Xanthoxyletin blocks the RANK/RANKL signaling pathway to suppress the growth of human pancreatic cancer cells

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ABSTRACT

Xanthoxyletin is a vital plant-derived bioactive coumarin. It has been shown to exhibit anticancer effects against different human cancers. Nonetheless, the anticancer effects of xanthoxyletin against human pancreatic cancer cells have not been evaluated. Against this backdrop, the present study was designed to evaluate the anticancer effects of xanthoxyletin in human pancreatic cancer cells and to decipher the underlying molecular mechanisms. The results revealed a significant ($p < 0.05$) upregulation of receptor activator of NF-kappaB (RANK), receptor activator of NF-kappaB ligand (RANKL) and osteoprotegerin (OPG) in human pancreatic tissues and cell lines at both transcriptional and translational levels. The administration of pancreatic cancer cells with xanthoxyletin diminished the viability of Capan-2 cells in a concentration-dependent manner and led to a significant decline in RANK, RANKL, and OPG expression. Silencing of RANK and xanthoxyletin treatment declined the viability of Capan-2 pancreatic cancer cells *via* induction of apoptosis. However, pancreatic cancer cells overexpressing RANK could rescue the growth inhibitory effects. Collectively, xanthoxyletin targets the RANK/RANKL signaling pathway in pancreatic cancer cells to induce cell apoptosis and may prove to be an important lead molecule.

Pancreatic cancer is counted amongst the fatal human malignancies and ranks fourth most lethal (1). In the USA, pancreatic cancer makes up to 3 % of cancer cases and its prevalence rates are around 12.5 per 0.1 million human population (1, 2). The disease diagnosis is one of the crucial factors responsible for higher mortality of pancreatic cancer (3). Although the mortality rates of pancreatic cancer have slightly declined during the past decade nevertheless the studies have suggested that pancreatic cancer might be the second most mortal cancer in the next decade, particularly in Western countries. Therefore suggest

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the inefficacy of the therapeutic modalities presently used against this deadly disease (4, 5). There are reports that the natural compounds and their semi-synthetic derivatives, seemingly with low toxicity against the normal human body cells, are the molecules of choice to act as led therapeutic agents against pancreatic cancer (6–8). Taking the lead from these findings and considering the utility of coumarins and their derivatives against different human cancer types, the present study investigated the efficacy of xanthoxyletin against pancreatic cancer (9). Xanthoxyletin is a plant-based pyranocoumarin whose anticancer effects have been reported in both *in vitro* and *in vivo* systems (10). However, the effects of this molecule and its mechanism of action have not yet been studied against pancreatic cancer cells.

Several studies have shown that RANK signalling plays a crucial role in a number of malignancies (11, 12). In the tumour microenvironment, RANK and RANKL-expressing cells are frequently present. In malignancies of the breast, endometrial, prostate, oesophagus, cervix, bladder, stomach and thyroid, the RANKL/RANK pathway is frequently over-expressed and is associated with a poor prognosis (13, 14). Osteoprotegerin interacts with RANKL and regulates RANK/RANKL signaling. Combining antibodies that block OPG or RANK with chemotherapy, hormone therapy, or targeted medications led to a larger decrease in tumour burden in bone tumors (12). The effects of xanthoxyletin on pancreatic cancer have not yet been studied. The present study was designed to study the effect of xanthoxyletin on RANK/RANKL signalling in pancreatic cancer cells.

EXPERIMENTAL

Tissue samples

After obtaining their written consent, the pancreatic cancer tissues and the normal adjacent tissues were procured from 30 patients who underwent surgery at the First People's Hospital of Lianyungang, Lianyungang, Jiangsu, China. The institutional ethics committee approved the study. Liquid nitrogen was used for quick freezing and transportation of the tissues, and then stored at –80 °C until use.

Cell lines and culturing

The pancreatic cancer cell lines (Capan-2 and MIA PaCa-2) and the normal pancreatic epithelial cells (H6c7) were procured from the cell bank of the Chinese Scientific Academy. The cells were maintained and cultured in RPMI-1640 medium (GIBCO, Life Technologies, USA) supplemented with 10 % fetal bovine serum (FBS; Thermo Fisher Scientific, USA). Cell culturing was performed at 37 °C with a 5 % atmosphere in a humidified incubator.

Transfection

The siRNAs targeting RANK transcripts, si-RANK and the respective negative control, si-NC; RANK overexpression plasmid (pcDNA-RANK) and the vector control (pcDNA3.1) were obtained from Ribobio (Guangzhou, China). The Lipofectamine 3000 reagent (Thermo Fisher Scientific) was used to transfect the Capan-2 cancer cells according to the manufacturer's instructions. The culture medium was replaced with fresh medium after 24 h of transfection.

Expression analysis

Total RNA was extracted from tissues and cell lines with the help of TRIzol Reagent (Thermo Fisher Scientific) as per the standard procedure. The RNA was purified by DNase (Invitrogen) treatment and subsequently converted into cDNA using the RevertAid cDNA synthesis kit (Thermo Fisher Scientific). qRT-PCR was performed on StepOnePlus Real-Time System (Thermo Fisher Scientific) to analyze the transcript levels *RANK, RANKL,* and *OPG* in the tissue samples and cell lines. The actin gene was used for normalizing the PCR template. The $2^{-\Delta\Delta CT}$ method was used for the determination of relative expression levels.

Western blotting analysis

The cell lysates or tissue fractions were treated with RIPA buffer (Sigma-Aldrich, USA) to extract the total proteins and then quantified by BCA protein assay kit (Beyotime, China). Equal proteins (30 µg) were resolved on 8–10 % SDS-PAGE gels and the latter were blotted onto the PVDF membranes (Bio-Rad). Membranes were blocked with 5 % skim milk (Sigma-Aldrich) for 45 minutes and subsequently incubated with specific primary antibodies at 4 °C overnight. This was followed by their incubation with an anti-rabbit secondary antibody (Abcam, UK; 1:4000) at room temperature for 2 h. Odyssey Imaging System was used for the detection of specific protein bands which were then analyzed using Odyssey v2.0 software (LICOR Biosciences, USA). β-Actin served as an internal control.

Immunohistochemical analysis

The immunohistochemical staining procedures were carried out as per the standard experimental protocols. The paraffin-embedded tissue sections were washed with ethylenediaminetetraacetic acid (EDTA) (pH = 9.0). The tissue sections were then exposed to a microwave for retrieving RANK, RANKL, or OPG antigens. Afterward, they were incubated with specific primary antibodies (Cell Signaling Technology, USA) at 4 °C overnight and then with the horseradish peroxidase-conjugated secondary antibody (DAKO, Denmark) for 30 min at 37 °C in an air oven. Lastly, the DAB solution (Sigma-Aldrich) was used to detect antibody binding and sections were stained with hematoxylin for 30 seconds. The immuno-histochemical staining was assessed with an image analysis workstation (Image Pro plus 6.0, Media Cybernetics).

MTT assay

Xanthoxyletin was prepared in dimethyl sulfoxide (DMSO) solution to obtain 1 mg mL^{-1} stock solution. 100 µL cell suspensions obtained from the xanthoxyletin-treated or transfected cells were plated in a 96-well plate at the concentration of 4000 cells per well. Control cells were treated with DMSO only. The cells were incubated at 37 °C with 5 % CO_2. Each well was added with 15 µL of MTT reagent (Sigma-Aldrich, Germany) at different culture durations. The cells were again incubated at 37 °C for 4 h. DMSO was added to each well to dissolve the formazan precipitate. Finally, the absorbance was calculated at 570 nm using a microplate reader (Multiskan^{TM} FC Microplate Photometer, Thermo Scientific).

EdU assay

The EdU assay was performed using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, China). The cancer cells were plated at a density of 10^5 cells per well in a 24-well plate. Following an initial incubation of 2 h with 50 mmol L^{-1} EdU, 4 % paraformaldehyde (Sigma-Aldrich) was used for fixing the cells. Afterward, the cancer cells were treated with Apollo Dye Solution. The nuclei of the cancer cells were stained using a DAPI solution. Fluorescent microscopy (Olympus, Japan) was used to visualize and photograph cells.

Annexin V-FITC/PI apoptosis assay

Capan-2 cancer cells, either xantoxyletin treated or transfected with si-NC along with respective negative controls, were lysed using 0.25 % trypsin (Gibco). The lysed cells were washed thrice with PBS. Then, the cells were mixed with 150 μL of binding buffer. A cell suspension was constituted with a density of 10^5 cells mL^{-1} and inoculated with annexin V-FITC (Yeasen Biotech Co., Ltd.) and PI solutions in dark at room temperature for 15 min. The cell apoptosis was analyzed with the help of an FC500MCL flow cytometer.

Statistical analysis

The experiments were conducted at least three times independently and final values were presented as mean ± SD. Data were normalized by descriptive statistics using a normality test. Student's *t*-test and one-way ANOVA were performed on GraphPad Prism 4 (GraphPad Software Inc, USA) to analyze the statistical significance. *p*-values < 0.05 were used to represent the statistically significant inter-value difference.

RESULTS AND DISCUSSION

Pancreatic cancer is associated with RANK/RANKL pathway upregulation

Human cancers are linked with the overexpression of signalling cascades like the RANK (receptor activator of nuclear factor-kB)/RANKL (ligand of RANK, a tumor necrosis factor-alpha superfamily cytokine) pathway. To confirm whether the same is true for pancreatic cancer, the expression analyses of signaling components like RANK, RANKL and OPG (osteoprotegerin) were performed. The pancreatic cancer tissues exhibited significantly higher expression of RANK, RANKL and OPG genes (Fig. 1a-c). The immuno-histochemical staining method was used to infer the expression of RANK, RANKL and OPG at the translational level. The staining study showed that the pancreatic cancer tissues express markedly higher RANK, RANKL and OPG protein levels (Fig. 1d-f). Overexpression of RANK, RANKL and OPG proteins was also noted from the pancreatic cancer cell lines (Capan-2 and MIA PaCa-2) compared to normal pancreatic epithelial cells (H6c7) (Fig. 2a). The qRT-PCR results were also consistent with the findings of Western blotting and RANK, RANKL and OPG transcript levels were shown to be remarkably higher in pancreatic cancer cell lines compared to normal pancreatic cells (Fig. 2b-d). The results suggest that pancreatic cancer has RANK/RANKL pathway upregulation.
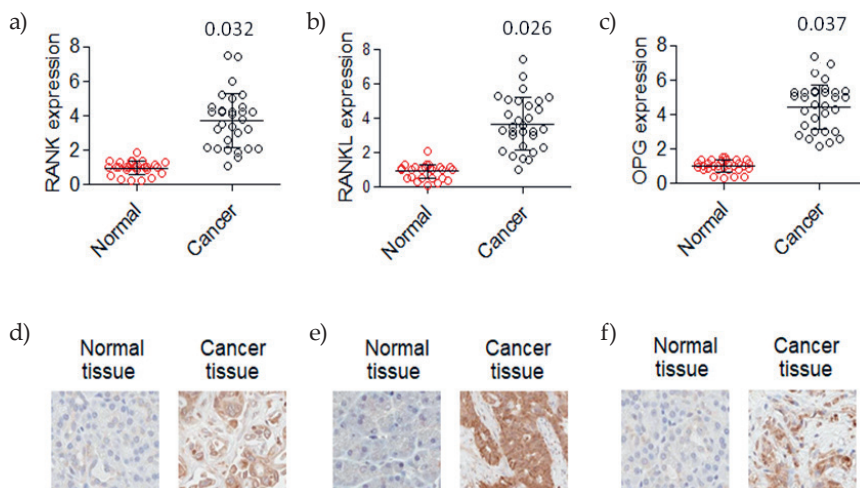
Fig. 1. RANK/RANKL pathway is upregulated in pancreatic cancer. a) Relative gene expression of receptor activator of NF-kappaB (RANK) in normal and cancerous pancreatic tissues; b) relative gene expression of receptor activator of NF-kappaB ligand (RANKL) in normal and cancerous pancreatic tissues; c) relative gene expression of osteoprotegerin (OPG) in normal and cancerous pancreatic tissues, d) immuno-histochemical staining of RANK from normal and cancerous pancreatic tissues; e) immuno-histochemical staining of RANKL from normal and cancerous pancreatic tissues; f) immuno-histochemical staining of OPG from normal and cancerous pancreatic tissues. The experiments were performed in triplicate and are expressed as mean ± SD.

Cancer cells evade the normal developmental program and proliferate in an uncontrolled fashion. Fate is achieved through the accumulation of a vast number of genetic mutations (15). Of note, several signaling pathways deviate at the molecular level and their altered expression mediates the cancer onset and unrestrained cell proliferation (16). Overexpression of the RANK/RANKL signaling pathway has been documented to be linked with different types of human cancers and the signaling components like RANK, RANKL and OPG have been deduced to act as key therapeutic targets in cancer management (17–19). Van Dam *et al.* have stressed the targeted inhibition of the RANK/RANKL signaling pathway as a breakthrough in cancer treatment (20). In the present study, pancreatic cancer was shown to be associated with the upregulation of the RANK/RANKL signaling pathway.

Xanthoxyletin exhibited antiproliferative effects against the pancreatic cancer cells via *the RANK/RANKL signaling pathway*

The H6c7 normal pancreatic cells and Capan-2 cancer cells were administered with different concentrations of xanthoxyletin for 24 h and the effects on cell viability were analyzed using an MTT assay. Xanthoxyletin treatment inhibited the growth of both normal and cancerous pancreatic cells; however the latter was affected more prominently (Fig. 3a). The IC_{50} value of xanthoxyletin was 110 µmol L^{-1} against H6c7 pancreatic cells, while it was significantly low against the Capan-2 cancer cells, *i.e.* 7 µmol L^{-1}. Interestingly, the Western blotting and qRT-PCR studies showed that xanthoxyletin repressed the RANK,
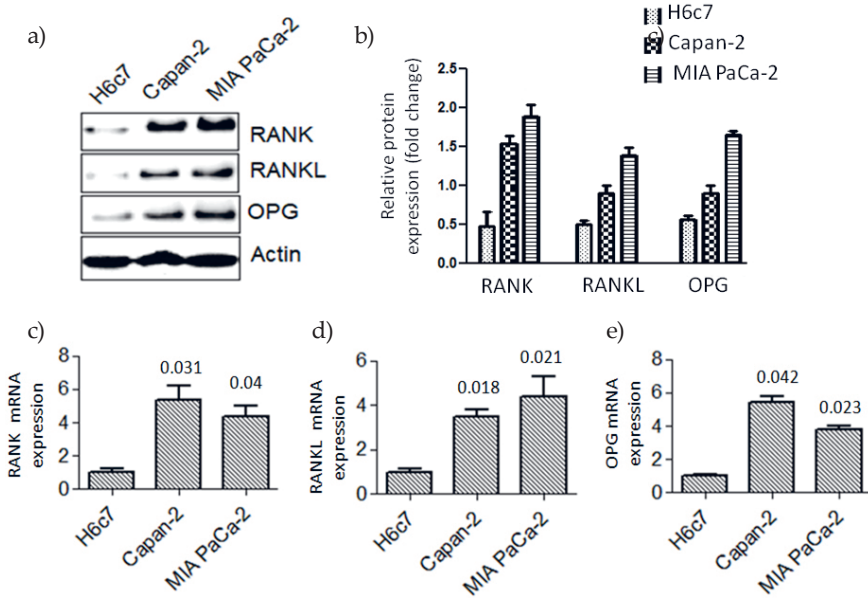
Fig. 2. RANK/RANKL pathway is overexpressed in pancreatic cancer cell lines. a) Expression levels of RANK, RANKL and OPG proteins in a normal epithelial cell line (H6c7) and pancreatic cancer cell lines (Capan-2 and MIA PaCa-2; b) densitometry analysis of Western blot; c) RANK mRNA levels exhibited by normal epithelial cell line (H6c7) and pancreatic cancer cell lines (Capan-2 and MIA PaCa-2); d) RANKL mRNA levels exhibited by a normal epithelial cell line (H6c7) and pancreatic cancer cell lines (Capan-2 and MIA PaCa-2); e) OPG mRNA levels exhibited by a normal epithelial cell line (H6c7) and pancreatic cancer cell lines (Capan-2 and MIA PaCa-2). The experiments were performed in triplicate and are expressed as mean ± SD.

RANKL and OPG in a dose-dependent manner at both translational and transcriptional levels (Fig. 3b-e). This indicates that xanthoxyletin inhibited pancreatic cancer cell growth *in vitro* by blocking the RANK/RANKL signaling pathway.

The administration of pancreatic cancer cells with xanthoxyletin significantly declined the RANK/RANKL signaling pathway and induced apoptosis in pancreatic cancer cells. The pro-apoptotic effects of xanthoxyletin have also been reported against oral cancer cells, together with its potential to induce the arrest of cell division (21). Furthermore, xanthoxyletin has been reported to inhibit the growth of gastric cancer cells by inducing apoptosis and cell cycle arrest. In a previous study, xanthoxyletin was reported to reduce the growth of gastric adenocarcinoma cells by triggering apoptosis and arrest of the cell cycle (22).

RANK gene silencing mimicked the antiproliferative effect of xanthoxyletin and induced apoptosis in pancreatic cancer cells

The RANK gene was silenced in Capan-2 cancer cells by transfecting them with small interfering oligos of RANK (si-RANK), while the si-NC transfected Capan-2 cells were used as negative control (Fig. 4a). MTT assay was performed to analyze the impact on cell
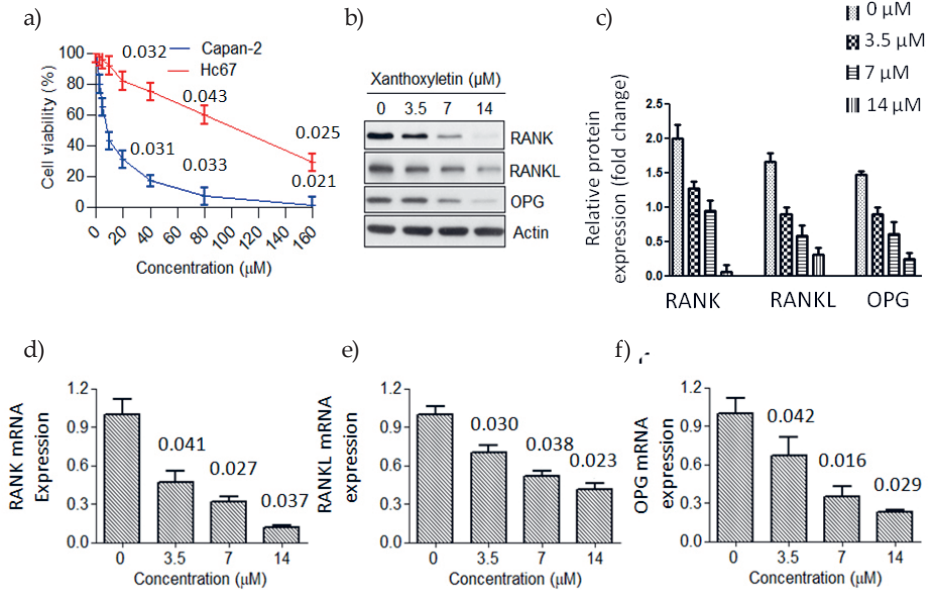
Fig. 3. Xanthoxyletin reduced the viability of pancreatic cancer cells and blocked the RANK/RANKL pathway. a) Viability assessment of normal H6c7 and cancerous Capan-2 pancreatic cells treated with different doses of xanthoxyletin; b) analysis of RANK, RANKL and OPG protein levels from Capan-2 cells treated with varying concentrations of xanthoxyletin; c) densitometry analysis of Western blot; d) mRNA level of RANK expressed by Capan-2 cells treated with varying concentrations of xanthoxyletin; e) mRNA level of RANKL expressed by Capan-2 cells treated with varying concentrations of xanthoxyletin; f) mRNA level of OPG expressed by Capan-2 cells treated with varying concentrations of xanthoxyletin. The experiments were performed in triplicate and expressed as mean ± SD.

viability of si-RANK transfected Capan-2 cancer cells compared to the negative control cancer cells. The si-RANK transfected cancer cells showed significantly lower cell viability rates when compared with the respective negative control cell viability rate at different time durations (Fig. 4b). The decline in pancreatic cancer cell viability also showed a similar trend under 7 µmol L^{-1} xanthoxyletin treatment (Fig. 4c). The EdU staining assay also revealed that RANK silencing and xanthoxyletin treatment led to a significant loss of proliferative viability in Capan-2 pancreatic cancer cells (Fig. 4d-e). The percentage of apoptotic (early and late) cells was significantly higher for si-RANK transfected or xanthoxyletin-treated Capan-2 cancer cells compared to the corresponding negative control cells (Fig. 4f-g). The percentage of apoptotic cells increased from 3.78 to 18.41 under RANK silencing and 3.38 to 17.25 under xanthoxyletin administration. The results thus indicate that Capan-2 cancer cells are inducted with apoptotic cell death by RANK gene silencing and xanthoxyletin treatment.

RANK was shown to be the main target of xanthoxyletin in pancreatic cancer modulating its antiproliferative effects, *in vitro*. Molecular targeting of RANK has already been shown to act as a vital lead against cancer, and the RANK-RANKL axis has been deduced to offer a critical paragon for the anticancer drug design (19, 23).
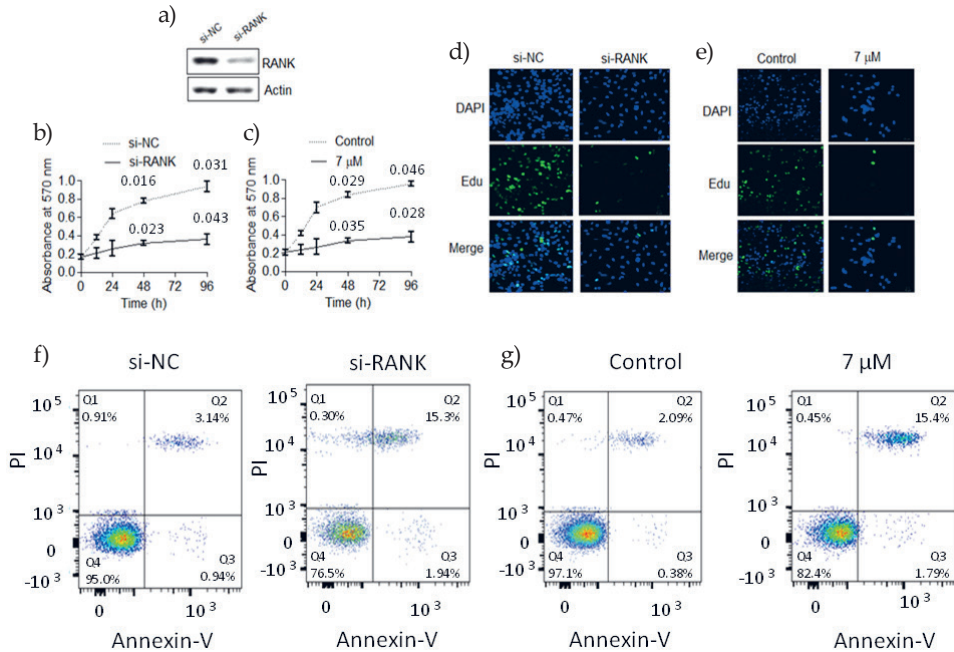
Fig. 4. Xanthoxyletin targeted RANK to induce apoptosis in pancreatic cancer cells. a) Confirmation of silencing of RANK protein in si-RANK transfected Capan-2 cancer cells; b) MTT assay-based viability analysis of si-RANK or si-NC transfected Capan-2 cancer cells; c) viability assessment of Capan-2 cancer cells treated with 7 μmol L^{-1} xanthoxyletin for varying durations; d) EdU staining assay of si-RANK or si-NC transfected Capan-2 cancer cells; e) EdU staining assay of Capan-2 cancer cells treated with 7 μmol L^{-1} xanthoxyletin; f) annexin V-FITC/PI apoptosis analysis of si-RANK or si-NC transfected Capan-2 cancer cells; g) annexin V-FITC/PI apoptosis analysis of Capan-2 cancer cells treated with 7 μmol L^{-1} xanthoxyletin. The experiments were performed in triplicate and expressed as mean ± SD.

RANK overexpression attenuated the antiproliferative effects of xanthoxyletin on pancreatic cancer cells

To gain more support regarding the targeting of RANK by xanthoxyletin in pancreatic cancer cells, RANK was experimentally overexpressed in Capan-2 cancer cells. RANK overexpressing cancer cells were administered with 7 μmol L^{-1} xanthoxyletin for 0, 24, 48, 72 or 96 h. MTT assay was performed to estimate the cell viability rate of xanthoxyletin administered RANK overexpressing cancer cells and compared with untreated and only xanthoxyletin (7 μmol L^{-1}) Capan-2 cancer cells. Surprisingly, the RANK overexpressing cancer cells proliferate as well as untreated cancer cells despite being treated with xanthoxyletin (Fig. 5a). Further confirmation was drawn from the EdU staining assay. The relative proportion of RANK overexpressing Capan-2 cancer cells treated with 7 μmol L^{-1} xanthoxyletin was almost the same as that of the untreated cells (Fig. 5b). The results are thus conclusive that xanthoxyletin treatment down-regulates the RANK gene in pancreatic cancer cells to induce apoptosis and therefore inhibit their growth and viability *in vitro*.
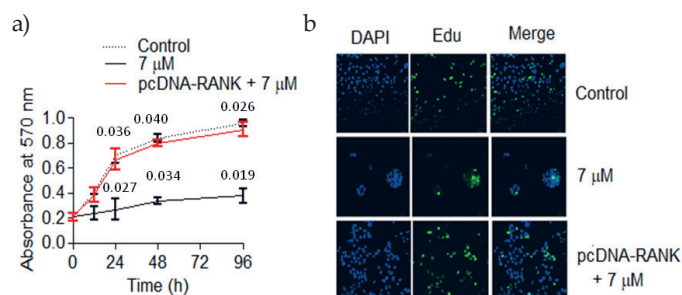
Fig. 5. RANK overexpression attenuated the antiproliferative effects of xanthoxyletin on pancreatic cancer cells. a) MTT assay-based cell viability analysis of Capan 2 cancer cells administered with 7 µmol L^{-1} xanthoxyletin alone or conjugated with RANK overexpression with reference to untreated or control cancer cells; b) proliferative viability analysis of Capan 2 cancer cells administered with 7 µmol L^{-1} xanthoxyletin alone or conjugated with RANK overexpression with reference to untreated or control cancer cells by EdU staining assay. The experiments were performed in triplicate and expressed as mean ± SD.

The inhibition of RANK has been earlier proven to induce apoptosis in cancer cells (24). Results similar to our present study have been reported by murrayanine treatment, which targets the RANK/RANKL pathway to inhibit breast cancer progression (25).

Summing up, the study based on *in vitro* experimentations established the anticancer potential of xanthoxyletin against pancreatic cancer and worked out the mechanism of its action; however same needs to be studied *in vivo* systems also.

CONCLUSIONS

Pancreatic cancer exhibits overexpression of the RANK/RANKL signaling pathway. The administration of pancreatic cancer cells with xanthoxyletin inhibited their growth and proliferative vigor *in vitro via* RANK/RANKL pathway blockade. The latter inducted the pancreatic cancer cells with programmed cell death. RANK was shown to be the primary target of xanthoxyletin. The current study proved the therapeutic importance of xanthoxyletin and might pay the way for understanding and exploring the anticancer potential of this vital compound against other types of human cancers in the future.

Conflict of interest. – All the authors declare that there is no conflict of interest.

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