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Cancer Lett. Author manuscript; available in PMC 2014 August 19.

Published in final edited form as:
Cancer Lett. 2013 August 19; 336(2): 270–280. doi:10.1016/j.canlet.2013.03.017.

Holy Basil Leaf Extract Decreases Tumorigenicity and Metastasis of Aggressive Human Pancreatic Cancer Cells *in vitro* and *in vivo:* Potential Role in Therapy

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Abstract

There is an urgent need to develop alternative therapies against lethal pancreatic cancer (PC). *Ocimum sanctum* ("Holy Basil") has been used for thousands of years in traditional Indian medicine, but its anti-tumorigenic effect remains largely unexplored. Here, we show that extracts of *O. sanctum* leaves inhibit the proliferation, migration, invasion, and induce apoptosis of PC cells *in vitro*. The expression of genes that promote the proliferation, migration and invasion of PC cells including activated ERK-1/2, FAK, and p65 (subunit of NF-κB), was downregulated in PC cells after *O. sanctum* treatment. Intraperitoneal injections of the aqueous extract significantly inhibited the growth of orthotopically transplanted PC cells *in vivo* ($p<0.05$). Genes that inhibit metastasis (*E-cadherin*) and induce apoptosis (*BAD*) were significantly upregulated in tumors isolated from mice treated with *O. sanctum* extracts, while genes that promote survival (*Bcl-2* and *Bcl-xL*) and chemo/radiation resistance (*AURKA*, *Chk1* and *Survivin*) were downregulated. Overall, our study suggests that leaves of *O. sanctum* could be a potential source of novel anticancer compounds in the future.

Keywords

Basil; Pancreatic cancer; therapy; apoptosis; tumorigenicity

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Conflicts of Interest Statement
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1. Introduction

Pancreatic cancer (PC) has a dismal prognosis with a median 5-year survival of about 5% [1]. This is in large part due to the intrinsic resistance of PC cells to chemo and radiation therapy [2, 3]. Thus, there has been a concerted effort towards the development of newer drugs that can overcome this inherent resistance. Plant-derived chemicals (phytochemicals) have emerged as a potential source of novel anticancer compounds. Several natural products including curcumin [4], fisetin [5] and thymoquinone [6] have been demonstrated to enhance the sensitivity of PC cells to chemotherapeutic agents. Although initially promoted as chemopreventive drugs, they have also shown significant pro-apoptotic, anti-proliferative and anti-metastatic effects on cancer cells, prompting a call for their introduction as therapeutic agents.

Ocimum sanctum (commonly known as “Holy Basil”) is a medicinal herb found in the semitropical and tropical parts of India. It has been used for thousands of years in the Ayurvedic and Siddha systems of medicine to treat diverse ailments including infections, skin and liver disorders and as an antidote for snake and scorpion bites [7]. It has been used as an anti-inflammatory, immunomodulatory, anti-infective, anti-stress, antipyretic, antitussive, anti-diabetic [8], cardioprotective, neuroprotective and hepatoprotective agent [9, 10]. Infusions of *O. sanctum* have been shown to protect human lymphocytes from genotoxic stress induced by Cyproterone acetate [4]. Although every part of the plant has been suggested to have therapeutic uses, the leaves (and leaf extracts) have been most extensively studied. The leaves of *O. sanctum* are the source of an essential oil which has numerous medicinal properties. Both ethanolic and essential oil basil extracts have previously been shown to have antioxidant effects [10–15]. Ethanolic extracts have been shown to promote epithelialization of wounds and to counteract the healing suppressant effect of dexamethasone in albino rats [16]. Eye drops containing *O. sanctum* leaf extract protected against ferric chloride-induced lipid peroxidation and exhibited significant antibacterial and antifungal activity [13]. Another study has shown that essential basil oil fed to male Wistar rats significantly decreased the levels of serum lipids [14], while supplementation of fresh basil leaves (2g/kg) daily for 30 days significantly lowered blood glucose and peroxidized lipid levels [17].

Few studies have also demonstrated the potential of *O. sanctum* as an anti-tumor agent [18]. In a comparison of the cytotoxic activity of essential oils from 17 Thai medicinal plants, *O. sanctum* was the most effective in inhibiting the proliferation of human oral squamous cell carcinoma (KB) and mouse leukemia cells (P388) *in vitro* [19]. Other studies have shown that its ethanolic extracts exhibited a cytotoxic effect against A549 lung cancer cells, cleaved the pro-apoptotic molecule poly-(ADP-ribose) polymerase (PARP), promoted the release of cytochrome C, increased the activity of caspases 3 and 9 and the ratio of Bax/Bcl-2 [20]. It also decreased the rate of proliferation as evidenced by a decrease in the percentage of cells in the G2/M phase. The ethanolic extracts of *O. sanctum* have also inhibited the invasion of murine Lewis lung cancer (LLC) cells *in vitro* associated with a decrease in the activity of matrix metalloproteinase-9 (MMP9) [20]. *In vivo*, it significantly reduced the number of metastatic lung nodules following injection of LLC cells through the tail vein [21]. Overall, these studies suggest that extract from the leaves of the plant can induce apoptosis, inhibit cell cycle progression and prevent metastasis.

In the present study, we investigated whether the essential oil or extracts prepared from commercially available dried leaves of *O. sanctum* could inhibit proliferation, survival and metastasis of PC cells. The results of our study reveal that both ethanolic extracts (EEOL) and the essential oil of *O. sanctum* leaves (EOOS) significantly inhibit the aggressiveness of

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PC cells and inhibit the growth of orthotopically implanted PC cells. Overall, our study is the first to suggest a potential role for *O. sanctum* in the therapy of PC.

2. Materials and Methods

2.1 Preparation of ethanolic extracts of *O. sanctum* leaves (EEOL)

We purchased capsules containing powdered dried leaves of *O. sanctum* from four vendors in the United States: New Chapter (NC), (New Chapter Inc., Bloomingdale, IL, USA), Club Natural (CN), (Club Natural Inc. Irvine, CA, USA), Superior Herbs (SH), (Swanson Health Products, Fargo, ND, USA) and Morpheme (Morph), (Morpheme Remedies Pvt. Ltd., Panchkula, Haryana, India). We also purchased the essential oil of *O. sanctum* leaves (EOOS) from Now Foods, (Bloomingdale, IL, USA).

Ethanolic extracts of *O. sanctum* leaves (EEOL) were prepared by dissolving the contents of a single capsule (400mg of powdered dried leaves per capsule for NC and SH, and 450mg per capsule for CN and Morph) in 10ml of filtered 100% ethanol. To ensure maximum dissolution, the tubes were vortexed for 10 minutes before being filtered through a 0.2μm filter. Extracts were prepared fresh every week and stored away from bright light at 4°C. For *in vivo* experiments, the extracts were prepared in the same manner but using double distilled water as the solvent in order to avoid ethanol toxicity. The concentrations of the extracts were expressed as μg of dried leaves per ml of solution.

2.2 Cell culture and chemicals

The human PC cell lines AsPC-1, MiaPaCa, and Capan-1 were purchased from the American Type Culture Collection (ATCC). The CD18/HPAF PC cell line was generated in Dr. Metzger's laboratory at Duke University Medical Center. The cell lines were validated and authenticated by short tandem repeat analysis. PC cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100μg/ml penicillin and streptomycin). Cells were grown at 37°C with 5% CO_2 in a humidified atmosphere.

2.3 Cell viability assay

The effect of Basil extract on cell viability was assessed by MTT assay. Briefly, 3×10^3 cells were seeded in 10% DMEM (n=6 replicates per dose) in a 96-well plate. After overnight incubation, the medium was replaced with fresh 10% DMEM containing various dilutions of either EEOL or Basil oil. The cells were then incubated for 48 hours (to determine IC-50) or up to 7 days (to assess the effect on growth kinetics). MTT was added to each well (final concentration per well: 1.2mM) and the plates were incubated for 4 hours at 37°C. Formed formazan crystals were dissolved with 100μl dimethyl sulfoxide (DMSO) and the absorbance of each well was read using a microplate reader (Molecular Devices Co, Sunnyvale, CA, USA) at 560nm with background wavelength at 670nm.

The IC-50's were determined by graphing the $\log_{10}$ (dose) on the x-axis and percentage inhibition of proliferation on the y-axis. The graphs were constructed employing the four parametric logistic regression models using the FindGraph software (version 2.291, Uniphiz labs, Vancouver, BC, Canada).

To assess the effect of EEOL/EOOS on growth kinetics, PC cells were seeded at a density of 1000 cells per well. After overnight incubation, the medium was replaced with media containing a fixed dose of EEOL, EOOS or vehicle. The cells were then returned to the incubator. Cell viability was then assessed by the MTT assay performed daily (starting from 24 hours post treatment) for seven consecutive days. The results were expressed as

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percentage viability (relative to the untreated cells). Absolute ethanol corresponding to the concentration of ethanol present in EEOL was added in the vehicle control wells.

2.4 Transwell migration and invasion assay

Following trypsinization, cells were washed twice with PBS and seeded in serum-free DMEM containing either EEOL (80μg/ml NC) or EOOS (0.1%v/v) on the top chamber of 8μm pore-size polyvinyl-pyrrolidone free polycarbonate (PVPF) filter. For invasion assay, the filter was coated with a 1:1 mixture of gelatin and Type IV collagen simulating the basement membrane. DMEM containing 50% FBS was placed in the lower chamber. The cells were allowed to migrate for 24 hours at 37°C. After removing the non-migrating cells from the top chamber, the cells that had migrated through the filter were fixed and stained using the Diff Quik Staining kit (Mertz-Dade AG, Dade International, Milan, Italy) and counted under a light microscope (100× magnification in 10 random fields). Each experiment was performed in triplicate. The results were expressed as the mean number of cells (±SE) migrating per high power field (HPF).

2.5 Wound healing assay

To confirm the effect of EEOL/EOOS on the migration ability of PC cells, we performed an *in vitro* wound healing assay. Cells were grown to 100% confluency in a 100mm dish. A series of linear wounds were made on the plate using a sterile 1000μl pipette tip. The cells were washed once with complete media to remove dead and un-adhered cells. The wound was then photographed at baseline (0h). Complete medium (10% DMEM) containing the EEOL, EOOS or ethanol (0.2% v/v) was added to the plates and the cells were incubated at 37°C. Photographs of the wound were taken at 24 hour intervals until the wound in one of the treated plates closed completely. The distance migrated was calculated according to the following formula: *distance between the edges of the wound at time point 1- distance between the edges of the wound at time point 2*. For each cell line, an average of eight wounds was taken to determine the average rate of migration for a given dose of EEOL/EOOS. Experiments were repeated at least three times.

2.6 Anchorage dependent clonogenicity assay

To examine the effect of EEOL/EOOS on the clonogenic potential of PC cells, we performed an *in vitro* colony formation assay. 250 cells were seeded in triplicate in a 12-well plate. After overnight incubation, the media was replaced with 10% DMEM containing the EEOL (or ethanol) or EOOS at various doses. The plates were incubated for a further 2 weeks. Thereafter, the colonies formed were fixed in methanol and stained with 0.5% crystal violet in PBS and counted using the colony counting function of the Quantity One software (BioRad, Hercules, CA, USA).

2.7 Cell cycle analysis

200,000 cells were seeded in complete medium. After allowing them to attach overnight, the media was replaced with either fresh medium (without drug) or media containing the EEOL, EOOS or the vehicle. After 48 hours of incubation, the cells were trypsinized, washed, fixed in 70% ethanol, stained with 500μl propidium iodide (1μg/ml) and analyzed by flow cytometry.

2.8 Apoptosis assay

Apoptosis was measured by using the Annexin V-FITC apoptosis detection kit (Roche Diagnostics, Indianapolis, IN). Briefly, 250,000 cells were seeded in triplicate in 10% DMEM. After overnight incubation, the media was replaced with either 10% DMEM containing EEOL, EOOS or the vehicle (ethanol). The cells were incubated for a further 48

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hours. Apoptosis was detected by staining the cells with Annexin V and propidium iodide solution followed by flow cytometry as described previously [22].

2.9 Western blotting

Cells were processed for western blotting as described previously [22]. In brief, a total of 20–40μg of protein from the cell extracts were resolved by electrophoresis on 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (PVDF). The membrane was probed with anti-phospho or total FAK, anti-phospho or total ERK, (all obtained from Santa Cruz, CA) anti-p65 Nf-κB (Cell Signaling Technology, Danvers, MA), and anti-βactin (1:10,000; Sigma, St. Louis, MO) antibodies. The membrane was then incubated with horseradish peroxidase–labeled appropriate secondary immunoglobulin, and the signal was detected using a chemiluminescence reagent kit (Amersham Pharmacia, Piscataway, NJ).

2.10 RNA extraction and Real-time PCR

Transcript levels of specific genes were measured in PC cells treated with the EEOL, EOOS and pancreatic tumors isolated from mice by using the SYBR® Premix *Ex Taq*™ kit. cDNA was synthesized using 2μg of total RNA, oligo$(dT)_{18}$ primer and Superscript RT (Invitrogen Corp.). Quantitative real-time PCR was performed using 1μl of a 1:5 dilution of first-strand cDNA using the SYBR Premix *Ex Taq* kit (Takara Bio, Madison, WI, USA) and specific primers (Table 1) on a Roche Light Cycler 480 system (Roche Diagnostics, Mannheim, Germany). Each cDNA sample was used in triplicate, and a reaction without any cDNA was used as negative control. The level of mRNA expression of each gene was normalized to that of *β-actin*. For cell lysates, the results were graphically represented as a fold difference in mRNA expression in the treated vs. control cells. Real time PCR data for tumor lysates is represented as the average relative mRNA gene expression of each experimental group.

2.10 Animals and treatments

The protocol for examining the anti-tumor effect of AEOL on PC cells *in vivo* was approved by the Institutional Animal Care and Use Committee (IACUC) at UNMC. Female athymic mice between 4–6 weeks of age were anesthetized and 2.5×10^5 AsPC-1 cells suspended in 50μl sterile PBS were injected orthotopically into the head of the pancreas, as described by us previously [22, 23]. The AsPC-1 cells were chosen after analysis of the results from *in vitro* experiments and taking into consideration that this cell line contains the four most common mutations in PC (i.e. *KRAS*, *TP53*, *CDKN2A/p16*, and *SMAD4/DPC4*) and produce well- to poorly-differentiated adenocarcinoma after transplantation in athymic mice [24, 25]. One week after surgery, the mice were weighed and either treated with AEOL (6mg/mouse) administered twice weekly by intraperitoneal injection or with an equivalent dose of double distilled water (control group). AEOLs from NC, SH and Morph were employed for the experiment. Prior to each injection, the mice were weighed. A decrease in weight of ≥10% compared to the previous weight was considered as the indication to euthanize the mice. The mice were fed *ad libitum* and monitored for any distress during each treatment. Following euthanization, pancreatic tumors from each mouse were dissected out, weighed processed for RNA extraction.

2.11 Statistical analysis

Data was analyzed using the Medcalc for Windows version 9•6•4•0 software (MedCalc Software, Belgium). Continuous variables were compared using the Student's two-tailed t-test assuming unequal variance, while the categorical variables were compared using the two-way analysis of variance (ANOVA). For the *in vivo* experiments, using a sample size of 5 mice per group gave us a power of 0.96 in detecting a difference of at least 50% between

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the animals treated with the crude extract and those treated with the vehicle control (water) (assuming a variance in tumor weight of 25% for a given treatment and a type-I error (α) of 0.05). A p-value ≤ 0.05 was considered significant.

3. Results

3.1 Determining the optimum dose of ethanolic extracts of *O. sanctum* leaves (EEOL) and essential oil of O. *sanctum* leaves (EOOS) to inhibit the proliferation of pancreatic cancer cells *in vitro*

We first sought to determine the optimum dose of EEOLs and EOOS for *in vitro* treatment of PC cells from various sources. For this, AsPC-1, MiaPaCa, CD18/HPAF, and Capan-1 PC cells were treated with EEOL (2-fold serial dilutions between 2mg/ml-1μg/ml) or EOOS (2-fold serial dilutions between 2%-0.001% v/v) for 48 hours. Cell viability was determined by MTT assay and the IC-50 was calculated as described in Materials and Methods. As shown in Figure 1A, the IC-50 of the EEOL from the basil leaves capsules purchased from New Chapter (NC) in the USA was 46μg/ml in AsPC-1 cells. In MiaPaCa cells, the EEOL from Morpheme capsules inhibited the proliferation at a higher rate (IC-50 69μg/ml) than the extracts from the other vendors. EEOL that were prepared from SH (IC-50 range 113–202.5μg/ml) and CN (IC-50 230–2,086μg/ml) capsules were less cytotoxic to PC cells. The IC-50 for EOOS ranged from 0.17%–0.20%v/v.

We also tested an arbitrarily chosen range of concentrations from 800μg/ml to 0.8μg/ml (for EEOL) and 1% to 0.001%v/v (for EOOS) to determine the most effective concentration for long term inhibition of PC cell proliferation. EEOL at a concentration of 800μg/ml (from all the four vendors) and EOOS at a concentration of 1% v/v completely inhibited the formation of colonies (100% inhibition) in all cell lines following incubation for 14-days after a single treatment (data not shown). A dose of 80μg/ml (of EEOL) and 0.1% v/v EOOS caused a significant reduction in the number of colonies, while no effect was observed at a dose ≤8μg/ml (of EEOL) or ≤0.01% (of EOOS) (Figure 1B).

From the preceding experiment, a dose of 80μg/ml of EEOL and 0.1% v/v of EOOS were determined to be the lowest effective doses that inhibited the clonogenicity of PC cells. To further confirm the effectiveness of these two doses in inhibiting the growth kinetics of PC cells, we investigated the inhibitory effect of 80μg/ml EEOL or 0.1%v/v EOOS (i.e. high concentration) versus 0.8μg/ml EEOL or 0.001%v/v EOOS (i.e. low concentration) on the growth of PC cells. As the EEOL of NC exhibited the lowest IC-50 value (Figure 1A) and a strong anti-proliferative effect (by clonogenicity assay, Figure 1B), we chose this extract for growth kinetics assay. As shown in Figure 1C, the highest dose of both EEOL and EOOS significantly inhibited the proliferation of PC cells over time. While both AsPC-1 and MiaPaCa cells were sensitive to the highest dose EOOS, only the proliferation of the former was inhibited by the highest dose of EEOL.

Based on the results of the clonogenicity assay and the effect on growth kinetics, we chose a dose of 80μg/ml and 0.1% v/v as the optimum concentration of EEOL and EOOS respectively for further *in vitro* functional studies. The *in vitro* results on this manuscript are focused on AsPC-1 and MiaPaCa PC cells. Nevertheless, similar results from all the *in vitro* experiments were also obtained in CD18/HPAF and Capan-1 PC cell lines (data not shown).

3.2 EEOL and EOOS inhibit the migration and invasion ability of pancreatic cancer cells

We next sought to investigate the effect of both the basil extract and essential oil on the behavior of PC cells through a series of *in vitro* functional assays. To test whether EEOL/EOOS can inhibit PC cell motility *in vitro*, we utilized the wound healing and transwell migration assays. Both EEOL (of NC) and EOOS significantly inhibited the ability of PC

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cells to migrate and close a scratch wound (Figure 2A) and inhibited their migration towards a chemotactic stimulus (Figure 2B). Both EEOL and EOOS also inhibited the invasion of PC cells through a gelatin-collagen coated filter towards a chemotactic stimulus (Figure 2C). EOOS (0.1% v/v) was more effective than EEOL (80μg/ml) in inhibiting both the migration and invasion of PC cells.

3.3 EEOL induce apoptosis of pancreatic cancer cells

The effect of EOOS and EEOL on PC cell cycle progression was evaluated (Figure 3A). Overall, the effect of basil extracts on cell cycle progression was not conclusive. In AsPC-1 cells, EOOS decreased the percentage of cells in the G2/M-phase, whereas there was an increase in the S phase and no significant effect was noted on the G1 phase. On the other hand, in MiaPaCa cells, both EOOS and EEOL extracts appeared to promote the progression through the cell cycle as evidenced by a decrease in the percentage of EOOS and EEOL treated cells in the G1 phase and an increase in the percentage in the S and G2/M phases. Nevertheless, the percentage of apoptotic and necrotic cells after basil extract treatment was analyzed after Annexin V-propidium iodide staining and the results revealed the percentage of apoptotic cells was significantly higher upon treatment with EEOL, revealing the cytotoxic mechanism of the extract on PC cells (Figure 3B).

3.4 EEOL/EOOS downregulates the expression of proteins that promote pancreatic cancer invasion and metastasis

As we had observed a significant inhibition of motility and invasion of PC cells by EEOL and EOOS, we also sought to investigate whether there was a change in the molecular pathways mediating these functions. For this, AsPC-1 cells were treated with either EEOL or EOOS for 48 hours followed by western blotting and Real time RT-PCR to examine the change in the expression of key proteins and genes. Both EEOL and EOOS significantly decreased the activation of the key mediators of PC cell survival, proliferation, metastasis, invasion and chemoresistance: the extracellular signaling regulated kinase (ERK-1/2) and focal adhesion kinase (FAK) (Figure 4A). Activation of NF-kB, specifically the p65 subunit, is known to be a key activator of pathways that promote proliferation, invasion and metastasis [26]. Both EEOL and EOOS downregulated the protein expression of the p65 subunit of NF-κB in PC cells suggesting that inhibition of NF-kB is one of the possible mechanisms by which basil oil or leaf extract inhibits the aggressive behavior of PC cells (Figure 4A).

Total RNA extracted from AsPC-1 cells was reverse transcribed and the expression of genes associated with apoptosis (*BAD*, *Bcl-xL*, *BAK*), metastasis and invasion (*E-cadherin*, *CEACAM6, Vimentin*), chemotherapy and radiation resistance (*AURKA*, *Chk1, Survivin*), and cell cycle (*Cyclin D1*, *Cyclin A*) were examined by Real time RT-PCR (Figure 4B). In correlation with the *in vitro* apoptosis results, AsPC-1 cells treated with both EEOL and EOOS showed a strong upregulation of the pro-apoptotic protein *BAD*. Nevertheless, there were no changes in the gene expression of *Bcl-xL* and *BAK*. There was a significant decrease in the expression of *AURKA*, *Chk1*, and *Survivin* in PC cells treated with both extracts, suggesting the potential use of basil in combination therapies against cancer. Unexpectedly, there was an increase in the expression of *CEACAM6*, *Vimentin* and *Cyclin D1* in EOOS-treated PC cells when compared to untreated cells.

3.5 Aqueous extracts of *O.sanctum* leaves (AEOLs) inhibit the tumorigenicity of orthotopically implanted AsPC-1 cells in athymic mice

Having demonstrated the activity of EEOL and EOOS on PC cells *in vitro*, we next investigated whether extracts of *O. sanctum* leaves (EOLs) could inhibit the tumorigenicity of PC cells *in vivo*. From our *in vitro* studies, we had observed that AsPC-1 cells were most

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sensitive to both EEOL and EOOS. Hence, these cells were chosen for investigating the effect of basil leaf extracts on the growth of PC cells *in vivo*. AsPC-1 cells were injected orthotopically into the pancreas of female athymic mice. Starting seven days post-injection, the mice were treated with AEOL of NC, SH, or Morph (Figure 5A). From a review of literature, we noted that the acute LD50 of AEOL has been reported to be 6g/kg body weight [27]. With a mean weight of 22.4g/mouse at the start of the study, each animal received a dose of 267.5mg/kg (i.e. 6mg/animal) body weight which was significantly lower (~23 fold) than the reported LD_{50}. AEOL was used (instead of EEOLs) as the volume of ethanolic extract required to achieve this dose produced significant intoxication (from alcohol) in the mice in a pilot experiment (data not shown). After 3 weeks of therapy, the mice were euthanized and the primary tumors were resected and weighed. Compared to control animals (tumor weight 609±55mg), AEOL treated animals had significantly smaller tumors (285.9±32mg, 284±64mg, and 398.5±60mg in animals receiving AOEL of NC, SH and Morph respectively) (Figure 5B). However, there was no significant difference in the incidence of visible metastasis in basil extract-treated animals (Figure 5C). A possible cause for the lack of an effect of AEOLs on the number of visible metastasis could be the difference in their potency compared to the EEOLs. To investigate this possibility, we compared the IC-50 of AEOL vs. EEOL of NC in AsPC-1 cells using the MTT assay. The experiment revealed that at each dose, the ethanolic extract was nearly 19-fold more effective at inhibiting AsPC-1 proliferation than the aqueous extract (data not shown).

Similarly to the *in vitro* studies, total RNA extracted from the tumor xenografts was reverse transcribed and the expression of genes associated with apoptosis (*BAD*, *Bcl-xL*, *Bcl2*), cell cycle (*Cyclin D1*, *Cyclin A*), metastasis and invasion (*E-cadherin*, *CEACAM6, Vimentin*), desmoplasia (*Gli1*, *Snail*), angiogenesis (*VEGFA, HIF-1α*), and chemo and radiation resistance (*AURKA*, *Chk1* and *Survivin*) was examined by Real time RT-PCR. In corroboration with the *in vitro* results, animals treated with AEOL of NC showed a strong upregulation of *BAD* with a downregulation of *Bcl-2* and *Bcl-xL* suggesting an increase in the rate of apoptosis (Figure 6). As opposed to *in vitro* studies, there was also a significant increase (nearly 30-fold) in the expression of *E-cadherin* mRNA in animals receiving AEOL (NC>SH>Morph). NC-treated animals also showed a significant decrease in the expression of *CEACAM6*, a gene that is known to promote invasiveness in PC cells [28]. *GLI1*, a transcription factor with prominent role in the desmoplastic reaction in PCs was downregulated nearly 4-fold in the animals treated with AEOL of NC. *Survivin* expression was significantly downregulated in animals treated with NC and SH, but not with Morph. We, however, observed an upregulation of *AURKA* and *Chk1. HIF-1α*, a major target of anti-PC therapy was significantly upregulated in NC-treated animals but significantly decreased in Morph and SH treated mice. Overall, there was no significant change in the expression of *Cyclin A*, *Snail*, *VEGFA*, *Vimentin* in the AEOL-treated tumors compared to tumors from the control animals.

4. Discussion

O. sanctum Linn., also known as *O. tenifluorum* or Holy basil, an herb belonging to the family *Lamiaceae,* is a well-known medicinal plant in use for several thousands of years in the traditional Indian system of medicine. While most studies have focused on the therapeutic effects of *O. sanctum* leaf extracts on benign diseases, studies of its anti-cancer effects are sparse.

To our knowledge, there are no published studies of the effect of basil extracts in PC cells. We observed that both EOOS and EEOL significantly inhibited the motility and invasion of PC cells *in vitro*. This was associated with a significant downregulation of activated FAK, a key mediator of both these processes in PC cells. *In vivo*, while AEOL significantly

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decreased tumor size, we did not observe any significant difference in the incidence of macroscopic metastases between animals treated with AEOL or vehicle (water). However, there was a strong upregulation of *E-cadherin* in the tumors of the AEOL treated mice (Figure 6). One possibility for the lack of observable effects on metastasis *in vivo* is that PC cells establish metastasis very early, when the tumor is still small. Since the mice were treated with AEOL after seven days post-orthotopic implantation, we hypothesize that micrometastasis had already been established by the time the therapy was started. A comparison of the IC-50 values between AEOL and EEOL revealed that the former was nearly 20–40 fold less effective at inhibiting PC proliferation *in vitro*. This suggests that a higher AEOL dose in our *in vivo* experiment may have inhibited the proliferation of metastatic deposits. Our future studies will aim to determine the effect of individual compounds present in EOOS in inhibiting the aggressive behavior of PC cells *in vivo*.

Additionally, we observed that *in vitro* incubation of AsPC-1 cells with EOOL and EOOS and *in vivo* treatment of orthotopically implanted AsPC-1 cells with AEOL downregulated the expression of *Survivin*, a gene that has been previously shown to be a key mediator of chemo- and radio-resistance in PC and other solid tumors [29–32]. Thus, its downregulation in PC cells suggests the possibility that compounds present in *O. sanctum* leaves could potentially sensitize PC cells to chemotherapy and radiation. We are currently evaluating the effect of EEOL (of NC) and EOOS on both the sensitization (by pre-treatment) and the efficacy (in combination with) of PC cells to conventional chemotherapy. We are also evaluating the role of major compounds found in basil [11, 33] in PC therapeutics. An analysis of essential oil from fresh leaves of *O. sanctum* identified nearly 16 different constituents [34]. Of these, methyl eugenol is the most abundant, with eugenol, linalool, apigenin, orientin and vicenin being some of the other components.

A limitation of our study is the use of dried vs. fresh basil leaves to prepare the extracts. While this might have resulted in the lower magnitude of effects in cells treated with EEOL/AEOL (compared to EOOS treated cells), it gave us valuable insight into the potential clinical benefits of commercially marketed basil supplements. Fresh basil leaves are difficult to obtain and store in quantities large enough for long-term studies and may show variation from batch to batch. On the other hand, we obtained the dried leaves from four independent vendors and used the same lot for the entire experiment. Given the lack of reliable scientific evidence to support the use of most health supplements, our study, through its unbiased and broad-based experimental strategy, provides the first evidence for the potential utility of basil extract for the treatment of one of the most lethal malignancies known to man.

In conclusion, we report that ethanolic extract and the essential oil of *O. sanctum* inhibit the proliferation, motility and invasive ability of PC cells. Intraperitonally injected aqueous extracts decreased the tumorigenecity of orthotopically implanted AsPC-1 cells significantly. This was associated with significant increase in the expression of pro-apoptotic genes with a concomitant decrease in the level of genes that inhibit apoptosis or promote PC cell proliferation or metastasis. Taken together, these results suggest that the extract or essential oil of basil leaves and the components present in them could be potentially useful as novel agents for the therapy and/or prevention of human PC. These results will form the basis for future studies to investigate the effect of individual components of *O. sanctum* leaves for the treatment of PC.

Acknowledgments

The invaluable technical support from Kavita Mallya is greatly appreciated. We also thank Janice A. Tayor and James R. Talaska of the Confocal Laser Scanning Microscope Core Facility at UNMC, Victoria B. Smith and Megan Michalak of the UNMC Cell Analysis Core Facility, and the Eppley Cancer Center for their support of the core facilities. The authors of this work are supported by grants from the National Institutes of Health: NIH-NCI

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Cancer Biology Training Grant UNMC T32CA009476 (MPT, JS), VA Career Development Award (AKG) and NIH grants U01EDRN CA111294, P50 SPORE CA127297 and U54 CA160163 (SKB).

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A.

Cell line	IC-50				
	Basil oil (%v/v)	NC (μg/ml)	CN (μg/ml)	SH (μg/ml)	Morph (μg/ml)
AsPC-1	0.20	46 [a]	2,086	202.5	180
MiaPaCa	0.17	127	230 [b]	113	69

Ethanolic extracts of *O.sanctum* leaves: NC-New Chapter, CN-ClubNatural, SH- Superior Herbs, Morph-Morpheme
[a] Corresponding IC50 for the aqueous extract was 852 μg/ml. .
[b] The extract stimulated proliferation of MiaPaca cells at all concentrations tested

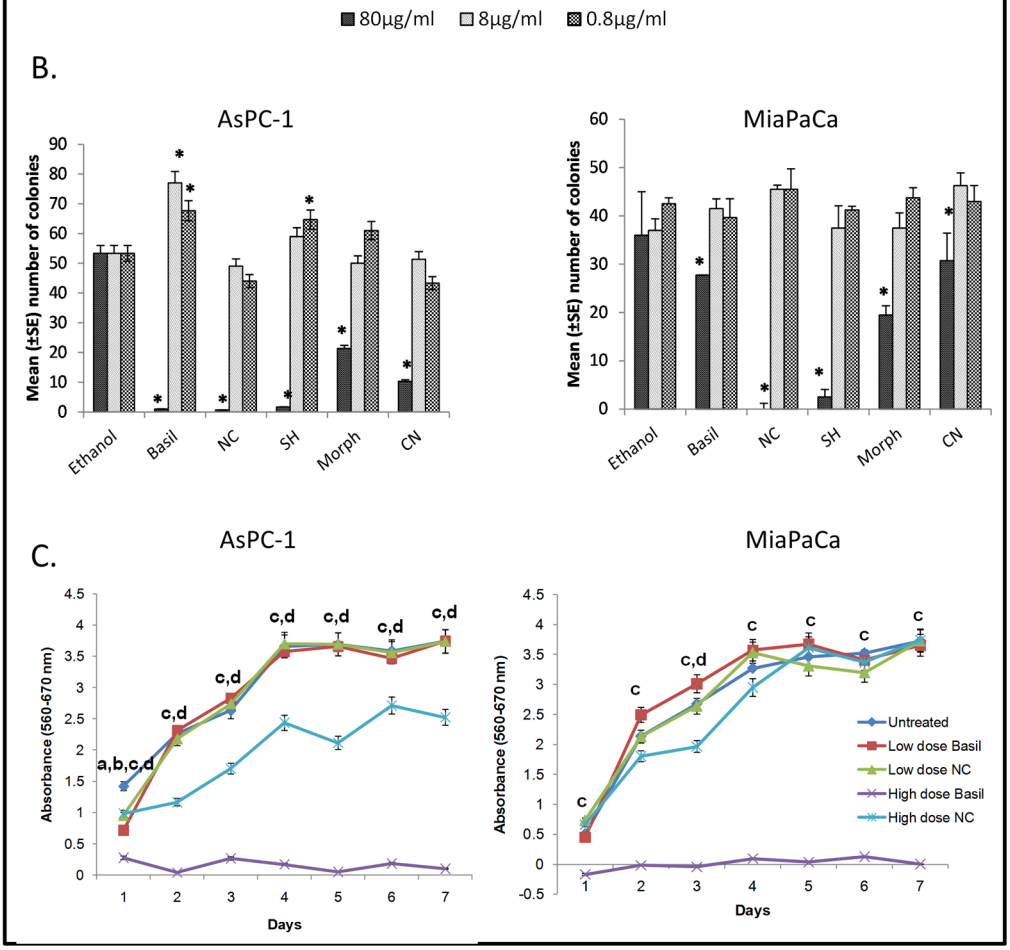

Figure 1. Ethanolic extract of *O. sanctum* (EEOL) and essential oil of *O. sanctum* leaves (EOOS) inhibit the proliferation of pancreatic cancer cells

AsPC-1 and MiaPaCa cells were seeded in triplicate in 10% DMEM. After overnight incubation, the cells were treated either with EOOS (denoted as Basil oil), New Chapter (NC), Club Natural (CN), Superior Herbs (SH) and Morpheme (Morph) at the indicated concentrations in 10% DMEM. (A) IC-50 of EEOL and EOOS in PC cells. After 48 hours of incubation with EOOL and EOOS the cytotoxicity and IC-50 values were evaluated from MTT assay (as described in Materials and Methods). (B) *In vitro* clonogenicity assay. After 14 days of incubation with the basil extracts, the colonies formed were washed with PBS, fixed in methanol and stained with 0.1% Crystal violet in PBS. The number of colonies (per

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well) were counted with the automatic colony counting tool of the Quantity One Imaging software. The graphs represent the mean number of colonies (±SE) for a given treatment. The experiment was repeated twice (*p-value<0.05). (C) Inhibition of PC cell growth *in vitro*. Cell viability was assessed every 24 hours beginning 24 hours after the addition of the basil extract for up to 7 days by MTT (as described in Materials and Methods). The results are expressed as the mean absorbance (A_{560nm}-A_{670nm}) versus the number of days after addition of Basil extract (or oil). Two doses of EEOL (from NC) were used- 0.8μg/ml (*low dose NC*) and 80μg/ml (*high dose NC*). Basil oil (EOOS) was also added at two doses- 0.001% v/v (*low dose Basil*) and 0.1% v/v (*high dose Basil*). [a- p-value<0.05 (Untreated vs. low dose Basil oil), b-p-value<0.05 (Untreated vs. low dose NC), c-p-value<0.05 (Untreated vs. high dose Basil oil), d-p-value<0.05 (Untreated vs. high dose NC)]

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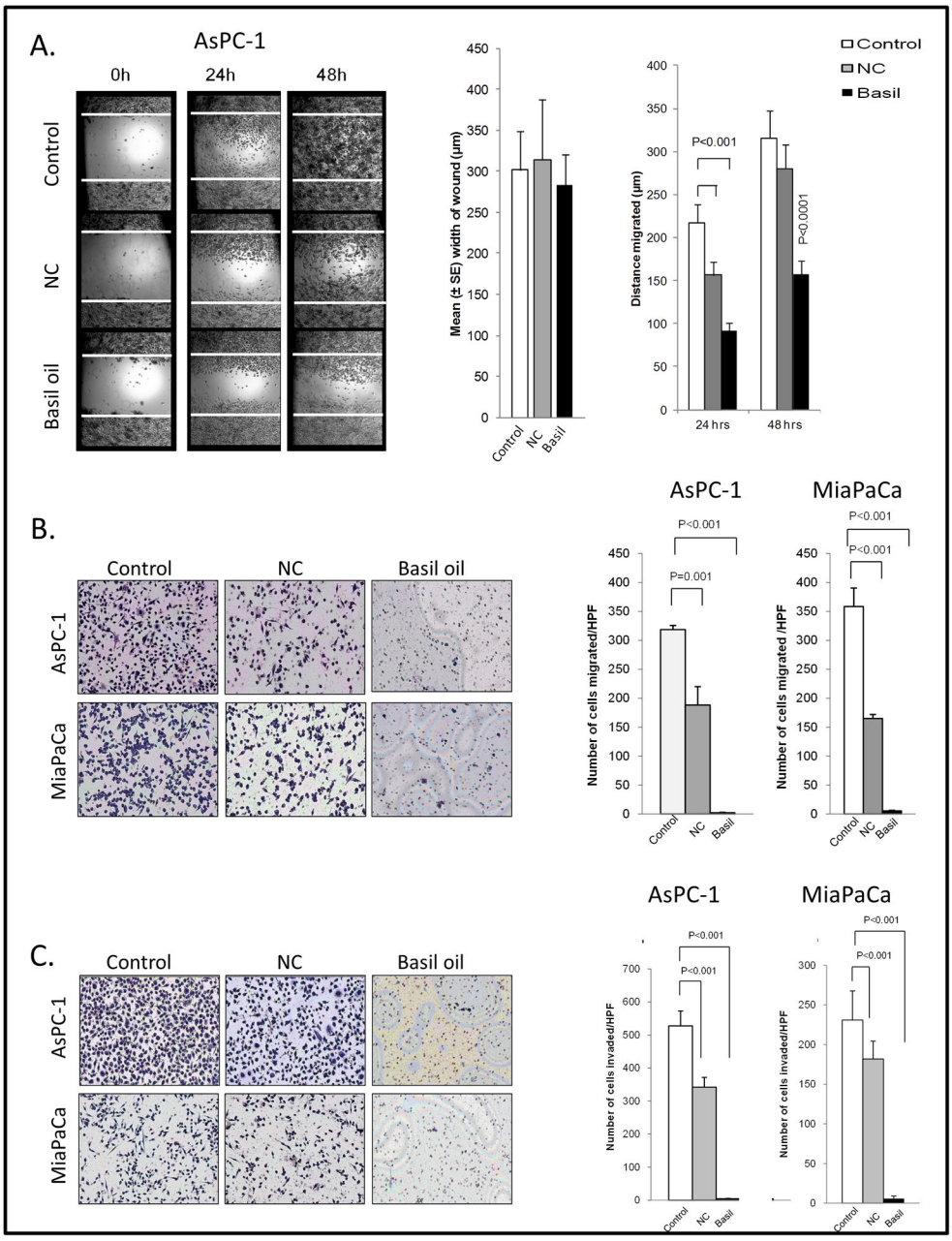

Figure 2. EEOL (of NC) and EOOS inhibit the migration and invasion of pancreatic cancer cells

(A) Wound healing assay. PC cells were plated in a 10cm dish in complete medium (10% DMEM) and allowed to grow until they formed a confluent monolayer. An artificial wound was induced on the center of the monolayer and the ability of the cells to close the wound after adding EEOL of NC (final concentration 80μg/ml) or Basil oil (final concentration 0.1% v/v) was analyzed. Images were taken immediately (t=0 hours) and after incubation for 24 hours and 48 hours. The results are expressed as the initial width of the scratch in μm and the distance migrated (calculated as the difference from the width of scratch at time t=24/48 hours and the width at t=0). The height of the graph represents the mean of six different scratches (±SE). **(B)** *In vitro* migration of PC cells towards a chemotactic stimulus. PC cells were seeded in serum free DMEM onto the top of a 6cm^2 sterile transwell insert with a pore

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diameter of 8μm (n=3 replicates). The bottom well of the transwell contained DMEM supplemented with 50% FBS. The cells were incubated for 24 hours after which the cells that migrated through the transwell were fixed and stained with hematoxylin and eosin. The cells were photographed under high magnification (100×) and the numbers of cells in at least 15 independent high power fields (HPF) were counted. Data is represented as the mean number of cells/HPF (±SE). **(C)** Ability of PC cells to invade through the basement membrane towards a chemotactic stimulus *in vitro*. PC cells were seeded in serum free DMEM onto the top of a 8μm pore-sized 6cm^2 sterile transwell insert coated with 1:4 volumetric ratio of rat tail collagen and 1% gelatin mimicking the basement membrane (n=3 replicates). The bottom of the transwell contained DMEM supplemented with 50% FBS (chemoattractant). The cells were allowed to invade through the coated wells overnight. The cells that had invaded through the transwell were first fixed (with methanol) and then stained with hematoxylin and eosin. Invading cells on the undersurface of the insert were photographed under high magnification (100×) and the numbers of cells in at least 15 independent high power fields (HPFs) were counted. Data is represented as the Mean number of invading cells/HPF (±SE). Representative images from the three assays (40× for wound healing and 100× for migration and invasion assays) and different treatment groups (Ethanol (control), EEOL of NC at 0.08μg/ml and Basil oil at 0.1% v/v) are shown.

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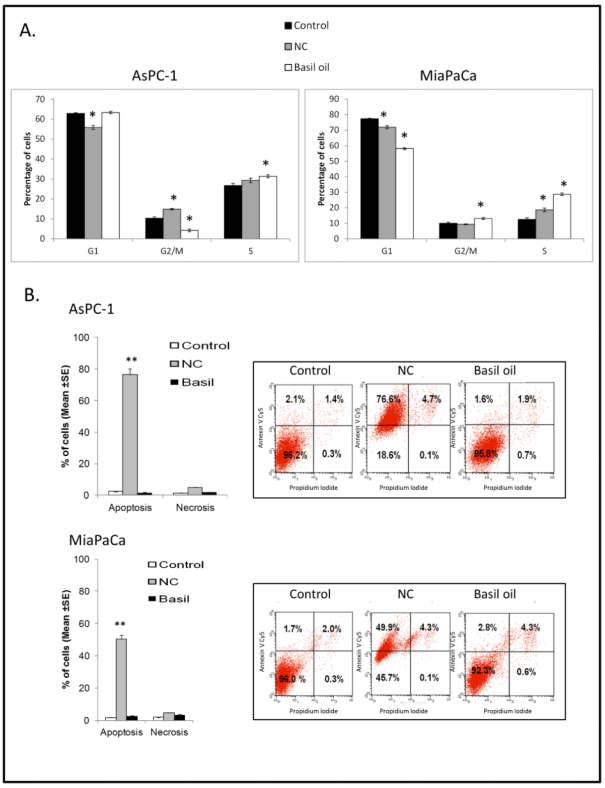

Figure 3. EEOL and EOOS induce the apoptosis of pancreatic cancer cells

(A) Cell cycle analysis. 2×10^4 cells were seeded in triplicate in complete medium in a 6-well plate. After incubating PC cells with Ethanol (0.1% v/v), EEOL of NC (80μg/ml) or EOOS (0.1% v/v) for 48 hours, the unsynchronized cells (including those in the media supernatant) were collected, stained with propidium iodide (PI) and analyzed by flow cytometry. The graphs represent the mean percentage of cells (±SE) in each phase of the cell cycle. (*p-value<0.05 when compared to control cells) **(B)** Quantification of apoptotic and necrotic PC cells. 2.5×10^5 cells were seeded in triplicate in 10% DMEM. After overnight incubation, the media was replaced with either 10% DMEM containing EEOL (80μg/ml NC), EOOS (0.1% v/v) or 0.2% v/v ethanol (vehicle control). The cells were incubated with the extracts for 48 hours. Apoptosis was detected by staining the cells with Annexin V and PI solution followed by flow cytometry. Representative results of flow cytometry density diagrams are shown: Live cells (Annexin V−/PI−), Early apoptotic cells (Annexin V+/PI−), Late apoptotic/ necrotic cells (Annexin V+/PI+). Data is represented as the mean number of cells (±SE). All experiments were done in triplicate and repeated at least twice. (** p-value≤0.001 when compared to control cells)

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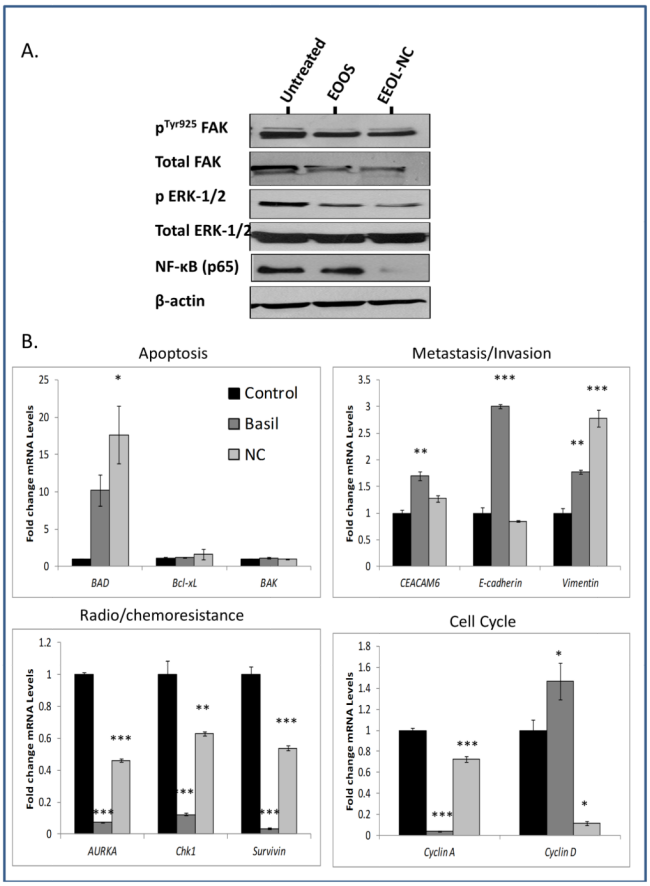

Figure 4. EEOL and EOOS treatment downregulates the expression of genes and proteins associated to metastasis and invasion of pancreatic cancer

AsPC-1 cells were treated with either EOOS (0.1% v/v) or EEOL of NC (80μg/ml) for 48 hours. Total protein and RNA was extracted and expression of key metastasis and invasion-associated proteins/genes were analyzed by **(A)** Western blotting and **(B)** Real time RT-PCR. Data is represented as the fold change mRNA level (±SE) of the respective gene in each treatment group. (*p-value≤0.05, **p-value≤0.005, ***p-value≤0.0001 when compared to control cells) β-actin was used both as the protein loading control and the housekeeping control gene for each experiment. Details of the experimental procedures are shown in Materials and Methods.

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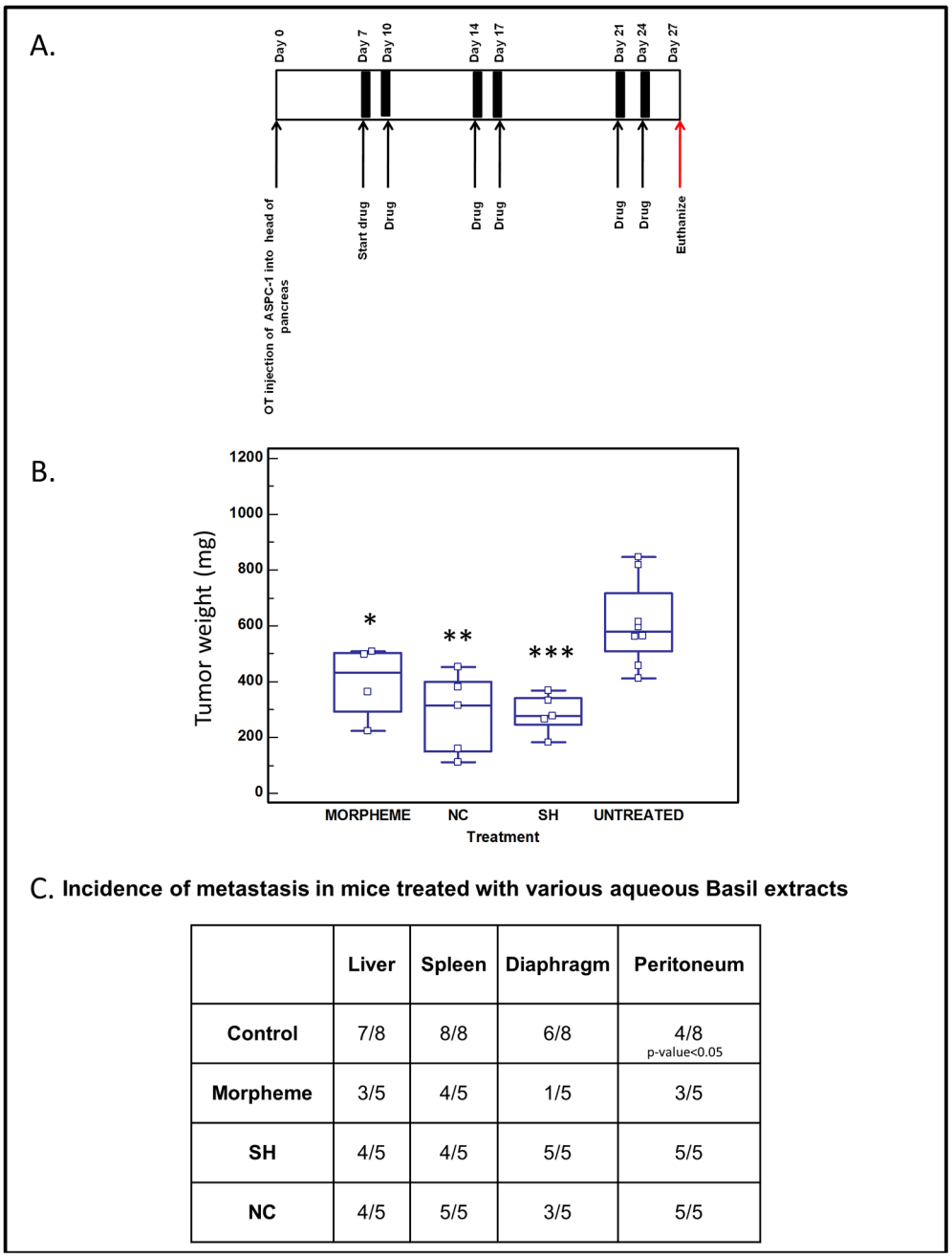

C. **Incidence of metastasis in mice treated with various aqueous Basil extracts**

	Liver	Spleen	Diaphragm	Peritoneum
Control	7/8	8/8	6/8	4/8 p-value<0.05
Morpheme	3/5	4/5	1/5	3/5
SH	4/5	4/5	5/5	5/5
NC	4/5	5/5	3/5	5/5

Figure 5. Effect of the aqueous extract of *O. sanctum* leaves (AEOL) on the tumorigenicity of AsPC-1 pancreatic cancer cells *in vivo*

In order to investigate the effect of AEOL on the tumorigenic potential of AsPC-1 cells *in vivo*, we injected 2.5×10^5 cells suspended in 50μl of PBS into the head of the pancreas of athymic female mice. Seven days post-implantation, the mice were treated either with AEOL (of NC, SH or Morph) intraperitoneally at a fixed dose of 6mg/mouse. The mice were treated two times a week for 3 weeks. (**A**) Schematic representation of the experimental strategy for assessment of the effect of AEOL on tumorigenicity of AsPC-1 PC cells. (**B**) Comparison of tumor weights (Average weight±SE) between untreated and AEOL treated mice. (*p-value=0.04, **p-value=0.004, ***p-value=0.0004) (**C**) Tabular

representation of the results of the effect of AEOL on the incidence of visible metastasis of AsPC-1 cells injected orthotopically into the head of the pancreas. Statistics were calculated in comparison with control group.

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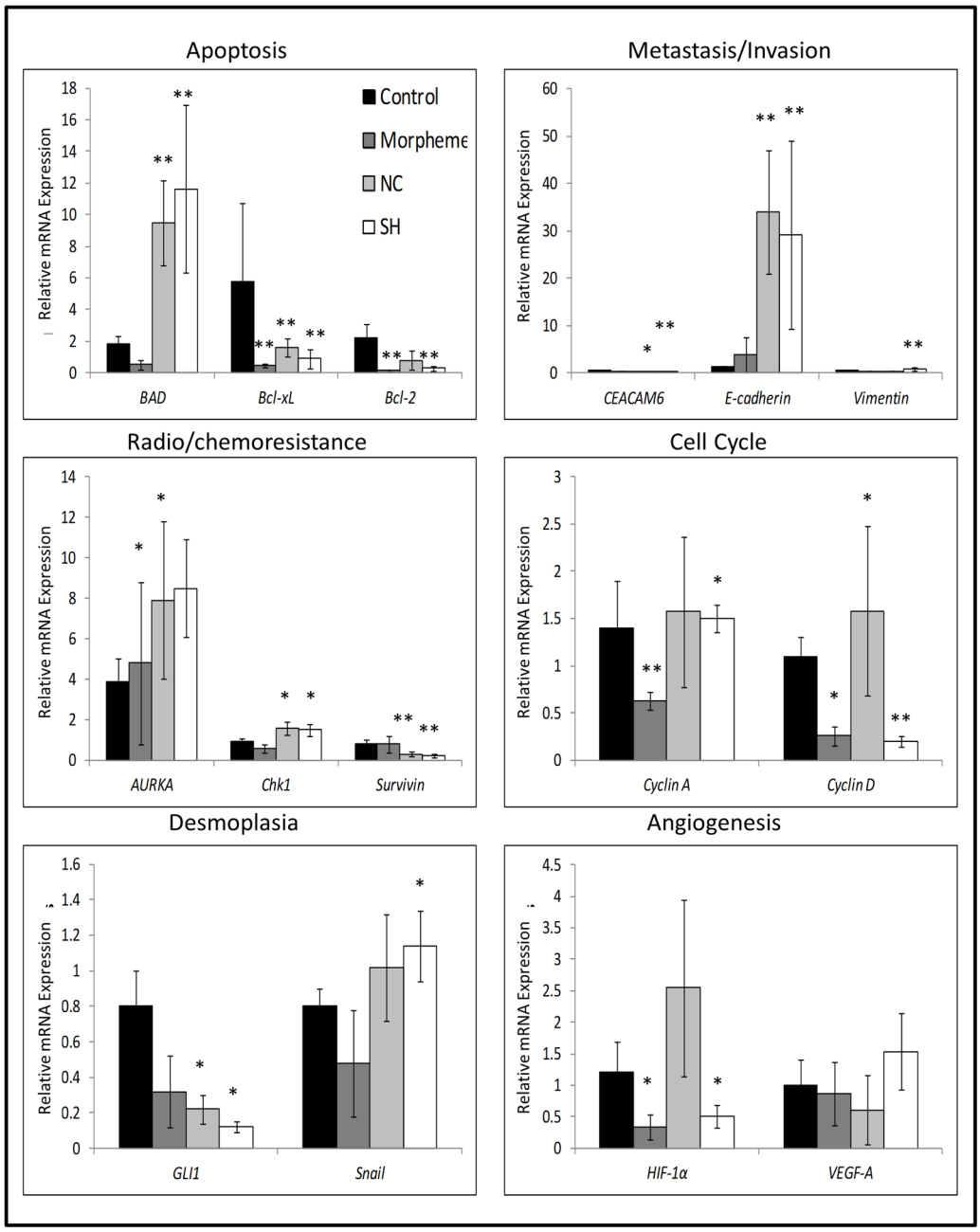

Figure 6. Analysis of AsPC-1 pancreatic tumors isolated from mice treated with the aqueous extract of *O. sanctum* leaves (AEOL)

RNA was extracted from pancreatic tumors from mice injected with either the vehicle or AEOLs and expression of key metastasis and invasion-associated genes were analyzed by Real time RT-PCR. Data is represented as the relative mRNA expression (±SE) in each treatment group. (*p-value<0.05, **p-value<0.005) β*actin* was used as the housekeeping control gene for each experiment. Details of the experimental procedures are shown in Materials and Methods.

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Table 1

Primers used for Real-time PCR in the study

Gene Symbol	Primer	Primer sequence	Accession number	Product length (bp)
AURKA	FP	GCTGGAGAGCTTAAAATTGCAG	NM_198433.1	220
	RP	TTTTGTAGGTCTCTTGGTATGTG		
BAD	FP	AACATCTTGTCCTCACAGCC	NM_004322.3	392
	RP	CACAAACTCGTCACTCATCC		
Bcl-xL	FP	AGGATACAGCTGGAGTCAG	NM_138578.1	416
	RP	TCTCCTTGTCTACGCTTTCC		
Beta actin	FP	AGCAAGAGAGGCATCCTC	NM_001101.3	474
	RP	GCACAGCTTCTCCTTAAT		
CEACAM6	FP	GAAATACAGAACCCAGCGAGTGC	NM_002483.4	226
	RP	CAGTGATGTTGGGGATAAAGAGC		
Chk1	FP	TGAGAATCCATCAGCAAGAATTACC	NM_001274.4	128
	RP	ATCCACTGGGAGACTCTGACACA		
Cyclin D1	FP	GTGACCCGGACTGCCTCCG	NM_053056.2	154
	RP	ACGTCGGTGGGTGTGCAAGC		
Cyclin A	FP	ACAGCCAGACATCACTAACAG	NM_001237.3	141
	RP	AGCACTGACATGGAAGACAG		
E-cadherin	FP	ATGAGTGTCCCCCGGTATCT	NM_004360.3	172
	RP	TCAGGGAGCTCAGACTAGCAG		
GLI1	FP	CTACATCAACTCCGGCCAAT	NM_005269.2	155
	RP	CGGCTGACAGTATAGGCAGA		
HIF-1α	FP	CCATTAGAAAGCAGTTCCGC	NM_181054.2	194
	RP	TGGGTAGGAGATGGAGATGC		
Snail	FP	TTCGGACCCACACATTACCT	NM_003068.4	122
	RP	GCAGTGAGGGCAAGAAAAAG		
Survivin	FP	GCCCTTCTTGGAGGGCTGCG	NM_001168.2	156
	RP	TGTTCCTCTATGGGGTCGTCATCTG		
VEGFA	FP	CCTCCGAAACCATGAACTTT	NM_001204385.1	412
	RP	TTCTTTGGTCTGCATTCACATT		
Vimentin	FP	GCAGCTCAAGGGCCAAGGCA	NM_003380.3	164
	RP	CCTGCAATTTCTCCCGGAGGCG		
Bax	FP	CTGGACCCGGTGCCTCAGGA	NM_138761.3	244
	RP	TGGTGCACAGGGCCTTGAGC		