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Inhibitory Effects of Plant Extracts on Postproline Cleaving Enzyme Activity in Human Breast Cancer Cells

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Effects of crude extracts from three herbs: *Rhaponticum carthamoides* (maral root), *Tanacetum vulgare* L. (tansy) and *Tribulus terrestris* L. (small caltrops) on the postproline-specific enzyme activity in the human triple negative breast carcinoma cell line MDA-MB-231were investigated. In all cases, a concentration-dependent inhibition was observed, with the degree of inhibition from *T. vulgare* L. being the highest. Different tansy extracts, also had a pronounced inhibitory dose-dependent effect on the enzyme activity in this cell line. The highest effect was observed using the ethyl acetate/aqueous extract from *Flores Tanaceti*. Since the proline specific enzymes are known to participate in different tumors growth, it could be concluded that the natural inhibitors from tansy have a potential to be used as therapeutic anti-cancer agents.

Key words: postproline-specific enzymes, *Tanacetum vulgare* L., *Tribulus terrestris* L., *Rhaponticum carthamoides*, MDA-MB-231cells

Introduction

Studies on the identification of proteolytic enzymes - markers of pathological processes, could lead to the development of innovative therapeutic strategies and agents. Those enzymes are known to be involved in the genesis and development of many types of tumors and are considered as potentially attractive therapeutic targets. Proline is an important amino acid in many biologically relevant polypeptide sequences. Its presence affects the peptide's interactions with other proteins and prevents their degradation by the most common proteases. Postproline proteases constitute a subset of serine proteases involved in the regulation of many signaling events and are emerging as promising therapeutic targets for prevalent diseases such as diabetes and cancer [14]. This protease subset belongs to the S9 family of serine peptidases and includes such diverse and important enzymes as prolyl oligopeptidase (POP; EC 3.4.21.26) [7], dipeptidyl peptidase

IV (DPP-IV, EC 3.4.14.5) [12] and fibroblast activation protein alpha (FAP, EC 3.4.21. B28) [6]. Increased activity levels of these enzymes are observed in various pathological conditions, including malignancies [2, 17]. Their inhibitors are potential therapeutic agents [13].

Plants produce a broad range of bioactive compounds via their secondary metabolism. These compounds elicit different effects on humans' and animals' organs and tissues. Recently, there is increasing interest towards substances of natural origin that are selective inhibitors of proline-specific enzymes. For example, it has been found that certain flavonoids and caffeoylquinic acids, as well as derivatives thereof, are POP inhibitors with good selectivity to DPP-IV [1, 5, 10, 11]. Some of the chemical constituents of *Rhaponticum carthamoides* (*Leuzea carthamoides*; maral root), *Tanacetum vulgare* L. (tansy) and *Tribulus terrestris* L. (small caltrops) are phenolic acids, flavonoids and their derivatives [3, 9, 18], which are potential inhibitors of these peptidases.

In the present study, the effect of the extracts from three herbs on the postproline-specific enzyme activity in the human tumor mammary gland cell line MDA-MB-231 was investigated. The IC_{50} values of inhibition from fractions were determined.

Materials and Methods

Chemicals and reagents. Ethyl acetate, diethyl ether, diisopropyl ether and hexane were from Fisher Chemical (UK). All reagents were of the highest purity available. The substrate Z-Gly-Pro-methyl coumaryl amide (Z-Gly-Pro-AMC) was from Bachem (Switzerland).

Plant extracts. The crude extracts of *T. terrestris* L. and *T. vulgare* L. and flowers of tansy were kindly provided by Vemo 99 Ltd (Sofia, Bulgaria). In the present study, we used a commercial extract from the roots of *R. carthamoides*.

Preparation of the ethyl acetate fraction from the crude extract (EACE). Twenty mL of water were added to 5 g of the powdered crude tansy extract while stirring. 6N HCl was then added in a dropwise manner until pH 3.0. Ethyl acetate (15 mL) was applied to the aqueous phase while stirring. The organic phase was separated and the aqueous phase was extracted with 10 mL ethyl acetate. The combined organic phases were filtered, washed with brine and dried by Na_2SO_4. Ethyl acetate was removed under vacuum and a small amount of diisopropyl ether was added. The formed dark yellow solid was filtered and dried out.

Preparation of dicyclohexylammonium salts fraction (DCHAS). Solid dicyclohexylammonium salts were obtained from the ethyl acetate extract, as follows: The volume of the ethyl acetate extract was reduced to 1/4 and dicyclohexylamine was added dropwise. The obtained precipitate was filtered, washed with diisopropyl ether and dried.

*Isolation of solid substance from diethyl ether/hexane (DEHS).*The filtrate obtained after the removal of dicyclohexylammonium salts was concentrated *in vacuo* to give a thick oily residue. Diethyl ether was added to this residue, followed by hexane, thus obtaining a dark yellow precipitate.

Preparation of ethyl acetate extract from Flores Tanaceti (EAFT). Sixteen mL of water and 48 mL ethyl acetate were added to 4 g of *Flores Tanaceti* while stirring. Then, 6N HCl was added dropwise until the aqueous phase reached a pH 3.0 and the mixture was stirred for an additional hour. Following filtration, the organic phase was separated and processed as above. Finally, diisopropyl ether was added and the obtained precipitate was filtered and dried.

Cell culturing. A permanent cell line was used – MDA-MB-231 (human tumor cells from triple negative mammary gland carcinoma). The cancer cells were cultured

in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle's Medium – high glucose 4.5‰ (DMEM), supplemented with 10% fetal calf serum and antibiotics in usual concentrations. Cell culture was maintained at 37.5°C in a humidified atmosphere and 5% CO_2 until 95% confluence was achieved. The cells were harvested by a rubber policeman and homogenized using homogenizer MSE (England) in 5 mL 0.1 M phosphate buffer (pH 7.4) with 0.1 M NaCl and 1 mM EDTA.

Enzyme activity measurement. Enzyme activity in the cell homogenate was measured in the presence of 0.1 or 0.2 mg/mL extracts or fractions in 0.1 M phosphate buffer (pH 7.4), containing 0.1 M NaCl and 1 mM EDTA at 37°C using 80 μM fluorogenic substrate Z-Gly-Pro-AMC. Enzyme assays were carried out in 96-well plates, in a multifunctional spectrofluorimeter Varioscan Fluorescence at 360 nm excitation and 460 nm emission every 3 minutes. The software program EnzFitter V2 was used for data processing. The enzyme activity was determined from the initial rate of the reaction.

IC_{50} determination. Enzyme activity in homogenate of cell line MDA-MB-231 was measured in the presence of different concentrations (5, 10, 15, 20, 25, 37.5 и 50 μg/mL) from highest activity fraction in 0.1 M phosphate buffer (pH 7.4), containing 0.1 M NaCl and 1 mM EDTA using 80 μM fluorogenic substrate Z-Gly-Pro-AMC at 37°C.

Column chromatography. The crude extract of *T. vulgare* was fractionated with chromatography system Grace Davison Purification (USA) Reveleris® Flash System. Chromatographic separation was performed on a Claricep C_{18} column (Bonna-Angela), 80 g, particle size 20-45 μm and pore size 100Å, ELSD detector: isopropanol, UV wavelength irradiation (UV1: 254 nm and UV2: 325 nm) using the following mobile phases: A: water and B: acetonitrile, at a flow rate of 40 mL/min and gradient (62 min): 0% B for 2 min, 23% B for 50 min, 90% B for 3 min, hold 90% B for 4 min and 0% B for 3 min.

Results and Discussion

The effect of the crude extracts from *R. carthamoides*, *T. terrestris* L. and *T. vulgare* L. on postproline-specific enzyme activity on the human cell line MDA-MB-231 was studied using the nonspecific fluorogenic substrate Z-Gly-Pro-AMC for post-proline cleaving enzymes. The tested extracts were with concentrations 0.1 mg/mL and 0.2 mg/mL respectively. In all three extracts, concentration-dependent inhibition was observed (**Fig. 1**). Degree of inhibition at concentration 0.1 mg/mL in the cell homogenates were 0.26 (*R. carthamoides)*, 0.32 (*T. terrestris*L.) and 0.40 (*T. vulgare* L.). The lowest degree of inhibition at concentration 0.2 mg/mL was observed for *R. carthamoides* (0.37) and the highest for *T. vulgare* L. (0.61). In view of the obtained results it can be concluded that crude extract of *T. vulgare* L. has the highest inhibitory effect on the enzymes' activity in breast cancer cells homogenates.

Cell line MDA-MB-231 (triple negative human mammary gland carcinoma) is usually used as a negative control for FAP, since it is known to lack this enzyme activity [4, 8]. The tansy extract inhibited mildly recombinant DPP-IV, specifying a high selectivity of the natural tansy inhibitors to the postproline endopeptidase activity. On the other hand, the MDA-MB-231 cell line is POP-positive [16], indicating that an inhibition of POP-activity was observed in our study.

Using the LC-HRMS method, we determined the major nonvolatile compounds in the extract of tansy. In the crude extract of tansy we detected the presence of 3-caffeoylquinic acid, 5-caffeoylquinic (chlorogenic) acid, 3,4-, 3,5- and 4,5-dicaffeoylquinic acids, O-glucuronides of apigenin, luteolin and quercetin and O-hexosides of luteolin and quercetin [15].

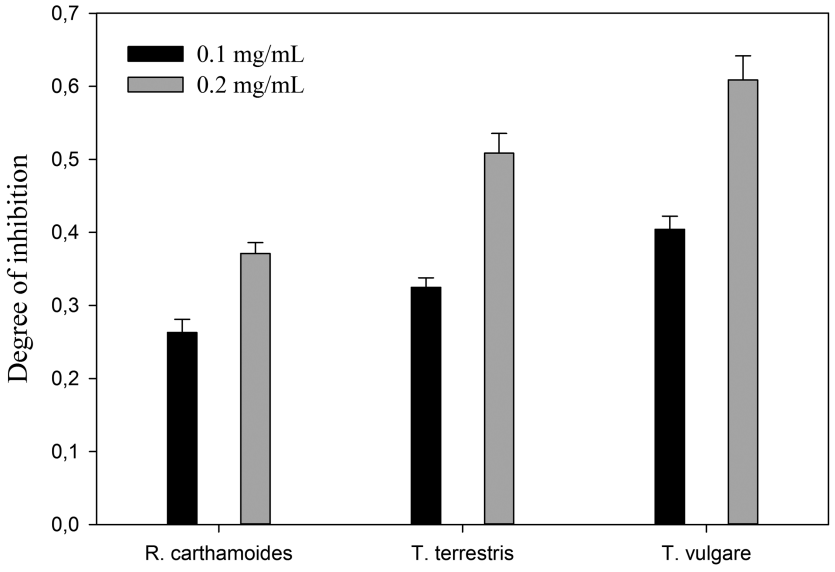

Fig. 1. The degree of inhibition of the postproline-specific enzymes' activity from the crude plant extracts in homogenates ofMDA-MB-231 human cells.

Further on, the crude extract was partitioned between water and ethyl acetate and the effect of the aqueous and organic fractions on postproline-specific enzyme activity in the homogenates of MDA-MB-231 cell line were investigated. Ethyl acetate fraction exhibited a higher activity (0.68 degree of inhibition at concentration 0.1 mg/mL and 0.83 at 0.2 mg/mL respectively) (**Fig. 2**) as compared to the crude extract (**Fig. 1**). On the other hand, aqueous fraction showed a very low activity.

From these data, we can assume that some of the above compounds are effective inhibitors of postproline endopeptidase activity, i.e. POPs activity. Dicyclohexylamine was added to the ethyl acetate fraction, whereby a precipitate of the dicyclohexylammonium salts of 3-caffeoylquinic acid, chlorogenic acid, isomeric dicaffeoylquinic acids and O-glucuronides of apigenin, luteolin and quercetin were formed. The inhibitory activity (**Fig. 2**) of these dicyclohexylammonium salts (degree of inhibition at concentrations 0.1 mg/mL and 0.2 mg/mL were 0.67 and 0.80 respectively) proved to be essentially the same as the activity of ethyl acetate fraction (0.68 and 0.83 respectively). After separating the precipitate from dicyclohexylammonium salts, the filtrate was concentrated and treated with diethyl ether and hexane, whereby a precipitate formed. The degree of inhibition by these substances is twice lower relative to of the ethyl acetate fraction (**Fig. 2**). These results confirm our hypothesis that some of the major components in the extract are potential selective inhibitors of the POP. The powder from the flower of the herb was extracted with two phase system water/ethyl acetate. The ethyl acetate extract from *Flores Tanaceti* (EAFT) obtained by using a two-phase water/ethyl acetate system showed the highest effect (0.85 and 0.97 degree of inhibition) on the postproline-specific enzyme activity (**Fig. 2**).

The crude extract from *T. Vulgare* L. was fractionated by flash chromatography. Some of the fractions showed inhibitory effect of 75-95% on the enzyme activity in the homogenate of MDA-MB-231 cell line.The dependence of the postproline-specific enzyme activity in the cell homogenate on the fraction concentration showing an inhibition rate of 0.95 was investigated. Results were presented in **Fig. 3.**

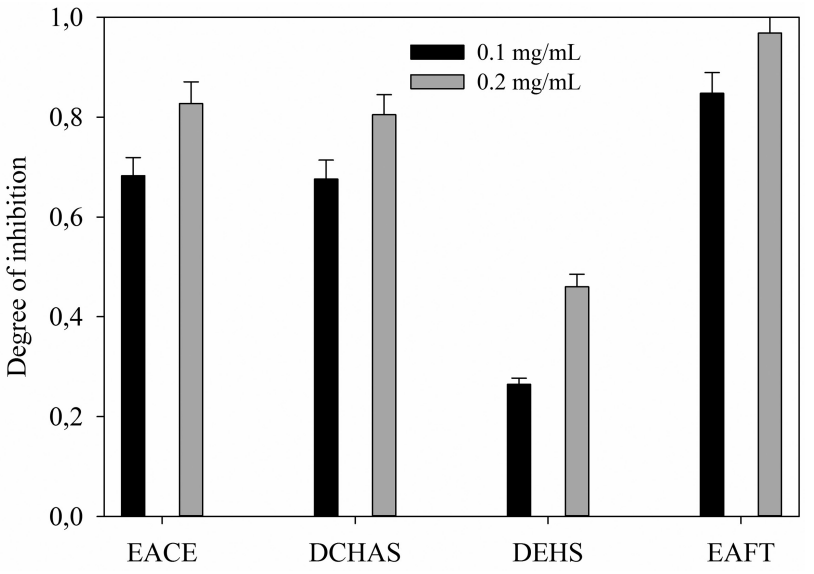

Fig. 2. The inhibition effect of the fractions of T. vulgare on postproline-specific enzyme activity in cell homogenates. EACE – ethyl acetate fraction of the crude extract of tansy; DCHAS – dicyclohexylammonium salts fraction; DEHS – solid substance from diethyl ether/hexane separation; EAFT – ethyl acetate extract from Flores Tanaceti.

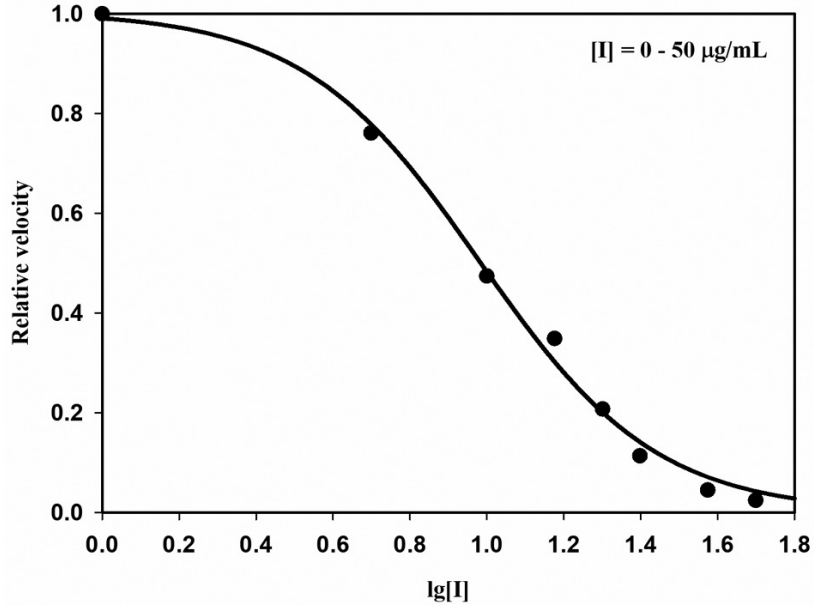

Fig. 3. Relative rate of hydrolysis of Z-Gly-Pro-AMC by homogenate of MDA-MB-231 cells in the presence of different concentrations of the fraction of the highest inhibition activity.

In **Fig. 3**, the dependency of the relative initial rate from the logarithm from concentration of fraction is shown. From the typical sigmoidal dependence obtained, calculated IC_{50} is 10 μg/mL.

Conclusions

The components of the ethyl acetate extracts from the flowers of *T. vulgare L.* demonstrate a high selectivity with respect to the inhibition of proline-specific endopeptidases – enzymes which are known to take part in the genesis and progression of many types of tumors. So, these natural inhibitors could be considered as potential therapeutic agents for the treatment at least of mammary gland carcinoma.

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