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Evening Primrose Oil improves chemotherapeutic effects in Human Pancreatic Ductal Adenocarcinoma cell lines. A preclinical study.

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Abstract: Evening Primrose Oil (EPO), obtained from the seeds of Evening Primrose (*Oenothera* L.), was largely used as dietary supplement and users increased after cancer diagnosis. Human Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive disease correlated with a poor clinical prognosis and a very low response rate to common chemotherapy. The aim of the work was to study the potential improvement of EPO for chemotherapeutics effects, in PANC-1 and MIAPaCa-2 cell lines. Cytotoxicity, cell death, ROS production and EPO anticancer activity associated with the main chemotherapeutic drugs commonly used in therapy were investigated. Results showed that EPO reduced PDAC cell viability and increased Paclitaxel (PTX) efficacy. These evidences suggested that EPO may be used as potential supplement to increase chemotherapeutic efficacy in PDAC therapy.

Keywords: pancreatic cancer, human pancreatic ductal adenocarcinoma, evening primrose oil, *Oenothera biennis*, chemoresistance, Paclitaxel chemoresistance, cytotoxicity.

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1. Introduction

Research of natural derivatives as adjuvant in cancer therapy is of current interest and several studies reported the potential anticancer activity of compounds derived from medicinal plants [1–5]. Different studies indicate that a large population of cancer patients make dietary changes following cancer diagnosis [6], as reported by the DietCompLyf study for breast cancer cases. Among the main supplements, Evening Primrose Oil (EPO) was largely used and the proportion of users significantly increased after diagnosis [7].

Evening Primrose (*Oenothera* L.) belongs to the Onagraceae family and *Oenothera biennis* is the most numerous and studied species. EPO, obtained from the seeds, is used as dietary supplement [8–10] and it is composed for a 98% of a mixture of triacylglycerols and for a 1-2% of non-saponifiable fraction, constituted of 53.16% of sterols. Among the triacylglycerols, a 70-74 % is composed by linoleic acid (LA) and another 8-10% by gamma-linolenic acid (GLA), which are essential polyunsaturated fatty acids (PUFAs), belong to omega-6 acids family [8–10]. Beneficial effects of EPO consumption are

associated with its high amount of PUFAs and several examples are well known such as effects on the skin, in different autoimmune diseases, premenstrual syndrome and in reducing low-density lipoprotein (LDL) levels [8–10]. Moreover, some effects are associated with the presence of sterols contained in the non-saponifiable fractions [8–10].

Pancreatic cancer, including Pancreatic Ductal Adenocarcinoma (PDAC), is one of the most aggressive and malignant solid cancer, with a 5-years survival of approximately 5–9%. PDAC is an infiltrating neoplasm with glandular differentiation derived from the pancreatic ductal tree [11,12]. It was demonstrated a family genetic predisposition, but also some precursor lesions within pancreatic tissue and somatic mutations of *KRAS* oncogene and *CDKN2A*, *TP53* and *SMAD4* suppressor genes are implicated in PDAC pathogenesis [13,14]. The main therapeutic approach in PDAC is the surgical resection with adjuvant chemotherapy, but surgery is not always possible, especially in case of metastases and, at the same time, PDAC response to chemotherapeutic drugs remains too low. Given that PDAC is still considered an incurable cancer, new drugs and adjuvant supplementation are necessary to reduce mortality and/or to improve therapeutical outcomes [4].

Until today, there are no preclinical evidences regarding potential EPO anticancer effects. In this study we evaluated the ability of EPO to promote cytotoxicity in human PDAC cell lines and its role as adjuvant for chemotherapeutic drugs activity, in order to support the use of EPO supplementation in the diet of PDAC patients.

2. Results

2.1. EPO reduces PANC-1 and MIAPaCa-2 cell viability

The cytotoxicy of EPO was investigated in PANC-1 and MIAPaCa-2 cells by MTT assay. Cells were treated with different doses of EPO (from 0.024 up to 50 μL/mL). After 72 hours, results showed a reduction of cancer cells viability in a dose-dependent manner with an IC_{50} of 1.37 ± 0.06 μL/mL for PANC-1 and an IC_{50} of 0.89 ± 0.02 μL/mL for MIAPaCa-2 **(Figure 1A)**. MIAPaCa-2 cells resulted more sensible to treatment than PANC-1.

The cytotoxic effect of EPO was subsequently evaluated in non-cancerous cell line such as human keratinocytes HaCaT (Figure 1B). Cells were treated with the same doses of oil used in PANC-1 and MiaPaCa-2 (dose range 0.024 - 50 μl/mL). After 72 hours the viability of HaCaT is reduced only for doses higher than 6.25 μl/mL. This value of IC_{50} confirms that HaCaT is more resistant than PANC-1 and MiaPaCa-2.

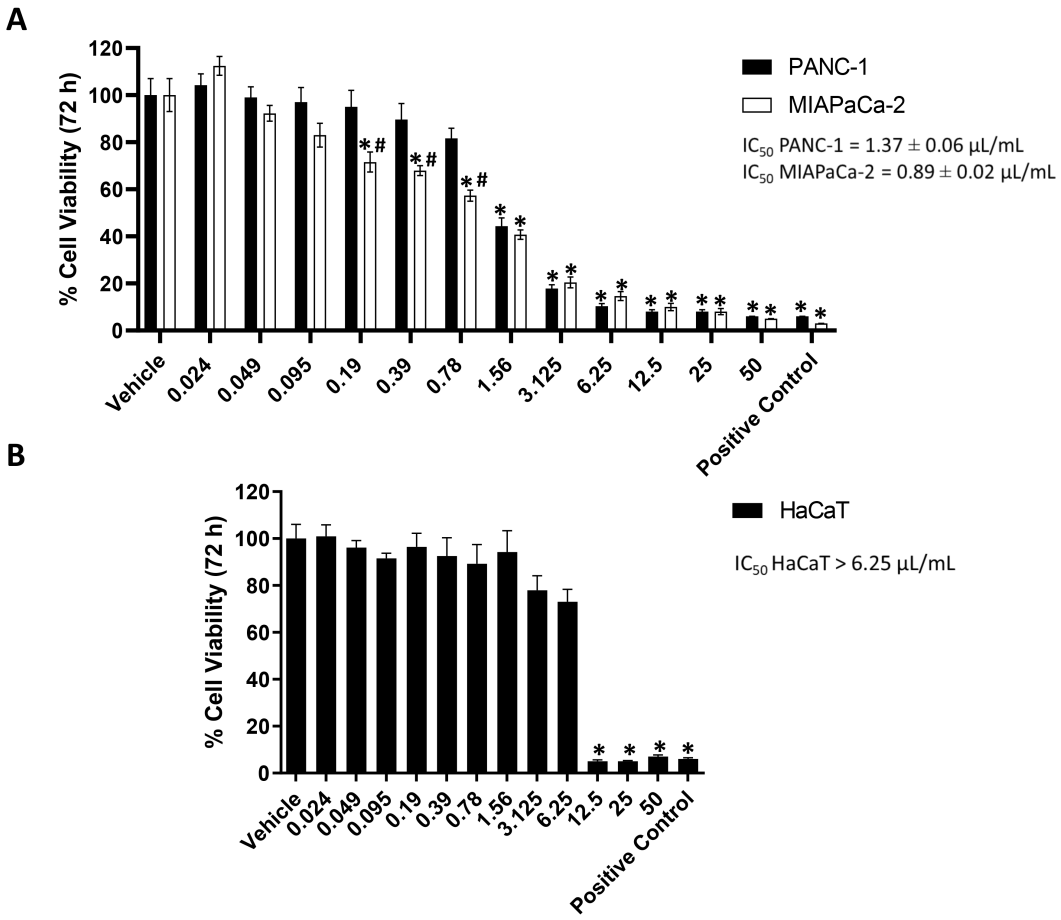

Figure 1. (A) PANC-1 and MIAPaCa-2 cell viability after treatment with different concentrations of EPO (µL/mL) for 72 hours**. Vehicle (DMSO) was used as negative control, while Paclitaxel (PTX) 23.9 µg/mL as positive control.** Data shown are expressed as mean ± SD of three separate experiments. * $p < 0.05$ treated PANC-1 or MIAPaCa-2 vs vehicle, # $p < 0.05$ treated PANC-1 vs treated MIAPaCa-2. **(B) HaCaT cell viability after treatment with different concentrations of EPO (µL/mL) for 72 hours. Vehicle (DMSO) was used as negative control, while PTX 23.9 µg/mL as positive control. Data shown are expressed as mean ± SD of three separate experiments. * $p < 0.05$ treated HaCaT vs vehicle.**

2.2. EPO induces cell death in PANC-1 and MIAPaCa-2 cell lines

Propidium Iodide (PI) staining were used to evaluate cancer cell death. Cells were treated with the lowest cytotoxic doses of EPO (0.39 µL/mL and 0.78 µL/mL) and after 48 hours it was observed an increase in cells undergoing cell death compared to the vehicle, in both cell lines. PANC-1 cell death was induced predominantly with the higher dose, while in MIAPaCa-2 was strongly induced already after treatment with 0.39 µL/mL EPO (Figure 2).

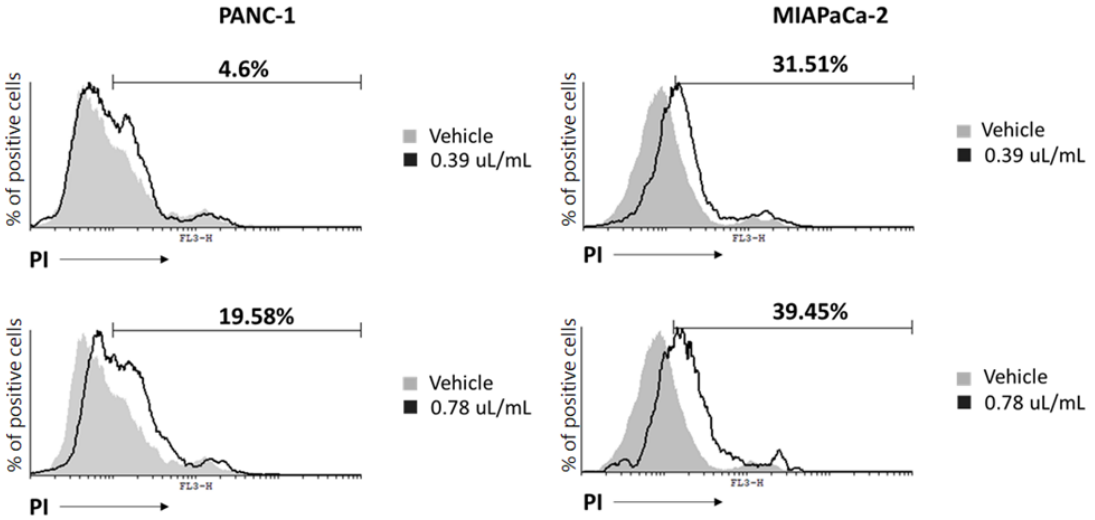

Figure 2. Cytotoxic doses of EPO induce cell death in PANC-1 and MIAPaCa-2 after 48 hours. Data represent the percentage of PI positive cells and are representative of one of three separate experiments.

To further confirm cell death EPO-dependent, Western Blot analysis was performed to evaluate H2AX presence as marker of DNA damage in PANC-1 and MIAPaCa-2 cell lines. PDAC cells were treated with EPO 0.39 μL/mL and 0.78 μL/mL for 48 hours. Results showed a significant increase of H2AX protein in MIAPaCa-2 with both doses, while in PANC-1 it was found an increase of H2AX only with 0.78 μL/mL, confirming a lower sensitivity of this cell line (Figure 3).

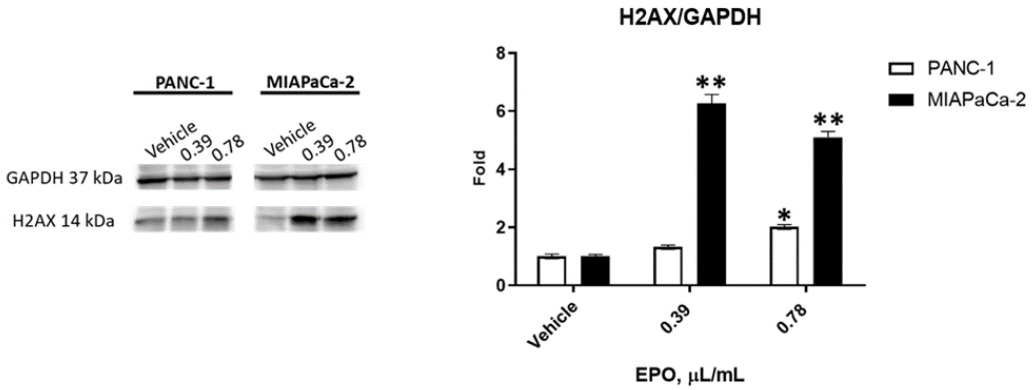

Figure 3. H2AX densitometric values were normalized to GAPDH used as loading control. Densitometric values shown are the mean ± SD of three separate experiments. * $p < 0.05$, ** $p < 0.01$, treated PANC-1 or MIAPaCa-2 vs vehicle cells.

2.3. EPO induces ROS production in PANC-1 and MIAPaCa-2 cell lines

Since EPO induced cell death, the involvement of intracellular reactive oxygen species (ROS) production was evaluated. Thus, PANC-1 and MIAPaCa-2 cells were treated with EPO at 0.39 μL/mL and 0.78 μL/mL and ROS production was analysed by cytofluorimetric analysis after 2 hours and 4 hours of treatment. Results showed that both EPO doses

increased ROS production in PANC-1 cells, while for MIAPaCa-2 the increase of ROS started with EPO 0.78 μL/mL. ROS formation was confirmed through the pretreatment of PANC-1 and MIAPaCa-2 cells with N-acetyl-l-cysteine (NAC) as inhibitor of ROS production. Indeed, NAC reverted this EPO-induced effect (Figure 4) suggesting that EPO-induced cytotoxicity is associated with ROS production.

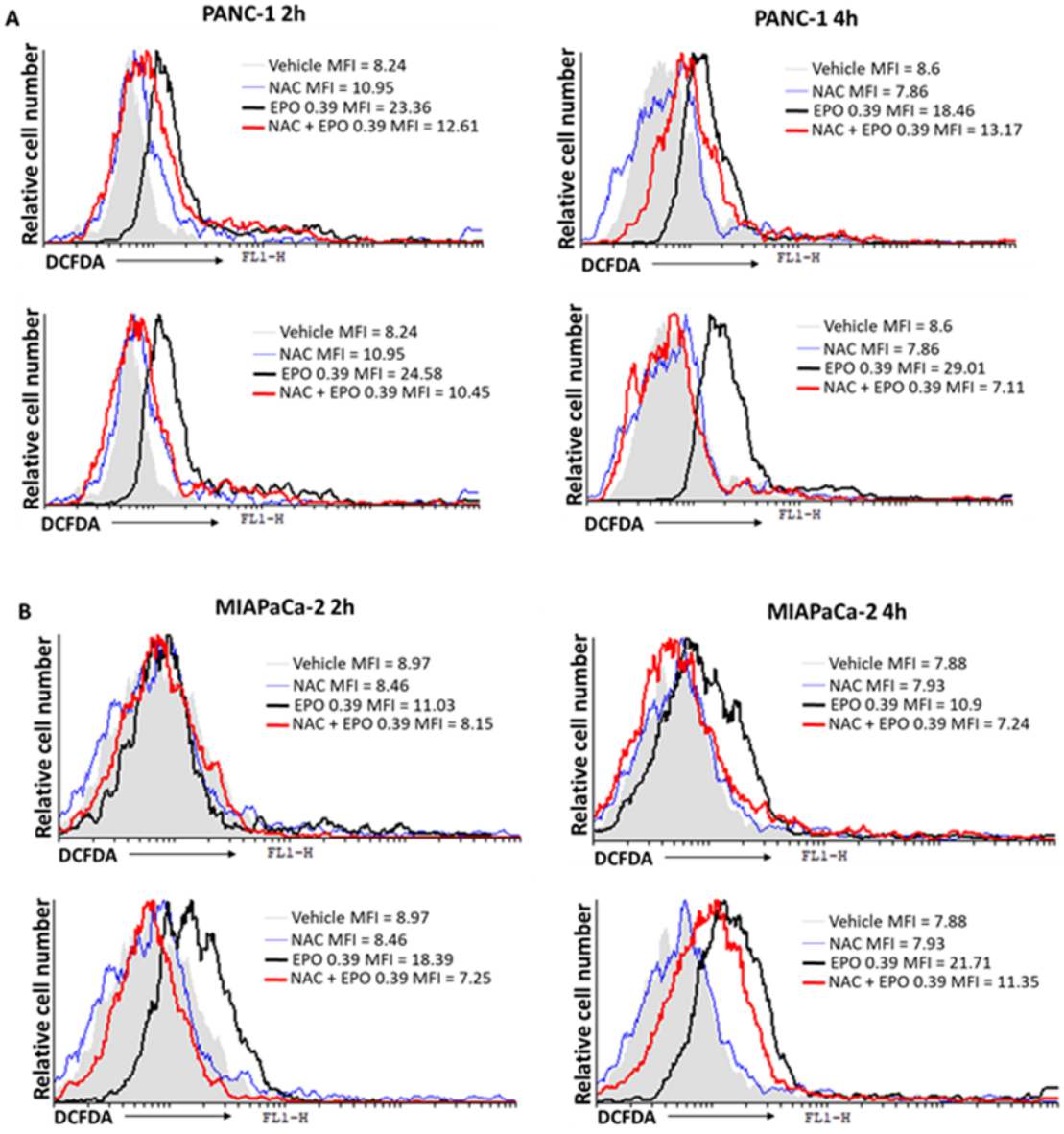

Figure 4. EPO effect on ROS production in (A) PANC-1 and (B) MIAPaCa-2 cells after 2 and 4 hours of treatment. Results are expressed as the mean fluorescence intensity (MFI).

2.4. EPO potentiates Paclitaxel efficacy in PANC-1 and MIAPaCa-2 cell lines

EPO cytotoxic effect was evaluated in combination with PTX and Gemcitabine (GEM), the main chemotherapeutic drugs used in PDAC therapy nowadays [13]. PANC-1 and MIAPaCa-2 cells were treated with different doses of EPO (from 0.095 up to 0.39 μL/mL), GEM (from 12.5 to 50 μg/mL) and PTX (from 0.75 to 3 μg/mL) alone or in combination for 72 hours.

Data showed that EPO was not able to increase the cytotoxicity of GEM (Figure 5) and according to isobologram (data not shown) there are not synergistic/additive effects.

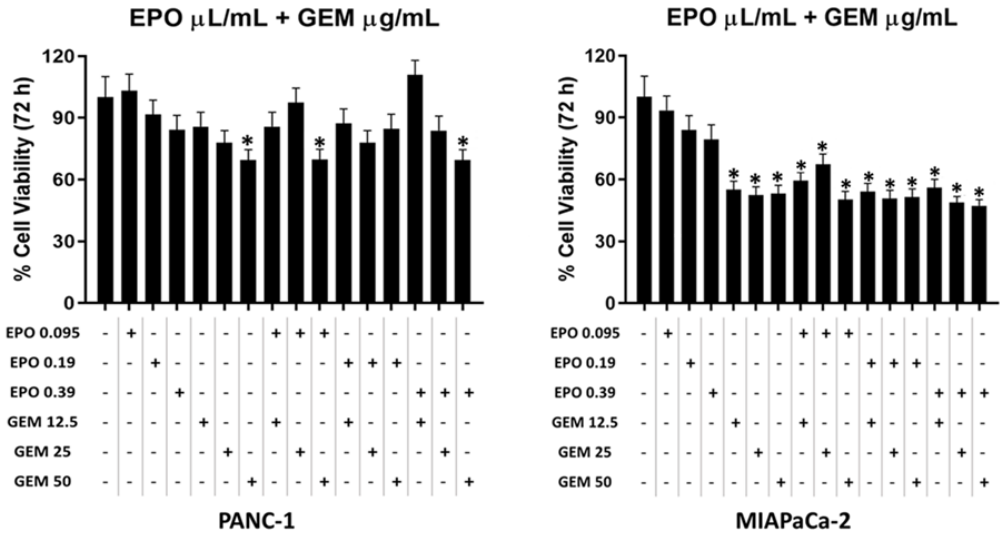

Figure 5. Evaluation of GEM and EPO combinations effect on PANC-1 and MIAPaCa-2 cell viability. Data shown are expressed as the mean ± SD of three separate experiments. * $p < 0.05$ treated PANC-1 or MIAPaCa-2 vs vehicle.

On the contrary, some combinations of EPO and PTX showed synergistic or additive effects (Figure 6). Indeed, combinations with PTX 3 µg/mL and each EPO dose showed a synergistic effect as suggested from CI values (0.16979, 0.1869 and 0.20108 for PANC-1, 0.1009, 0.14078 and 0.19799 for MIAPaCa-2). Further, two synergistic combinations with PTX 1.5 µg/mL and EPO 0.19 µL/mL or 0.39 µL/mL (CI = 0.78758 and CI = 0.84974 respectively) and an additive effect by using PTX 0.75 µg/mL combined with EPO 0.19 µL/mL (CI = 1.0412) were found for PANC-1 cells.

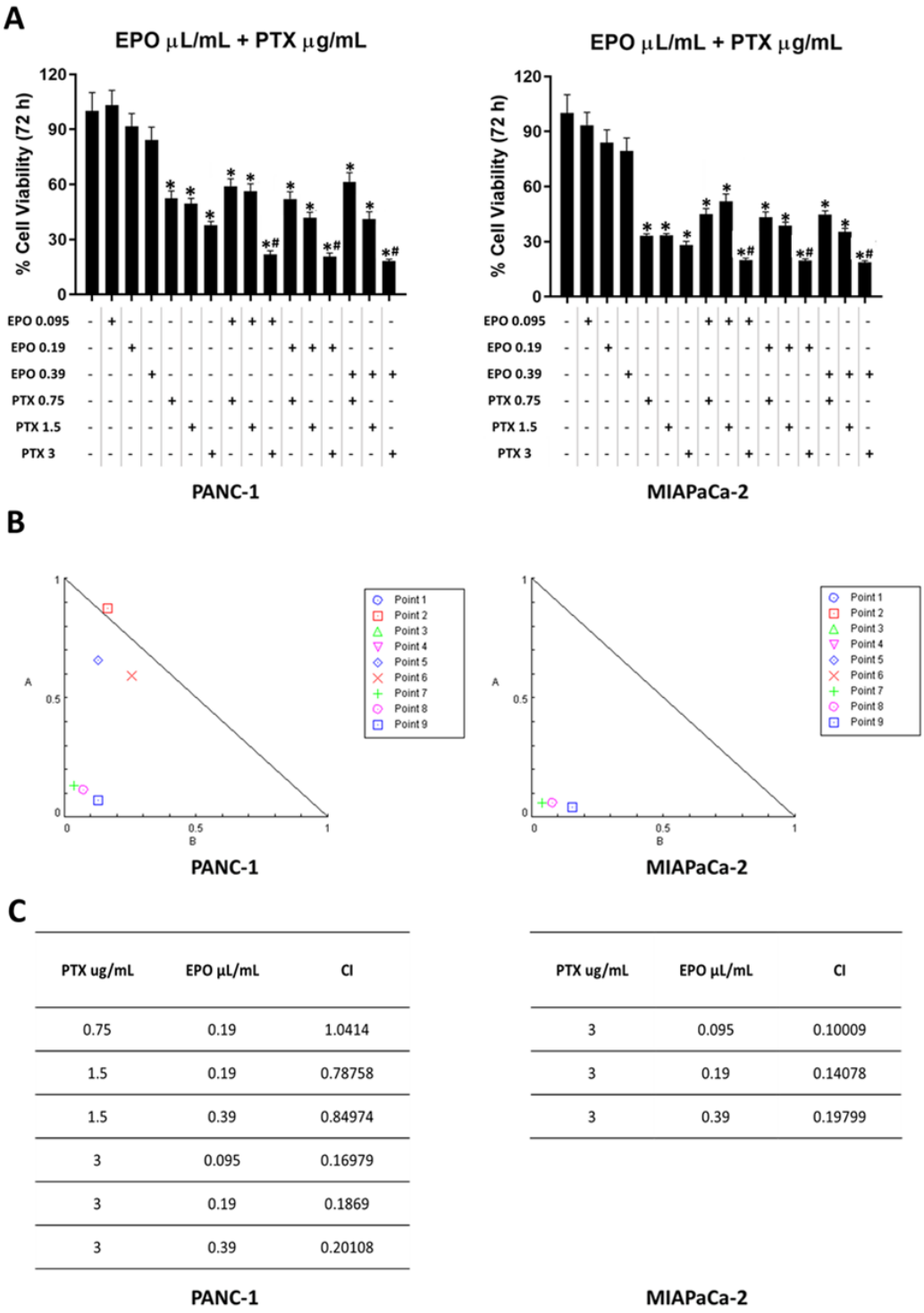

Figure 6. Evaluation of PTX and EPO combination effect in PANC-1 and MIAPaCa-2 cells. (A) PANC-1 and MIAPaCa-2 cell viability after treatment with different combinations of PTX and EPO. Data shown are expressed as the mean ± SD of three separate experiments. * $p < 0.05$ treated PANC-1 or MIAPaCa-2 vs vehicle, # $p < 0.05$ treated PANC-1 or MIAPaCa-2 vs PTX alone. (B) Isobologram plots for combination treatments of PTX and EPO in PANC-1 and MIAPaCa-2 cell lines. Lower left of the hypotenuse synergism, on the hypotenuse additive effect; upper right, antagonism. Synergistic activity of PTX-EPO was calculated by CompuSyn software. (C) CI values for PANC-1 and MIAPaCa-2 cell line.

2.5. EPO influences pERK/ERK protein levels and activation

It is demostrated that the Ras-ERK pathway contributes in oncogenic processes leading to cancer progression [15] and reduced response to chemotherapeutic drugs [16]. Therefore, the expression of ERK protein and its active phosphorylated form, pERK, was evaluated by Western Blot analysis after treatment with EPO. Cancer cells were treated with EPO 0.39 μL/mL and 0.78 μL/mL for 12 and 24 hours.

In PANC-1 cells, data evidenced a slight reduction of pERK starting from 12 hours, followed by a significant decrease of total ERK after 24 hours post-treatment with EPO 0.78 μL/mL. (Figure 7).

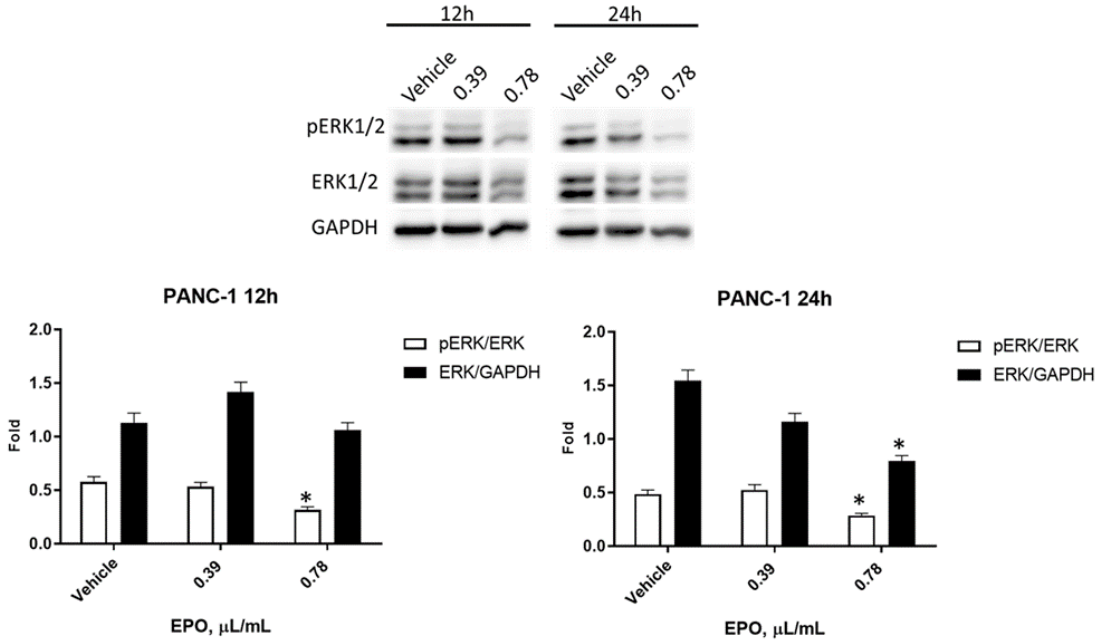

Figure 7. Evaluation of EPO effect in PANC-1. Western blot analysis of pERK and ERK levels in PANC- 1 cells after 12- and 24-hours treatment with EPO. ERK protein expression was normalized to GAPDH protein expression used as loading control, pERK protein expression was normalized to ERK protein expression. Densitometric values shown are the mean ± SD of three separate experiments. * $p < 0.05$ treated vs vehicle cells.

In MIAPaCa-2 cells, a reduction in pERK was detected at 24 hours post treatment with EPO 0.78 μl/mL, while a significant reduction of total ERK was observed after 24 hours of incubation with both doses of EPO (Figure 8).

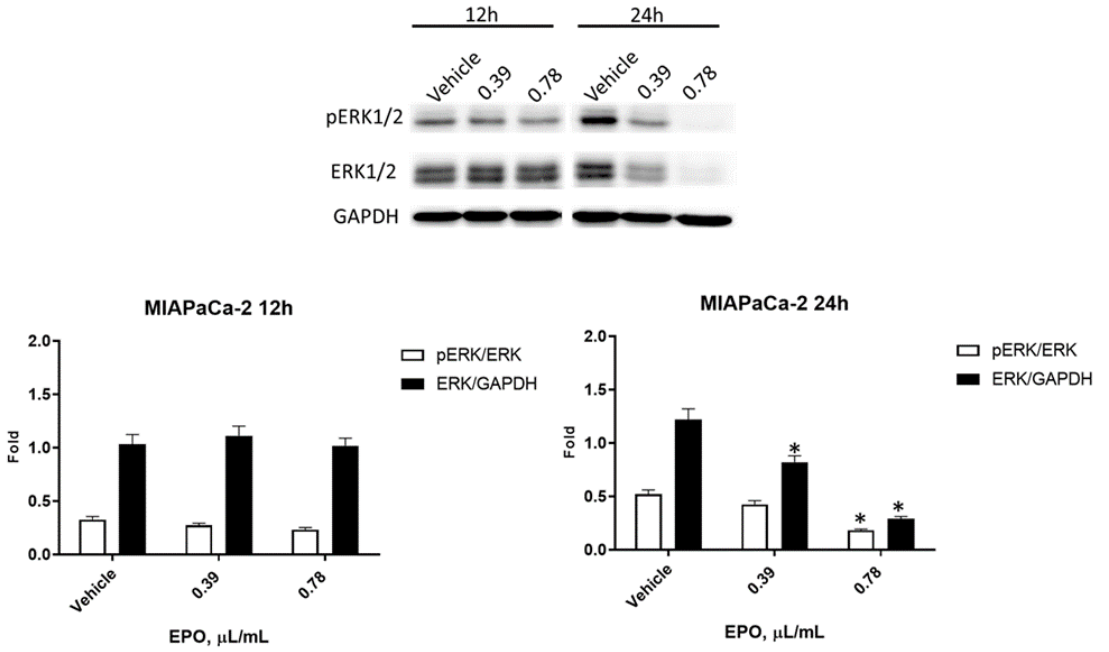

Figure 8. Evaluation of EPO effect in MIAPaCa-2. Western blot analysis of pERK and ERK protein levels in MIAPaCa-2 cells after 12 and 24 hours treatment with EPO. ERK ERK protein expression was normalized to GAPDH protein expression used as loading control, pERK protein expression was normalized to ERK protein expression. Densitometric values shown are the mean ± SD of three separate experiments. * $p < 0.05$ treated vs vehicle cells.

3. Discussion

Recently, plant derivatives are extensively studied to characterize their ability in improving therapy efficacy for many chronic diseases and cancer [17]. According to these evidence, *Oenothera* L. plants family is studied for its biological properties and EPO, obtained from Evening Primrose seeds, is rich in γ-linolenic and linoleic acid. However, in contrast to the high number of studies about GLA anticancer effect [18,19], data regarding the anticancer effect of EPO are few.

EPO was reported to reduce many inflammatory conditions, such as skin disorders, atopic dermatitis and rheumatoid arthritis, and its activity was also demonstrated in diabetes and in premenstrual syndrome [8,20,21]. Furthermore, some evidence showed its potential efficacy in reducing side effects associated with chemotherapy. Indeed, in *in-vivo* model EPO pre-treatment reduced the cyclophosphamide-induced hepatic and pancreatic toxicity, ameliorating biochemical parameters and histopathological alterations [20]. In addition, it reduced skin reaction induced by bortezomib injection in multiple myeloma patients [22].

We evidenced that EPO reduced PANC-1 and MIAPaCa-2 cell viability in a dose-dependent manner after a treatment of 72 hours. Similarly, different extracts obtained from another variety of Evening Primrose induced reduction of cancer cell viability. An extract obtained from the defatted seeds of *Oenothera paradoxa* reduced malignant pleural mesothelioma cell viability and invasiveness, and induced apoptosis in Caco-2 cells [17,23]; while phytosterols isolated from EPO and its main components (β-sitosterol and campesterol) decreased human colon adenocarcinoma cell proliferation [24]. Then, our results showed that EPO induced a cell death in PDAC cells confirmed by an increase of PI positive cells and H2AX protein expression. It has been demonstrated that Evening Primrose seeds extract induces apoptosis in Ehrlich ascites tumor cells [21] and cell death

in HT-29 cells [24]. In agreement, we demonstrated that cancer cell death was more evident in MIAPaCa-2 cells as shown by the lower IC_{50} value obtained from the cell viability assay.

Moreover, to examine the mechanism of EPO-induced cell death, we evaluated if EPO enhances the ROS production. ROS are often associated with cell damage, and, in cancer, their increase is often associated with cell death given that cancer cells are more sensitive to oxidative stress [25,26]. Our data showed that EPO induced ROS production in both cell lines.

Since the aim of integrated therapy is to improve the efficacy of common chemotherapy, we investigated EPO effect in combination with PTX and GEM. EPO showed synergistic and/or additive effect in combination with some PTX doses; in particular, synergism was evident with the higher dose of PTX after 72 hours of administration on PANC-1 and MIAPaCa-2. On the other hand, EPO was not able to increase the efficacy of GEM. Among the main pathways that mediate the response to chemotherapeutic drugs, RAS/RAF/MEK/ERK are attractive targets for cancer therapy [27]. Indeed, ERK is involved in many biological processes including cancer progression and resistance [28].

There are not previous data about the combined effect between EPO and chemotherapeutic drugs. Studies reported that the combination between Trametinib, a selective inhibitor of MEK1/2 kinase activity, and GEM does not lead to an efficient clinical response in PDAC phase I clinical trial, while in non-small-cell lung cancer (NSCLC) Selumetinib, another MEK inhibitor, demonstrated some benefits combined with Docetaxel. This supports the potential synergistic effect of MEK inhibitors with taxane derivatives [16]. Similarly, we demonstrated that EPO reduced ERK phosphorylation after 24 hours and total ERK levels in both cell lines and this may justify the increasing of PTX efficacy.

Furthermore, EPO is rich in PUFAs and it was demonstrated that they are able to affect MEK/ERK pathway; indeed, dietary long-chain n-3 PUFAs are able to inhibit the activation of this pro-survival pathway in *in vitro* and *in vivo* breast cancer models [29], supporting our evidences.

4. Materials and Methods

4.1. Cell Lines

Human Pancreatic Ductal Adenocarcinoma (PANC-1 and MIAPaCa-2) cell lines were obtained from Sigma Aldrich (Milan, Italy) **and Immortalized human keratinocytes cell line (HaCaT), furnished by IFOM (Institute of Molecular Oncology, Rome, Italy), were** maintained in DMEM glucose ^{high} medium (EuroClone, Milan, Italy) supplemented with 100 IU/ml penicillin, 100 mg streptomycin, 10% of fetal bovine serum (FBS), 1 mM sodium pyruvate and 2 mM L glutamine. Cell lines were cultured at 37 °C with 5% CO_2 and 95% of humidity.

4.2. Reagents

Evening Primrose oil (EPO), derived from *Oenothera biennis*, was purchased (Product n° PHR 2978; Sigma Aldrich, Milan, Italy) as pharmaceutical standard, certified reference material (composition described in Table 1) and diluted in DMSO 1:2 and it was freshly prepared for each experiment. Gemcitabine (GEM; 50 mg/mL) and Paclitaxel (PTX; 6 mg/mL) were acquired from Sigma Aldrich (Milan, Italy), solubilized in water and stored at −20 °C.

Fatty Acid Methil Esters (FAME)	USP Comp %
Methyl palmitate	6.00
Methyl stearate	1.91
Methyl oleate	6.93
Methyl linoleate	74.18
Methyl-y-linolenate	9.98
Methyl-o-linolenate	0.22
Methyl arachidate	0.49
Methyl eicosenoate	0.16

Table.1. Evening Primrose oil major component, as described by the manufacturer.

4.3. MTT Assay

In 96-well plates, cells were seeded at concentration of 3×10^4 cells/mL in a final volume of 100 μL/well. After 24 hours of incubation, treatments were administrated in six replicates for each. After 72 hours, cell viability was investigated by adding 0.8 mg/mL of MTT obtained from Sigma Aldrich (Milan, Italy) to the media, according to the protocol previously described [4].

4.4. Cell Death Assay

To evaluate cell death on PANC-1 and MIAPaCa-2 cells, PI staining was used and analyzed by FACScan. Cells were plated at a density of 5×10^4 cells/mL and two doses of EPO were added for 48 hours. After treatment, cells were stained with 20 μg/mL PI for 10 minutes at room temperature and washed. CellQuest software was used to determine the percentage of positive cells. All experiments were repeated three times.

4.5. Western Blot Analyses

Lysates of PANC-1 and MIAPaCa-2 cell lines, untreated or treated with EPO for 12 and 24 hours, were obtained with lysis buffer, composed as previously described [4]. Lysates were separated on a SDS polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare, Chicago, IL, USA), blocked with 5% of Bovine Serum Albumin (BSA) in PBS-Tween 20, immunoblotted with rabbit anti-phospho-histone H2AX (Ser139) (1:1000, #9718, Cell Signaling Technology, Danvers, MA, United States), mouse anti-pERK (1:2.000, Cell Signaling Technology, Danvers, MA, United States) and rabbit anti-ERK (1:1.000, Cell Signaling Technology, Danvers, MA, United States) Abs incubated overnight, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz, California, USA) Abs incubated one hour and then all incubated with their respective HRP-conjugated anti-rabbit secondary Abs (1:5.000, Jackson Immuno Research Europe Ltd, Cambridge, UK) and anti-mouse secondary Abs (1:2.000, Cell Signaling Technology, Danvers, MA, United States) for one hour. The detection was performed using the LiteAblot ®PLUS or the LiteAblot ®TURBO (EuroClone, Milano, Italy) kits and a Chemidoc and densitometric analysis was carried out using the Quantity One software (BioRad, Hercules, USA). H2AX and ERK densitometry values were normalized to GAPDH used as loading control and normalized with vehicle, while pERK densitometry value was

normalized to ERK. Densitometric values shown are the mean ± SD of three separate experiments.

4.6. ROS Assay

In order to assess the oxidative stress it was used a fluorescent probe dichlorodihydrofluorescein diacetate (DCFDA). Briefly, 5×10^4 cells, seeded on a 12-well plate and treated with two EPO doses, were incubated with 20 μM DCFDA (Life Technologies Italia, Italy) 20 min prior to the time point. The harvest was carried out after 2 and 4 hours. As control it was used 10 mM N-acetylcysteine (NAC) with preincubation of 3 hours. After washing the intensity of the fluorescence was analyzed using FACScan and CellQuest software.

4.7. Statistical Analysis

Mean and standard deviation are the result of three independent experiments. The statistical significance was determined by **One-Way ANOVA test with a Tukey's multiple comparisons post-hoc test**. Synergistic and additive effects were calculated by the Chou-Talalay method as previously described [4]. CompuSyn Software (ComboSyn, Inc., Paramus, NJ, USA, 2007) was used for automatically determining synergism and antagonism. The statistical analysis of IC_{50} was performed by Prism 5.01 (Graph Pad).

5. Conclusions

In conclusion, EPO significantly reduced PDAC cells viability, inducing cell death and improving PTX efficacy. These evidences suggest its potential use as an adjuvant in PDAC therapy. Further studies could be performed to elucidate other molecular mechanisms involved in EPO-induced anticancer activity.

Author Contributions: Conceptualization, M.N. and M.L.; methodology, O.M. and M.N.; validation, L.Z., C.A. and G.V.; formal analysis, C.A. (Consuelo Amantini); investigation, L.Z. and C.A.; resources, M.L.; data curation, O.M.; writing—original draft preparation, L.Z and G.V.; writing—review and editing, M.B.M., G.S. and F.M.; visualization, M.N.; project administration, M.N.; funding acquisition, M.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bojková, B.; Winklewski, P.J.; Wszedybyl-Winklewska, M. Dietary Fat and Cancer-Which Is Good, Which Is Bad, and the Body of Evidence. *Int. J. Mol. Sci.* **2020**, *21*, 1–56, doi:10.3390/IJMS21114114.
2. Lal, S.; Shekher, A.; Puneet; Narula, A.S.; Abrahamse, H.; Gupta, S.C. Cannabis and its constituents for cancer: History, biogenesis, chemistry and pharmacological activities. *Pharmacol. Res.* **2021**, *163*, doi:10.1016/J.PHRS.2020.105302.
3. Marinelli, O.; Morelli, M.B.; Annibali, D.; Aguzzi, C.; Zeppa, L.; Tuyaerts, S.; Amantini, C.; Amant, F.; Ferretti, B.; Maggi, F.; et al. The Effects of Cannabidiol and Prognostic Role of TRPV2 in Human Endometrial Cancer. *Int. J. Mol. Sci.* **2020**, *21*, 1–23, doi:10.3390/IJMS21155409.
4. Luongo, M.; Marinelli, O.; Zeppa, L.; Aguzzi, C.; Morelli, M.B.; Amantini, C.; Frassineti, A.; Di Costanzo, M.; Fanelli, A.; Santoni, G.; et al. Cannabidiol and Oxygen-Ozone Combination Induce Cytotoxicity in Human Pancreatic Ductal Adenocarcinoma Cell Lines. *Cancers (Basel).* **2020**, *12*, 1–24, doi:10.3390/CANCERS12102774.
5. Brunetti, A.; Marinelli, O.; Morelli, M.B.; Iannarelli, R.; Amantini, C.; Russotti, D.; Santoni, G.; Maggi, F.; Nabissi, M. Isofuranodiene synergizes with temozolomide in inducing glioma cells death. *Phytomedicine* **2019**, *52*, 51–59, doi:10.1016/J.PHYMED.2018.09.220.

6. Vernieri, C.; Nichetti, F.; Raimondi, A.; Pusceddu, S.; Platania, M.; Berrino, F.; de Braud, F. Diet and supplements in cancer prevention and treatment: Clinical evidences and future perspectives. *Crit. Rev. Oncol. Hematol.* **2018**, *123*, 57–73, doi:10.1016/J.CRITREVONC.2018.01.002.
7. Velentzis, L.S.; Keshtgar, M.R.; Woodside, J. V.; Leathem, A.J.; Titcomb, A.; Perkins, K.A.; Mazurowska, M.; Anderson, V.; Wardell, K.; Cantwell, M.M. Significant changes in dietary intake and supplement use after breast cancer diagnosis in a UK multicentre study. *Breast Cancer Res. Treat.* **2011**, *128*, 473–482, doi:10.1007/S10549-010-1238-8.
8. Timoszuk, M.; Bielawska, K.; Skrzydlewska, E. Evening Primrose (Oenothera biennis) Biological Activity Dependent on Chemical Composition. *Antioxidants (Basel, Switzerland)* **2018**, *7*, doi:10.3390/ANTIOX7080108.
9. Montserrat-De La Paz, S.; Fernández-Arche, M.A.; Ángel-Martín, M.; García-Giménez, M.D. Phytochemical characterization of potential nutraceutical ingredients from Evening Primrose oil (Oenothera biennis L.). *Phytochem. Lett.* **2014**, *8*, 158–162, doi:10.1016/J.PHYTOL.2013.08.008.
10. Montserrat-De La Paz, S.; Fernández-Arche, Á.; Ángel-Martín, M.; García-Giménez, M.D. The sterols isolated from Evening Primrose oil modulate the release of proinflammatory mediators. *Phytomedicine* **2012**, *19*, 1072–1076, doi:10.1016/J.PHYMED.2012.06.008.
11. Luchini, C.; Capelli, P.; Scarpa, A. Pancreatic Ductal Adenocarcinoma and Its Variants. *Surg. Pathol. Clin.* 2016, *9*, 547–560.
12. Adamska, A.; Domenichini, A.; Falasca, M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. *Int. J. Mol. Sci.* **2017**, *18*, doi:10.3390/IJMS18071338.
13. Ansari, D.; Tingstedt, B.; Andersson, B.; Holmquist, F.; Sturesson, C.; Williamsson, C.; Sasor, A.; Borg, D.; Bauden, M.; Andersson, R. Pancreatic cancer: yesterday, today and tomorrow. *Future Oncol.* **2016**, *12*, 1929–1946, doi:10.2217/FON-2016-0010.
14. McGuigan, A.; Kelly, P.; Turkington, R.C.; Jones, C.; Coleman, H.G.; McCain, R.S. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J. Gastroenterol.* **2018**, *24*, 4846–4861, doi:10.3748/WJG.V24.I43.4846.
15. Mendoza, M.C.; Er, E.E.; Blenis, J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem. Sci.* **2011**, *36*, 320–328, doi:10.1016/J.TIBS.2011.03.006.
16. Awasthi, N.; Monahan, S.; Stefaniak, A.; Schwarz, M.A.; Schwarz, R.E. Inhibition of the MEK/ERK pathway augments nab-paclitaxel-based chemotherapy effects in preclinical models of pancreatic cancer. *Oncotarget* **2017**, *9*, 5274–5286, doi:10.18632/ONCOTARGET.23684.
17. Chmielewska-Kassassir, M.; Sobierajska, K.; Ciszewski, W.M.; Bukowiecka-Matusiak, M.; Szczesna, D.; Burzynska-Pedziwiatr, I.; Wiczkowski, W.; Wagner, W.; Wozniak, L.A. Polyphenol Extract from Evening Primrose (Oenothera paradoxa) Inhibits Invasion Properties of Human Malignant Pleural Mesothelioma Cells. *Biomolecules* **2020**, *10*, 1–17, doi:10.3390/BIOM10111574.
18. Whitehouse, P.A.; Cooper, A.J.; Johnson, C.D. Synergistic activity of gamma-linolenic acid and cytotoxic drugs against pancreatic adenocarcinoma cell lines. *Pancreatology* **2003**, *3*, 367–374, doi:10.1159/000073651.
19. Yang, X.; Xu, Y.; Gao, D.; Yang, L.; Qian, S.Y. Dihomo-γ-linolenic acid inhibits growth of xenograft tumors in mice bearing human pancreatic cancer cells (BxPC-3) transfected with delta-5-desaturase shRNA. *Redox Biol.* **2019**, *20*, 236–246, doi:10.1016/j.redox.2018.10.001.
20. Khodeer, D.M.; Mehanna, E.T.; Abushouk, A.I.; Abdel-daim, M.M. Protective Effects of Evening Primrose Oil against Cyclophosphamide-Induced Biochemical, Histopathological, and Genotoxic Alterations in Mice. *Pathog. (Basel, Switzerland)* **2020**, *9*, doi:10.3390/PATHOGENS9020098.
21. Arimura, T.; Kojima-Yuasa, A.; Suzuki, M.; Kennedy, D.O.; Matsui-Yuasa, I. Caspase-independent apoptosis induced by evening primrose extract in Ehrlich ascites tumor cells. *Cancer Lett.* **2003**, *201*, 9–16, doi:10.1016/S0304-3835(03)00440-3.
22. Auberger, J.; Vogt, S.; Hopfinger, G.; Clausen, J.; Greil, R. Topical evening primrose oil for reduction of bortezomib-induced

skin reactions. *Ann. Hematol.* **2013**, *92*, 995–996, doi:10.1007/S00277-012-1656-2.

23. Gorlach, S.; Wagner, W.; Podsędek, A.; Sosnowska, D.; Dastych, J.; Koziolkiewicz, M. Polyphenols from evening primrose (Oenothera paradoxa) defatted seeds induce apoptosis in human colon cancer Caco-2 cells. *J. Agric. Food Chem.* **2011**, *59*, 6985–6997, doi:10.1021/JF200639E.
24. Montserrat-de la Paz, S.; Fernández-Arche, M.A.; Bermúdez, B.; García-Giménez, M.D. The sterols isolated from evening primrose oil inhibit human colon adenocarcinoma cell proliferation and induce cell cycle arrest through upregulation of LXR. *J. Funct. Foods* **2015**, *12*, 64–69, doi:10.1016/J.JFF.2014.11.004.
25. NavaneethaKrishnan, S.; Rosales, J.L.; Lee, K.Y. ROS-Mediated Cancer Cell Killing through Dietary Phytochemicals. *Oxid. Med. Cell. Longev.* **2019**, *2019*, doi:10.1155/2019/9051542.
26. Lu, C.C.; Yang, J.S.; Huang, A.C.; Hsia, T.C.; Chou, S.T.; Kuo, C.L.; Lu, H.F.; Lee, T.H.; Wood, W.G.; Chung, J.G. Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. *Mol. Nutr. Food Res.* **2010**, *54*, 967–976, doi:10.1002/MNFR.200900265.
27. Samatar, A.A.; Poulikakos, P.I. Targeting RAS-ERK signalling in cancer: Promises and challenges. *Nat. Rev. Drug Discov.* 2014, *13*, 928–942.
28. Lanfredini, S.; Thapa, A.; O'Neill, E. RAS in pancreatic cancer. *Biochem. Soc. Trans.* **2019**, *47*, 961–972, doi:10.1042/BST20170521.
29. Serini, S.; Calviello, G. Modulation of Ras/ERK and Phosphoinositide Signaling by Long-Chain n-3 PUFA in Breast Cancer and Their Potential Complementary Role in Combination with Targeted Drugs. *Nutrients* **2017**, *9*, doi.org/10.3390/NU9030185.