 50% Et. O.s, regulated some of the molecular players differently from Et. O.s, for example, the EMT, cell cycle, apoptosis, and Notch pathways. However, rosmarinic acid failed to show any beneficial effect on its own or in combination with gemcitabine which might be due to the formation of inactive complexes. Therefore, keeping in view the established non-toxic nature of chemo-herbal combination, it is concluded that 50% ethanolic extract of O.s has the potential to be developed as a complementary agent to gemcitabine for the treatment of pancreatic cancer. Data obtained from this study is imperative to form the basis of dose selection for future pre-clinical studies. On-going work includes the investigation of the effects of Et. O.s and gemcitabine combination in pancreatic tumour xenograft models. Findings from this work may provide the fundamentals for product formulation and manufacturing of botanical drugs as well as to pave ways for clinical trials in pancreatic cancer patients in the future. It is justifiable to carry out future studies on rosmarinic acid and its new derivatives efficacy when used alone or in combination with other chemotherapeutic drugs in pancreatic cancer.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bj.2020.05.015>.

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