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Investigation of the Immunological Effect of Fermented *Epilobium Angustifolium* Extracts at the Cell Level.

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ABSTRACT

Epilobium angustifolium has been traditionally used to treat a number of diseases. The mode of action of *Epilobium angustifolium* extracts is still unknown. The aim of this study is to identify the influence of various commercially available extracts of fermented *Epilobium angustifolium* at the cellular level. Water and ethanol extracts of *Epilobium angustifolium*, as well as polar and nonpolar fractions were tested on cell level in the range of concentrations between 3 and 1000mg / ml. It was shown that various fermented *Epilobium angustifolium* extracts could affect on cell proliferation and apoptosis of primary human lymphocytes.

Keywords: *Epilobium angustifolium*, extracts, proliferation, apoptosis, koporski tea

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INTRODUCTION

Preparations based on medicinal herbs have been used to treat a wide range of diseases from ancient times. Over the past decade, interest in the use of drugs herbal medicine has increased significantly. Moreover, the interest of the scientific community in the explanation of their mode of action increased. According to the literature [1-3], about 25% of all modern medicines are directly or indirectly derived from higher plants. Currently, about 60% of anticancer drugs that are on the market or in the late stages of clinical trials, its natural products derived mostly from higher plants [3]. According to the World Health Organization definition herbal drugs contain as active ingredients plant parts or plant materials in the crude or processed state plus certain excipients, i.e., solvents, diluents or preservatives. Usually, the active principles responsible for their pharmacological action are unknown.

In most cases the mode of action of these drugs is not fully understood. Plants of the *Epilobium* genus are traditionally used as a source of raw materials in the phytotherapeutic drugs production. According to the literature, extracts *Epilobium* plants have various medicinal activities, such as anti-inflammatory, anti-androgenic, anti-proliferative, anti-fungal, antimicrobial, antioxidant et al. [4-7]. Extracts of *Epilobium* plants are used in the treatment of different diseases such as prostatic hyperplasia, rectal bleeding, stomach ulcers, prostate, liver and kidney diseases, gastritis and sleep disorders, as well as a variety of skin infections [8, 9]. In the Russian folk medicine above ground fermented part of the plant (*Kaporskaya* tea, Russian tea) was used for kidney diseases, gastritis, and sleep disorders. The active components which are responsible for the biological activity *Epilobium* plants still not completely defined. Flavonoids (kaempferol, quercetin, myricetin, etc.), Phenolic acids and their derivatives (ellagic acid, chlorogenic acid, benzoic acid, cinnamic acid and derivatives thereof), tannins (tannins) [10], steroids triterpenes (cholesterol, campesterol, stigmasterol), as well as fatty acids (oleic, linoleic, palmitic and stearic acid) [11, 12] could be identified as the main biologically active components. The vegetative part of *Epilobium* contains large amounts of aspartic and glutamic acid, which have a positive effect on the cardiovascular system [13]. The mode of action of *Epilobium* plants extracts is not fully understood.

The purpose of this study is to investigate the mechanism of action of the fermented extracts of *Epilobium angustifolium* (*Koporsky* tea) at the cell level.

MATERIAL AND METHODS

Plant material

Commercially available *Kaporskiy* tea (fermented *Epilobium angustifolium*) were produced by "Promed", Perm, Russia.

Extracts preparation

Water soluble extract

Powdered commercially available plant material (100 g) was weighed on the scales (Sartorius LC 6201) and extracted with boiled water for 1 h at 80 C by using Buchi HB 1400 Water Bath (Gemini BV, Netherlands). The solid residue was filtrated through bandage.

Ethanol extract

Powdered plant material (100 g) was extracted with Ethanol (Alcool Ethylique Ethanol Absolute, J.T. Baker) (1:5) for 15 h at 25 C. The ethanol residue was evaporated at 80 C by using Buchi HB 1400 Water Bath (Gemini BV, Netherlands) with Rotavapor M (Gemini BV, Netherlands).

Polar and non-polar fractions preparation

1,7 g. of solid residue of ethanol extract was divided into polar and non-polar fraction by using Ethyl acetate (Sigma-Aldrich, USA), Butanol-1 (Carl Roth GmbH, Germany) and bidistilled water in Separatory

Funnels 250 mL, (Schott DURAN, Germany) with Buchi HB 1400 Water Bath (Gemini BV, Netherlands) with Rotavapor M (Gemini BV, Netherlands).

Ethics statement

All experiments conducted on human material were approved by the local Ethics Committee of the University of Freiburg (55/14; 11.02.14).

Preparation and cultivation of human peripheral lymphocytes

Human peripheral lymphocytes were isolated from the blood of healthy adult donors obtained from the Blood Transfusion Centre (University Medical Center, Freiburg, Germany). Venous blood was centrifuged on a LymphoPrep™ gradient (density: 1.077 g/cm³, 20 min, 500 x g, 20°C; Progen, Heidelberg, Germany). Cells were washed twice with medium and cell viability as well as concentration was determined using the trypan blue exclusion test. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria), 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (all from Life Technologies, Paisley, UK). The cells were cultured at 37°C in a humidified incubator with a 5% CO₂/ 95% air atmosphere.

Activation and treatment of immunocompetent cells

Lymphocytes were incubated for 72 h in the presence of medium alone (NC) or in the presence of anti-human CD3 (clone OKT3) and anti-human CD28 (clone 28.2) mAbs (each 10 or 3.3 ng/mL; both from eBioscience, Frankfurt, Germany) (PC 10; PC 3.3) in the presence of camptothecin (CPT; 100µM; Tocris, Bristol, UK) or different concentrations of the different extracts. The extracts were dissolved in sterile DMSO (Sigma-Aldrich, USA). After cultivation, the cells were used for biological tests as described.

Determination of lymphocyte apoptosis using annexin V

Apoptosis was determined by using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (eBioscience, Frankfurt, Germany) according to the manufacturer's instructions followed by flow cytometric analysis using a BD FACSCalibur flow cytometer and BD CellQuest Pro Software to quantify the levels of apoptosis.

Determination of proliferation and cell division

For cell proliferation and cell division tracking analysis of different T cell subsets, cells were harvested and washed twice in cold PBS and resuspended in PBS at a concentration of 5 x 10⁶ cells/mL. Cells were incubated for 10 min at 37°C with 5 µM 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich, St. Louis, MO). The staining reaction was quenched by washing twice with complete medium and the cell division progress was analyzed using flow cytometry.

RESULTS

The influence of tea Koporsky extracts on the proliferation of primary human lymphocytes.

Firstly the influence of Koporsky tea in various concentrations on proliferation of primary human lymphocytes was evaluated. Experiments were conducted with increasing of Koporsky tea extract concentrations (from 3 to 1000 µl) in the presence of CFSE. The CFSE dye is inherited from daughter cells after cell division and each dividing cell loses fluorescence intensity without affecting cell viability. The obtained data are shown in Fig. 1.

The flow cytometry analysis (Figure 1) demonstrated that the proliferation of CFSE⁺ mitogen-activated lymphocytes was inhibited in the presents of ethanol extract, lyophilizate and polar fractions in the concentration range between 1000 and 100 µg / ml, whereas in concentration range between 3 and 30 µg / ml we can observe a slight increase in the value of cells proliferation.

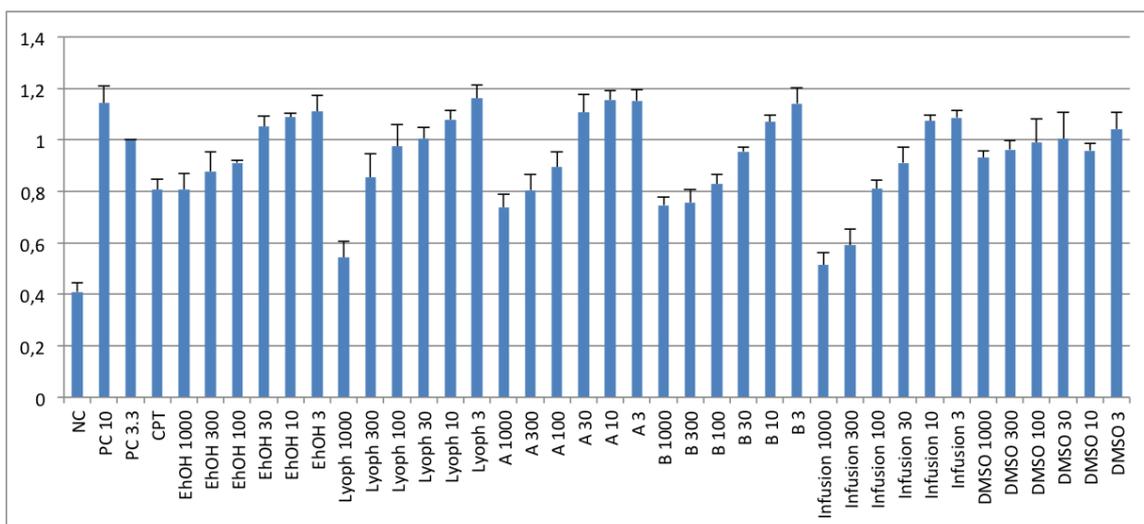


Fig 1: The effect of Koporsky tea extracts on the proliferation of primary human lymphocytes

(where EhOH - ethanol extract, Lyoph - lyophilisate, A - polar fraction, B- non-polar fraction, Infusion - aqueous extract).

In the case of non-polar fraction and water extract the inhibition of primary human lymphocytes proliferation was observed at concentration range between 1000 and 30 $\mu\text{g} / \text{ml}$. There is a slight increase in proliferation of primary human lymphocytes in the concentration range from 30 to 3 for Lyophilized of non-polar fraction and water extract in comparison with positive control (3.3 ng/mL anti-bodies).

The influence of Koporsky tea extracts on the induction of apoptosis of primary human lymphocytes

In a further step, we aimed to identify the mechanism behind the reduced cell proliferation observed, and quantified apoptotic effect on activated lymphocytes in the presence of high concentrations (1000 - 100 $\mu\text{g}/\text{mL}$) of the Koporsky tea extracts. As positive controls for apoptosis, we used camptothecin (CPT).

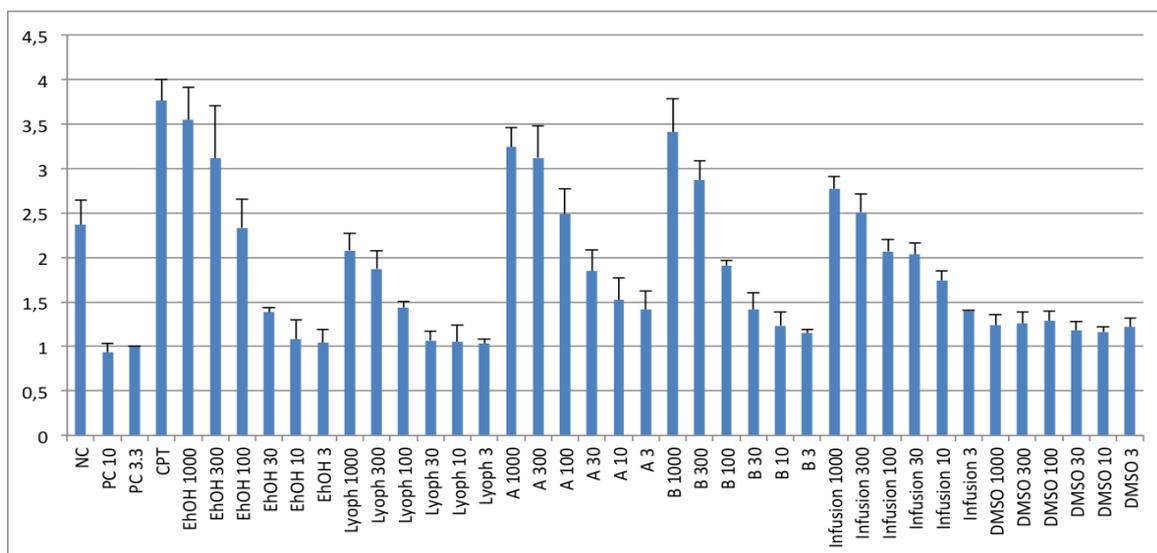


Fig 2: The effect of Koporsky tea extracts on the apoptosis of primary human lymphocytes

(where EhOH - ethanol extract, Lyoph - lyophilisate, A - polar fraction, B- non-polar fraction, Infusion - aqueous extract).

As shown in Figure 2, lyophilisate, ethanolic extract, an aqueous extract as well as polar and nonpolar fractions product induce an apoptosis of human lymphocytes in dose dependent way in comparison with positive control (3.3 ng/mL anti-bodies) on the whole concentration range of 3 - 1000 µg / ml. In the presence of higher concentrations of all factions we can observe more intense level of apoptosis of primary human lymphocytes.

CONCLUSION

Using of Koporsky tea (fermented *Epilobium angustifolium*) has a long tradition in Russian folk medicine. Extract of fermented *Epilobium angustifolium* leaves is used to treat such diseases as ulceration of the stomach, gastritis, as well as sleep disturbances, but still the mechanism of action at the cell level is poorly understood. For this reason, study of the effect of different Koporsky tea extracts is interesting to justify the rationality of its use. It has been shown that the ethanol extract, polar fraction and the lyophilisate of Koporsky tea can inhibit lymphocyte proliferation at concentrations above 100 mg / ml, in the case of a non-polar fraction and an aqueous extract the inhibition of proliferation was detected at concentrations above 30 µg / ml. Furthermore, it was found that the lyophilisate, ethanolic extract, an aqueous extract, as well as polar and nonpolar fractions of Koporsky tea can induce apoptosis in human lymphocytes. The intensity of the induced apoptosis is dose-dependent. So it could be assumed that the inhibition of primary human lymphocytes proliferation occurs due to the fact that Koporsky tea has the ability to induce apoptosis.

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