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ORIGINAL PAPERS

Entomological Compounds Impact on Key Factors of Prostate Adenocarcinoma Progression

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Abstract

Insect's metabolism produces a wide range of protein and peptides as a first defence line against pathogen infection. It has been proved by scientific literature that this class of compounds could impact viruses or tumours, as promising therapies for serious human diseases. We focused our study on the in vitro highlighting of anti-tumour properties of a Lymantria sp. extract on metastatic prostate adenocarcinoma. The experimental models were designed on standardized cell line DU-145, investigating through flow cytometry relevant anti-cytokines mechanisms (IL-6, IL-8, VEGF), correlated with pro-apoptotic and anti-proliferative phenomena. The entomological tested compounds inhibit with up to 40% the IL6 release, a morbidity mediator in prostate cancer, with maximum 16% for extracellular IL8, a modulator factor of tumour invasion and chemo-resistance, and with over 50% the VEGF signalling, the angiogenesis promoter in metastatic processes. Results show the in vitro inhibition of DU-145 metastasis tumour, on anti-cytokine pathway, completing thus the anti-proliferative and pro-apoptotic effects. One of the innovative applications that we propose as a consequence of this research is in the field of drug development focused on theranostic strategies for cancer treatment. The signal transduction pathways that we prove for the entomological extract could be co-modulated with theranostic nanostructures, reducing their toxicity and improving the efficacy.

Keywords: prostate adenocarcinoma, entomological compounds, cytokines, IL-6, VEGF

Introduction

Prostate cancer is the most common neoplasm in 50-year-old men and the second cause of cancer death in developed countries, thus representing a major socio-medical problem for this category of patients. A serious public health issue is emerging globally because the incidence of prostate cancer is high and the mortality rate of the population due to this disease, unfortunately not observed in time, is steadily increasing. According to the World Health Organization data, after cardiovascular disease, prostate cancer is one of the most common causes of death, accounting for 11% of malignant tumors and 9% of deaths [5, 21, 22].

Complexity of triggering and propagation mechanisms of prostate neoplasm, reveals the heterogeneity of this type of cancer, in which gene changes following the oxidative stress induced by the xenobiotic and chronic inflammatory processes of prostate mediated by T-lymphocytes, mast cells and macrophages, induce morpho-functional imbalances with repercussions on all cellular components (proteins, lipids, DNAs), causing epigenetic and genetic changes altered [14, 22].

In the biology of prostate cancer growth and progression are involved many mechanisms that interact under the influence of androgen and other hormonal factors [4]. Sometimes these changes occur due to the loss or mutation of androgen receptors in the prostate tumor cell, thus facilitating the progression of neoplasm as a result of changing the cellular development from an androgen dependent state to an androgen independent one. Studies on cell cultures have revealed that in androgen-independent tumors both autocrine and paracrine pathways are upregulated and can replace androgens as primary growth enhancing factors in cancer progression. [15].

Recent studies have shown that elevated serum levels of proinflammatory cytokines (key mediators of inflammation with an important roles in inflammation-cancer), including IL-1α, IL-1β, IL-6 and TNF-α (all of these are important members of the pro-inflammatory cytokine family), indicating that chronic inflammation and proinflammatory cytokines might be associated with carcinogenesis, including prostate cancer [11, 24]. The expression of this cytokine has been shown to be involved in the carcinogenesis of various tumor forms such as epithelial including breast, lung, ovarian and prostate [6, 16, 18] and is predominantly secreted by macrophages, T cells, adipocytes. For this reason, IL-6 occurs abundantly in periprostatic tissue [7, 16]. Recent studies have highlighted

for IL-6 a multitude of roles within the local tumor microclimate, including the possibility of creating an autocrine functional loop as a result of both increased ligand and receptor production in tumor cells. "In vitro" tests have shown that by activating cross-autocrine IGF-IR by activating STAT3 under the action of cytokine IL-6, an favorable environment to the development of a more aggressive form of prostate cancer is created [6, 7, 18, 24].

Increase in VEGF (an endothelial cell mitogen synthesized by epithelial cells and myofibroblasts) levels is associated with progression of prostate tumor, from a local to advanced metastatic stage, accompanied by the development of androgen resistance [10, 24]. Studies conducted in 2003 by Chung L.W.R. together with collaborators confirmed that the VEGF expression in prostate cancer is correlates to a prostate – specific antigen (PSA) level and Gleason score. Both IL-6 and VEGF secretion from prostate cancer cells may also increase secondary to tumor hypoxia [6, 10, 24], facilitating a feedback loop stimulating the increased vascularity. According to Hua Xu study their levels were elevated to different degrees in High Fat Diet TRAMP (transgenic adenocarcinoma mouse prostate) mice accompanied with promoted prostate cancer development and progression [24]. Based on this finding, inhibiting this cytokines may be a therapeutic approach to stop the metastatic invasion.

Sometimes, during chemotherapy, certain low-immune patients develop side effects that decrease the chances of successful treatment. In recent years owing to the fear of side effects of synthetic drugs, there has been a tendency for people to prefer more and more to use of natural products for cancer. Medicinal plants and insects are potent natural sources of drugs to treat different human inflammations since ancient time. The therapeutic possibilities offered by plants and insects have been known to mankind for centuries; the pharmaceutical companies continue to invest enormous resource in identifying agents, to define and understand their most appropriate therapeutic uses.

The aim of our researches was to investigate the therapeutic properties of Ento-P, an biological complex previously obtained from entomological source in order to elaborate pharmaceuticals with high efficiency and minimum side effects. Our studies proposed experimental models concerning the pro-inflammatory stimulation of DU-145 cells in order to start the expression of IL-6, IL-8 and VEGF, as the representative parameters for the prostate adenocarcinoma progression. The *Lymantria dispar* extract is a bioactive compound, whose cellular effects have been sustained especially on apoptotic and cell division mechanisms, by previous research [13]. We design these studies to complete the in vitro efficacy spectrum by monitoring the extracellular dynamics of secretion of tumor progression and metastasis mediators (IL-6, IL-8 and VEGF)

dramatically changed under inflammatory conditions imitated by two types of stimuli: PMA and TNFα.

Materials and methods

Materials

Insect material: A species of the genus *Lymantria dispar* was used for the extraction procedures for obtaining the active biocompound used in this study, named fallow Ento-P. The obtained extract is rich in essential fatty acids (1-2% ω-6), essential amino acids (2-6.5%), proteins (30%) and mineral salts (Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si).

Standardized cell lines: Human prostate cancer cell lines DU-145 (an androgen independent cell lines, with high proliferative capacity, cells culture reach confluency relatively quickly – 80% confluence in 3 days, purchased from ATTC) was used for in vitro tests. Cells were maintained under standard culture condition (21% O_2 and 5% CO_2, 37^0C) and DMEM (Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 Ham, code: D8437, Sigma-Aldrich) containing 1% antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (code: F7524, Sigma-Aldrich). Cells were analyzed in normal development conditions in the presence and absence of investigated compounds and also in an nonspecific inflammation model induced by TNF–α (15ng/mL, 24h, 37^0C) and PMA (50 mg/ml, 1h, 37^0C).

Chemical Substances: The control agents used was dexamethasone (code: D4902, Sigma-Aldrich) as anti-inflammatory agent. For the determination of cytokines for extracellular cytokines by flow cytometry, the BD Cytometric Bead Array (CBA) - Human Inflammatory Cytokines kit (BD Pharmingen) kit was used.

Methods

Expression of IL-6, IL-8: In order to examine the effect of Ento - P on pro-inflamatory cytokines in vitro under normal and proinflammatory conditions, were conducted by using a multiplex technique that simultaneously detects cytokine levels (IL-6 and IL-8) and proangiogenic factor (VEGF) in the cell culture supernatant DU-145 by flow cytometry. This technique has been adapted and applied successfully by our research group in the study of various biologically active complexes obtained from various plant, animal or entomological sources [12].

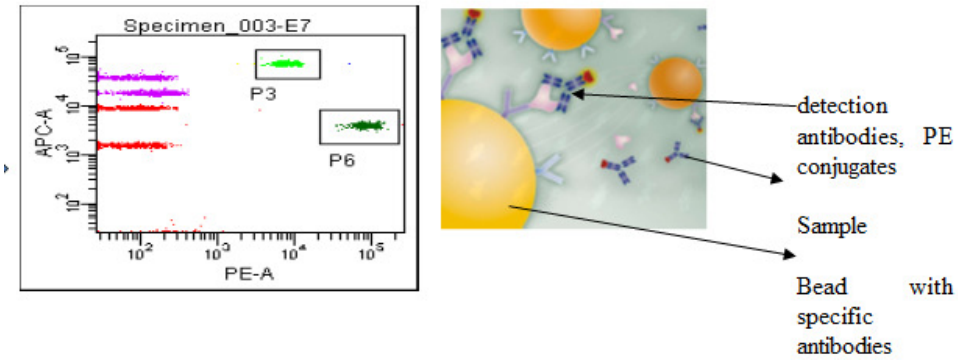


Fig. 1. Simultaneous detection of cytokine (IL-6 and IL-8) and proangiogenic factor (VEGF) levels by flow cytometry [2]

The assay uses a series of discrete fluorescence intensity particles for the simultaneous detection of several soluble analytes (inflammatory cytokines). Each particle (beads) of the kit has a capture surface coated with antibodies specific for IL-8, IL-6 and VEGF [2]. The detection reagent provided in the kit is a mixture of phycoerythrin (PE) – conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte (in our particular case IL-6, IL-8, and IL1-α). The capture beads, the conjugated detection antibodies and the recombinant standards or assay samples are incubated together to form a sandwich complex that is visualized in APC - A / PE - A coordinates following acquisition of flow cytometry. Analysis of fluorescence histograms and interpolation of values on calibration curves is performed with FCAP Beads Array software [12].

Expression of pro-angiogenic VEGF factor: Extracellular level of VEGF was evaluated through a flow cytometric beads based assay similar with that for extracellular cytokines: BD Cytometric Bead Array (CBA) - Human Cytokines kit (BD Pharmingen) for the simultaneous detection of soluble factors – VEGF flex set, Protein Master Buffer Kit.

Equipments: Flow cytometer FACS CANTO II with DIVA 6.1 and FCS Express software.

Statistics: Values are presented as mean ± standard deviation (SD), averaged over at least three independent experiments for normally distributed data. Comparisons of treatment outcome were tested for significant difference by Repeated Measures ANOVA, Dunnett's Multiple Comparision Test. Statistical significance was assumed at a P value of less than 0.05. Data were examined for extreme values, which were defined as values outside the mean ± 3 times the s.d. (Peirce's criterion); extreme values were excluded from analysis as described.

Results and discussions

Cancer remains one of the leading causes of morbidity and mortality globally and even if great advancements have been made in the treatment and control of cancer progression, significant deficiencies and room for improvement remain [3]. Currently, pharmaceutical companies are looking for new ways to explore the therapeutic value of insects and characterization of biologically active compounds that can cause them to develop cancer drugs and appropriate cancer treatment.

It is well known from both literature and clinical practice that tumor cell populations are characterized by the possibility of self-renewal, resistance to stress conditions and metastasis potential, favoring aggressiveness and cell migration [1]. The tumor stroma plays an essential role in the process of tumorigenesis, its components having a key role in the progression of prostate cancer by stimulating angiogenesis and promoting the survival, proliferation and invasion of cancer cells [1; 17]. Thus, chemokines determine the directed migration of leukocytes along a concentration gradient, leading to the accumulation of migrating cells at the source of chemokine production. Cytokine secreted by the tumor can act on the normal surrounding stroma, recruiting stromal cells to promote tumor migration and metastasis [1, 8, 9, 14, 17]. An identified metabolic pathway that substantially alters the infiltration of leukocytes into the tumor resulting in the accumulation of immunosuppressive and pro-tumorigenic cells is the cytokine / receptor pair (IL-8 / IL-8R) [1; 17]. As we have seen in the beginning, overexpression of cytokine IL-6 is associated with biochemical recurrence of prostate cancer [14,16] and reduced life expectancy [9,19]. IL-6 mediated the Janus kinase pathway (JAK), the signal transducer and the transcriptional activator 3 (STAT3) and the mitogen-activated protein kinase (MAPK), which induced androgen receptor-mediated gene expression and, Androgen-dependent PCs [9, 19, 20]. Immunotherapy pursuing IL-6 has opened up a promising prospect in the treatment of cancer, and a randomized Phase II study (CNTO 328), in which siltuximab (the anti-IL-6 monoclonal antibody) is applied in the treatment of cancer of metastatic prostate [8, 19, 20].

Previous experiments on Ento-P have revealed a potential antitumor effect, demonstrating its action in various convergent biological processes that inhibit the mechanisms of initiation of tumour transformations. Thus, the anti-proliferative action of Ento-P compared to methotrexate was demonstrated by regulating cell cycle sequencing, reducing the synthesis phase of DNA and inducing apoptotic processes [13]. The complementary studies that focused on the dynamics of enzyme activity of proteolytic enzymes involved in tumour progression as a consequence of the extracellular matrix degradation under the action of

proteolytic enzymes and the migration of malignant cells to new metastatic sites revealed the potential inhibitor of Ento-P on MMP-2 and MMP-9 [13].

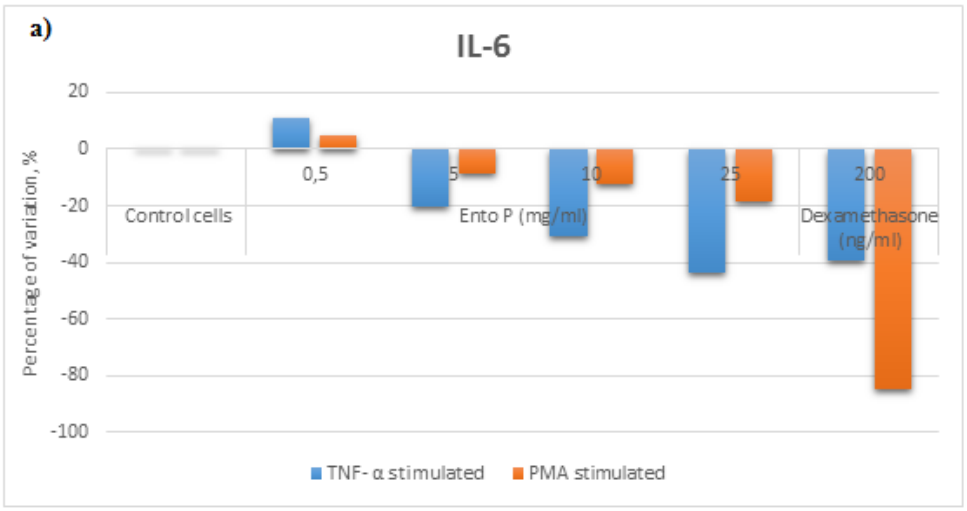
This study aims is to highlight the "in vitro" antitumor properties of the Ento-P complex at the level of metastatic prostate adenocarcinoma through a screening of cytokine signaling factors involved in tumor progression and metastasis (VEGF, IL6, IL8). Evaluation of the extracellular release dynamics of acute phase proinflammatory cytokines (IL-6 and IL-8) and vascular endothelial growth factor (VEGF) was performed on the DU-145 independent androgenic prostate carcinoma cell line by six successive experiments, between passages 5 and 16 in the range of 0.5-25 mg / ml of Ento-P, based on cytotoxicity studies (not presented in this paper). The experiments evaluated and compared the effects of Ento-P and Dexamethasone 200 ng/ml. Their effect was studied in two special stimulation conditions, specific for prostate cancer pathology, using 15 ng/ml of TNFα - systemic stimuli, the main generator of pro-inflammatory cascades and 50mg/ ml of PMA (a reversible, highly potent activator of PKC) [23].

Our results on metastatic DU 145 cells, pro-inflammatory stimulated with TNF alfa, show a significant anti-inflammatory effect of Ento-P by reducing extracellular release of both IL6 and IL8 (see Figure 2, a and Figure 2,b). Inhibition occurs by reducing the secreted IL6 in the extracellular medium by up to 44% over the control culture, over a large range of active doses (16 μg / ml - 0.2 μg / ml). The effect of Ento-P on IL8 is slightly weaker than on IL6, the presence of IL8 in the extracellular medium under the influence of Ento P is reduced by up to 16% compared to the culture blank for doses ranging from 5 μg / ml to 1 μg / ml.

Table 1 presents values with statistical significance as the mean ± standard deviation of the experimental data obtained after the statistical processing and application of the inclusion / exclusion criteria.

Table 1. The variations of IL-6, IL-8 and VEGF extracellular levels on Ento-P action

		IL6 (pg/ml)		IL8 (pg/ml)		VEGF (pg/ml)	
		TNF- α stimulated	PMA stimulated	TNF- α stimulated	PMA stimulated	TNF- α stimulated	PMA stimulated
Control cells		933,75 ± 58,15	740,04 ± 50,52	43012,68 ± 825,52	21034,52 ± 946,67	638,76 ± 49,51	1857,49 ± 94,20
Ento P (mg/ml)	0,5	1034,62 ± 49,39	776,42 ± 83,57	45927,4 ± 1000	24558,87 ± 1115,34	651,33 ± 43,03	2006,77 ± 150,22
	5	745,93 ± 52,08	673,83 ± 84,58	22993,4 ± 414,10	19415,05 ± 1098,93	358,31 ± 52,10	1792,04 ± 139,69
	10	647,46 ± 64,05	647,46 ± 71,48	23535,66 ± 498,99	19886,36 ± 883,10	424,37 ± 38,75	1694,93 ± 96,28
	25	525,19 ± 100,01	602,31 ± 56,42	11958,62 ± 632,39	14633,98 ± 1349,25	336,96 ± 51,33	1489,18 ± 91,62
Dexamethasone (ng/ml)	200	568,96 ± 73,64	113 ± 18,00	547,51 ± 34,48	1033,65 ± 47,13	274,99 ± 50,60	493,25 ± 79,57



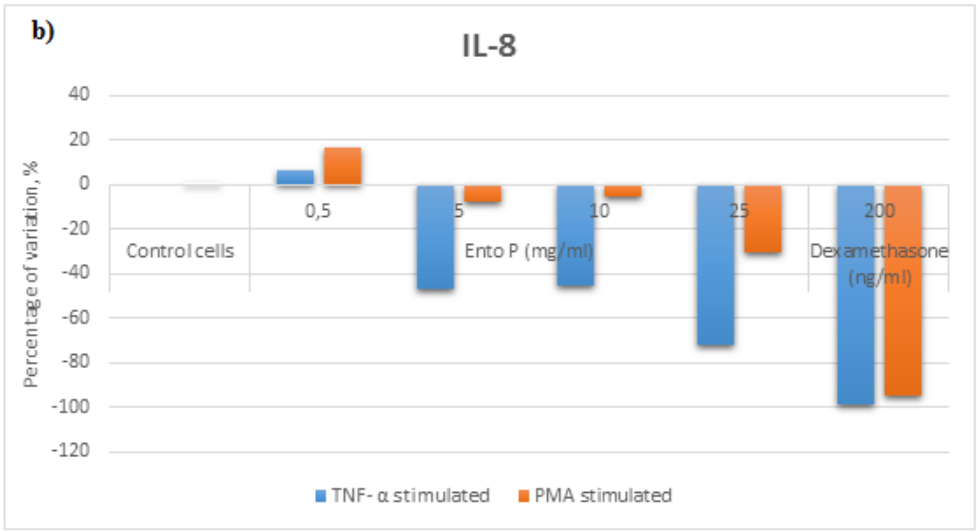


Fig. 2. Dynamics of extracellular release of tumor progression factors: a) IL-6 and b) IL-8

For tumors, sprouting new blood vessels is essential for growth and metastasis (solid tumors recruit blood vessels from surrounding tissues, by expressing and secreting growth factors that target endothelial cells). VEGF is one of the most important factors, promoting cell migration and proliferation, and it was detected extracellularly on DU-145 cell line. The rise of VEGF level is associated with prostate cancer progression, from a local event to an advanced metastatic one, accompaigned by the androgenic resistance development. In our study, Ento-P has a very good anti-angiogenic effect, especially on pro-inflammatory stimulated cells, suggesting the inhibition of tumour invasion and development, in a dose-effect manner see Figure 3. Evaluation of Ento-P effect on the main signaling factor on the onset of metastasis by stimulating tumor angiogenesis revealed significant compound activity, reducing this signal molecule in the extracellular environment by up to 59% from the untreated control. The effect occurs at 10 µg / ml and is maintained at all lower test concentrations, with a peak at 1.6 µg / ml.

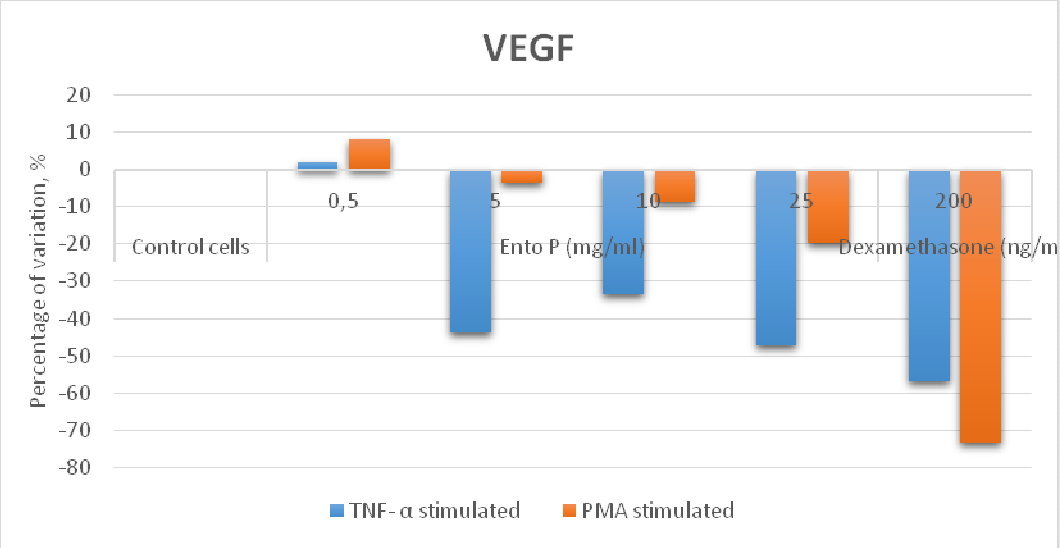


Fig. 3. The dynamics of extracellular release of VEGF, a proangiogenic factor in metastasis

Taking into account the role of signal molecules of IL-6 and IL-8 cytokines in stimulating prostate cancer tumour growth, the results presented in Figure 2 and 3 and Table 1 confirm the role of antitumor agent of Ento P, particularly active on IL-6, a mediator of morbidity in prostate cancer.

Conclusions

The entomological extract, through its unique chemical composition (proteins, essential amino acids, essential fatty acids and micro-elements: Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si) induces cellular effects targeted on tumor prostate cells survival mechanisms: *IL6 –morbidity mediator, IL8 – tumor growth mediator and angiogenic factor VEGF pathways, completing the anti-proliferative and pro-apoptotic effects previously proved.* As well as, the entomological complex was active in pro-inflammatory stimulated cells, suggesting its counteracting effect in inflammation that sometimes accompaigned the neoplasm pathology.

The results highlighted by *in vitro* experiments show that this type of extracts can be successfully used individually or synergistically with other bio products as medicinal active substances with valuable therapeutic properties.

One of the innovative application that we propose as a consequence of this research is in the field of drug development focused on theranostic strategies for cancer treatment. The signal transduction pathways that we prove for the entomological extract could be co-modulated with theranostic nanostructures, reducing their toxicity and improving the efficacy. Further studies will be designed for demonstrating the mechanisms of action of this complex and define its use as therapeutic tool.

Acknowledgment

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Notations and/or **Abbreviations**

IL-6 – Interleukin 6

IL-8 – Interleukin 8

VEGF - Vascular endothelial growth factor

PMA - Phorbol-12-myristate-13-acetate

TNFα – Tumor necrosis factor alpha

PKC - Protein kinase C

R E F E R E N C E S

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