Acta Veterinaria (Beograd), Vol. 52. No. 5-6, 345-354, 2002.

UDK 619:616.5-002

THE INDUCTION OF INFLAMMATION IN MOUSE TAILS WITH *ASPERGILLUS ORYZAE* PROTEASE AND THE INHIBITORY EFFECTS OF SYNTHETIC AND NATURAL SUBSTANCES *IN VITRO*

ŠUTIAK V*, KORENEK M**, ŠUTIAKOVA IRENA***, KORENEKOVA BEATA***, SOBEKOVA ANNA**, VAŠKO L** and NEUSCHL J*

**Department of Pharmacology, **Department of Biochemistry, Biology and Biophysics and ***Research Institute of Veterinary Medicine at The University of Veterinary Medicine, Komenskeho Košice, Slovakia*

(Received, 23 June 2002)

Four groups of male mice were injected with doses from 0.005 to 1.0 mg of protease ex Aspergillus oryzae (PAO) in 20.0 µl of a 0.9 % physiological saline solution (PSS) per mouse s.c. into their tails. The control mice received 20.0 µl of 0.9 % PSS only. The inflammation of the tails was measured volumetrically before injection and 1, 2, and 3 days afterwards. The clinical responses of the mice to PAO were observed up to 30 days. The development of inflammation corresponded well with the quantity of enzyme used. The data obtained indicated the smallest dose (0.005 mg) of PAO induced oedemization of the tail ($P<0.01$) and more significant and longer lasting tail changes were observed at the higher doses. For inhibitory studies in vitro, we used the following conditions: 450 µg PAO. 0.1 ml^{-1}, 20 mmol . 0.1 ml^{-1} chromogenic substrate Suc-$(Gly)_2$-Phe-NAn and Tris/HCl buffer in the volume of 0.7 ml. Incubation was carried out at 37 °C for 15 min. The results obtained demonstrated that all three synthetic compounds (I 1 = $(Ala)_2$-Leu-NH-EtPh, I 2 = Suc-$(Ala)_2$ -Pro-NH-EtPh and I 3 = Glt-$(Ala)_2$-Pro-NH-EtPh) exhibited inhibitory effects and four natural preparations of tannin (Tanin plv., Tanifarm plv.sol. a.u.v., Farmatan cps. a.u.v., and Pycnogel tbl.) had even more effective inhibitory action ($P<0.01$) on PAO.

Key words: inflammation, inhibitors, measurements, mice, oedema, protease

INTRODUCTION

There are many factors, which can induce inflammatory and other dangerous processes in mammals: bacterial and viral microbes, microbial endotoxins and exotoxins, allergens, xenobiotic agents, and some other compounds (Tsao *et al.*, 1990, Lainee *et al.*, 1991, Šutiak *et al.*, 1994, 1997, Shah *et al.*, 2001, Castino *et al., 2002).*

In the last two decades many new generations of various synthetic anti-inflammatory inhibitors, remedies, and drugs have been developed (Šutiakova and Šutiak 1991a, b, Korenek *et al.,* 1993, Šutiak 1997, Lees 1998, Chu 1999). Although many of them were quite effective in vitro, some of them failed to act under in vivo conditions (Šutiakova and Šutiak 1991a, b). Besides new agents, many classic herbal drugs (Šutiak *et al.,* 2001) containing various active substances (chamazulenes, phenolic agents, etc.) are still used. However, some of these agents have not been sufficiently effective against inflammation in animal tissues, especially when administered in the advanced stages of the inflammatory process. It is well known that many pathological processes and disturbances in living creatures begin with inflammation. This is the reason why many pharmaceutical firms and their research teams concentrate their interests on inflammatory processes, as we do.

The aim of this presentation is to inform interested parties about our experience with artificial induction of the inflammatory process, with protease ex *Aspergillus oryzae* (PAO) in mice. Apart from that we also want to share our experience regarding the effect of selected synthetic peptides, as well as some natural tannin preparations on the enzymatic activity of the above-mentioned enzyme under *in vitro* conditions. We decided to study their inhibitory effect against PAO, because they seemed to be promising agents for anti-inflammatory studies.

MATERIAL AND METHODS

Male mice (strain ICR) with a mean body weight of 34.3 ± 5.9 g, were used for our experiments. They were adapted to the experimental conditions 7 days before the start and kept on the Larsen diet with water *ad libitum*. The inflammatory process was induced by the enzyme protease ex *Aspergillus oryzae* (PAO) administered to 4 groups (A to D), of 5 mice each, with a Microsyringe Hamilton 700-200 s.c. 40-50 mm from the tips of their tails on the dorsal side (Šutiak *et al.,* 1997). A control group (K) of mice received 20 µl of 0.9 % physiological solution of NaCl (PSS) and the experimental groups A to D: 1.0, 0.1, 0.05 and 0.005 mg of PAO in 20 µl of 0.9 % PSS. Changes in tail volumes were measured in glass tubes with a diameter of 5 mm (r = 2.5 mm) filled with water using a 60 mm length of tail from the tip, before and after the PAO injection for a period of 3 days . The height of the water column displaced from the tube by the oedematous tails was measured with callipers and the volumes obtained were calculated, using the formula $V = \pi . r^2 . h$.

Kinetic studies of the effect of selected agents on the activity of PAO were performed *in vitro* according to Bartok *et al.,* (1990), and by a modified method according to Rosival et al., (1993). We studied the action of three synthetic inhibitors: *(Ala)$_2$ - Leu - NH - EtPh* (I 1); *Suc-(Ala)$_2$ - Pro - NH - EtPh* (I 2); *and Glt - (Ala)2 - Pro - NH - EtPh* (I 3) using the following conditions. Inhibitors I 1; I 2; and I - 3 were added at the concentrations: 37.5; 75.0; 150.0 and 300.0 µg . 0.1 ml^{-1}. PAO was used at 450.0 µg. 0.1 ml^{-1}, chromogenic substrate Suc-(Gly)$_2$-Phe-NAn at the concentration of 20 mmol . 0.1ml^{-1} and Tris/ HCl buffer in the volume of 0.7 ml. For the study of the inhibitory effect of four natural preparations on the action of PAO *in vitro*, we used the following pharmaceuticals: Tanin plv. (active substance tannin with PhBS IV pharmaceutical purity; Lekaren s.e. Košice, Slovakia), Tanifarm

plv.sol. a.u.v. (55 % of tannin; Pharmagal Nitra, Slovakia), Farmatan cps. a.u.v. (55 % of tannin; Sevnica, Slovenia), and Pycnogel tbl. (14 % of pycnogenol® ; Slovakofarma Co.Ltd. Hlohovec, Slovakia), all at concentrations of 4.8; 9.4; 18.75; and 37.5 μg. 0.1 ml^{-1}. Other ingredients and conditions were the same as in the experiment with the synthetic inhibitors. Incubations were carried out at 37°C for 15 min. (PAO was supplied by Lečiva Praha s.e., Dolno Mecholupy, Czech Republic. The synthetic substrate and inhibitors were received from Ing. E. Kasafirka, CSc., Research Institute for Pharmacy and Biochemistry, Prague, Czech Republic. Other preparations were bought in Lekaren s.e. Košice, Slovakia.) All our results were statistically evaluated using Student's paired t test in accordance with the Microsoft Excel Program on the PC Celeron Intel Inside. Values of $P<0.05$ for differences between means, were considered statistically significant results, although we preferred the values of $P< 0.01$.

RESULTS

In contrast to the control, PAO was found to induce a significant inflammatory process, depending on the dose of the enzyme administered to the experimental mice (Table 1.). As well as significant oedemization of tail tissue after PAO administration ($P < 0.01$), we also registered redness of the tail skin and an in-

Table 1. Changes of the tail volumes in control and experimental mice after s.c. administration of *Aspergillus oryzae* protease

Groups of mice and statistics		Volume of tails in mm^3 and on the days of experiments			
		Before administration of PAO	After administration of PAO 1st day	2nd day	3rd day
Control	Ø	371.9	388.5***	379.2	383.2
(K)	±s	28.5	43.1	37.2	43.6
Exp. group	Ø	364.6	444.5***	431.2***	427.3***
(A)	±s	37.8	30.7	38.6	22.6
Exp. group	Ø	379.8	408.6***	410.3***	404.9***
(B)	±s	21.3	41.0	34.8	20.7
Exp. group	Ø	368.8	376.6**	382.9***	360.3**
(C)	±s	18.7	17.5	50.0	17.8
Exp. group	Ø	372.2	384.6***	385.9***	387.9***
(D)	±s	20.9	35.7	23.4	37.9

Comments: Ø = mean values; ± s = standard deviation; (K) = animals given 20 μl of 0.9 % saline solution (PSS) of NaCl /head; (A) = animals given 1.0 mg of PAO in 20 μl of PSS of NaCl / head; (B) = animals given 0.1 mg of PAO in 20 μl of 0.9 % PSS of NaCl/ head; (C) = animals given 0.05 mg of PAO in 20 μl of PSS/ head; (D) = animals given 0.005 mg of PAO in 20 μl of a 0.9 % PSS of NaCl/ head; (E) PAO = protease ex Aspergillus oryzae * $P<0.05$, ** $P<0.02$, *** $P<0.01$, P = statistical significance of comparison of meanvalues with values before the administration (F).

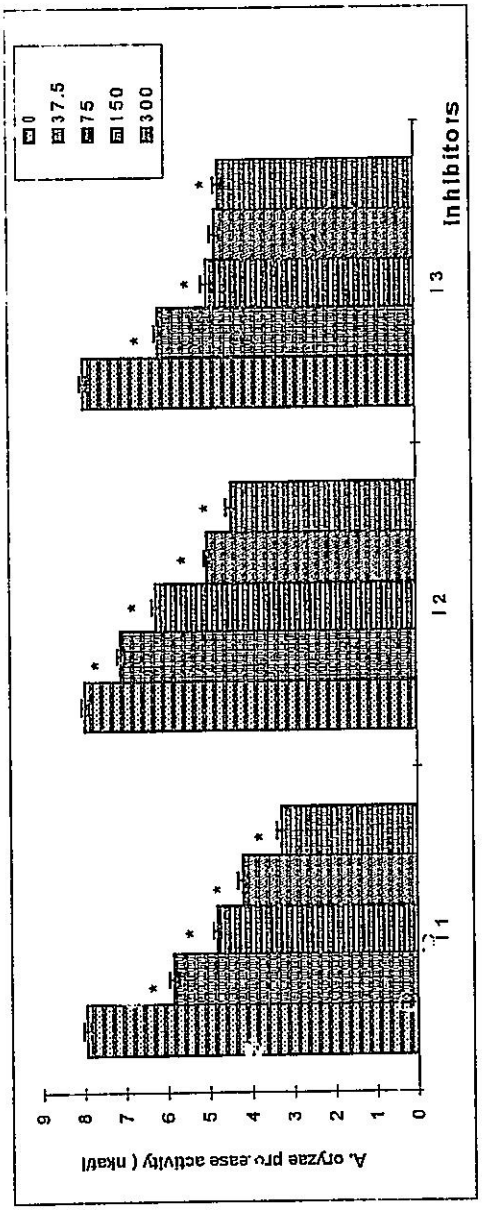

Fig.1 The effects of three synthetic inhibitors on *A. oryzae* protease activity *in vitro*

Comments: the following conditions were used: Inhibitors I - 1 = $(Ala)_2$-Leu-NH-EtPh, I - 2 = Suc-$(Ala)_2$-Pro-NH-EtPh and I - 3 = Glt-$(Ala)_2$-Pro-NH-EtPh, all at concentrations from 0 to 300.0 µg. 0.1 ml^{-1}. *A. oryzae* protease 450.0 µg . 0.1 ml^{-1}, chromogenic substrate Suc-$(Gly)_2$-Phe-ANn at 20 mmol . 0.1 ml^{-1} and buffer Tris/HCl (0.7) ml. Incubation was at 37 °C for 15 min.

Figure. 2 The inhibitory effects of four natural preparations of tannin on the activity of A. oryzae protease *in vitro*

Comments: the following conditions were used : preparation Tanin plv. (active substance tannin with a pharmaceutical purity of PhBS IV, Lekaren s.e. Košice, Slovakia; Tanifarm plv.sol a.u.v. (55 % of tannin) of the firm Pharmagal Nitra, Slovakia; Farmatan cps.a.u.v. (55 % of tannin, Sevnica Slovenia)and Pycnogel tbl. (14 % of pycnogenol©) at different concentrations from 0 to 37.5 µg . 0.1 ml^{-1}). *A. oryzae* protease 450 µg . 0.1 ml^{-1}, chromogenic substrate Suc-$(Gly)_2$-Phe-ANn at the concentration 20 mmol . 0.1 ml^{-1} and buffer Tris/HCl (0.7 ml) Incubation was at 37° C for 15 min.

crease in both tail temperature and sensitivity to touch roughly in direct proportion to the quantity of agent used. Thus, higher quantities of PAO induced more significant oedemization of tail tissues, which lasted for a proportionally longer time ($P < 0.01$). The highest dose (1.0 mg of PAO per tail) also induced necrosis of tissues, which persisted on the tails for more than 21 days, after which they were scarred. The redness and local alopecia persisted for a longer period. The detailed dynamics of the induction and development of the oedema can be seen in table 1.

The synthetic peptidic inhibitors: *(Ala)$_2$ - Leu - NH - EtPh* (I 1); *Suc-(Ala)$_2$ - Pro - NH - EtPh* (I 2); *and Glt - (Ala)$_2$ - Pro - NH - EtPh* (I 3) significantly decreased the activity of PAO *in vitro* ($P<0.01$) even at the lowest dose (37.5 μg of agent . 0.1 ml^{-1}). After the addition of higher doses (75.0, 150.0 and especially 300μg of agent . 0.1 ml^{-1} of solution) the inhibitory effect was still higher and also more accentuated (Figure 1).

Our study of four natural preparations: Tanin plv., Tanifarm plv.sol. a.u.v., Farmatan cps. a.u.v. and Pycnogel tbl. demonstrated an even more effective inhibition ($P<0.01$) of PAO action at lower doses than for the synthetic compounds, i. e. in concentrations from 4.8 to 37.5 μg. 0.1 ml^{-1} of additives (Figure 2).

DISCUSSION

It own that proteases may quite easily induce many physiologically useful reactions (e.g. fermentative transformation of grass or some other feeds to very valuable nutritious ingredients for animal diets etc.). However, they may also induce many pathological and deleterious processes, including very serious infective and parasitic diseases (Wretlind and Pavlovskis 1983, Casino 2002). The purpose of our experiments was to positively influence pathological states and processes including induced inflammation and especially some of its manifestations. Our experiments indicated (Table 1) that the induction and duration of the inflammatory process very significantly depended on the quantity of enzyme injected. Thus, 0.005 mg of PAO induced less oedemization of tail tissues and no necrotization, while the highest quantity (1.0 mg of PAO) induced greater oedemization and tissue necrosis for a longer duration. Although protease from *Pseudomonas aeruginosa* and also some other proteases may be more active than PAO, we used this enzyme deliberately as a mild model agent to minimise the suffering of animals from stress and pain. (e.g. elastase may induce also pancreatitis). Oedemization and dangerous inflammatory processes may also be induced by other agents (Lungarella *et al.*, 1980, Karlinsky *et al.*, 1985, Kida *et al.*, *1985, Miyazaki 1984, Oikarinen et al.*, 1986, Shah et al., 2001), with more drastic final effects, even involving the death of the animals. In our further experiments we again employed an alternative model of study (using an enzymatic model) in *in vitro* conditions. Our purpose in verifying the possible inhibitory effect of synthetic peptides and natural agents against inflammatory process induced with PAO was motivated mainly by the observation of Oh *et al.*, (1980) and Hagerman and Butler (1981). They registered that tannin forms so-called tannin-protein complexes via the induction of an interaction between proteins and tannin. It is well known that enzymes are proteins and so it was a good occasion to verify this complex-forming effect directly with PAO. However there were also some unknown factors. We did not know if these tannin-protein complexes would be stable

enough after the reaction, because it is known that tannin may be in monomeric and polymeric forms and may form soluble and insoluble complexes. Which of these complexes would be more effective and safe enough was unknown. Mitaru *et al.*, (1982) showed that condensed tannin isolated from rapeseed hulls did not inhibit α-amylase. Although this enzyme has completely different biochemical properties from proteases, we added this information to our database on enzymes. A further stimulus for our decision to verify the effect of the naturally occurring agent - tannin and also the synthetic peptides against the PAO were the observations of Martin-Tanguy *et al.*, (1977), Kumar and Singh (1984) as well as Barry (1985). They registered that a high content of tannin in the food (e.g. from the horse bean, *Lotus pedunculatus,* and from some other plant components) may be detrimental in diets for sheep and other ruminants and also in nonruminant animals (e.g. poultry), because it decreases the nutritional value of feeds. Further strong support for our decision to examine the effect of tannin against PAO was our experience with the use of tannin against the very potent poisonous agent, strychnine (Šutiak *et al.*, 1994 and 1997). Methylated tannin was preventively very effective against strychnine poisoning in mice. Our *in vitro* experiments here demonstrated that not only synthetic peptides (fig 1), but also tannin from natural sources induced significant inhibitions of PAO (Fig.2). Although we observed very significant inhibitory action of all three compounds, individual agents had different inhibitory actions. For example the best inhibitory effects were induced by I 1 ,intermediate inhibitory action by peptidic I 3, and the lowest action was shown by I 2. When we compared peptidic agents with natural tannin sources, tannin preparations had a quantitatively stronger inhibitory action against PAO than peptidic compounds. However, in this case also there were, different inhibitory actions for various preparations of tanin. The inhibitory action of the tannin preparations decreased in the following order: 1) Tanin plv, 2) Tanifarm plv.sol, 3) Farmatan cps. and 4) Pycnogel tbl.

Although favourable results were obtained in the *in vitro* studies, we do not want to overestimate their importance, because it is necessary to obtain more detailed information from *in vivo* studies, as it is known that results obtained *in vitro* may not be entirely valid for *in vivo* conditions (Šutiakova and Šutiak 1991a, b).

Address for correspondence:
Šutiak V.
Department of Pharmacology,
University of Veterinary Medicine,
Komenskeho 73, 041 81 Košice,
Slovakia

REFERENCES

1. *Bartik M, Šutiakova I, Kasalirek E, Šutiak V.* 1990, Some aspects of nutritional biochemistry and adequate animal feeding. In: Bartik M, editor, Final information of the SRP No. VI-4-08/04, *Košice: UVM*, 27 - 65.
2. *Barry TN.* 1985, The role of condensed tannins in nutritional value of Lotus pedunculatus for sheep. 3. Rates of body and wool growth. *Br J Nutr*, 54, 211 - 7.
3. *Casino R, Pace D, Damoz M, Gargiulo M, Ariatta, C, Raiteri E, et al.* 2002, Lysosomal proteases as potential targets for the induction of apoptic cell death in human neuroblastomas. *Int J Cancer*, 97, 775 - 9.

4.*Chu A,* 1999, Fluticasone propionate, In: Dollery C, Boobis A, Rawlins M, Thomas S, Wilkins M, editors, Therapeutic Drugs. 2nd ed, Edinburgh-London etc.: *Churchill Livingstone,* F-132 - F-135.
5.*Hagerman AE, Butler L.* 1981, The specificity of proanthocyanidin-protein interaction. *J Biol Chem,* 256, 4494 - 7
6.*Karlinsky J, Fredette J, Davidovitz G, Gatanase A.* 1983, The balance of lung connective tissue elements in elastase-induced emphysema. *J Lab Clin Med,* 102, 151 - 62.
7. *Kida K, Yasui S, Utsu Y, Oflue AF, Thuslabeck M.* 1984, Lung changes resulting from intraperitoneal injections of porcine pancreatic elastase in suckling rats. *Am Resp Dis,* 1111 - 7.
8.*Koraeek M, Šulik E, Koranekova A.* 1993, Endogenic and exogenic enzyme inhibitors in living organisms. *Veterinarstvi,* 43, 180 - 3.
9.*Kumar R, Singh.* 1984, Tannins: their adverse role in ruminant nutrition, *J Agric Food Chem.* 32, 447 - 53
10. *Lainee P, Robineau P, Gisittin P, Coq H, Benchetrit G.* 1991, The mechanism of pulmonary oedema induced by an organophosphorus compound in anaesthetised dogs. *Fund Appl Toxicol,* 17, 177 - 85.
11.*Lees P.* 1998, Anti-inflammatory Agents, In: Aiello SE, Mays A, Amstutz HE, Anderson DP, Armour DP, Jeffcott LB, Loew FM, Wolf AM, editors, The Merck Veterinary Manual. 8th ed, Whitehouse: Merck and Co. Inc, 1817 - 31.
12.*Lungarella G, Gardi G, De Santi MM, Luci P.* 1980. Pulmonary vascular injury in pancreatitis: Evidence for a major role played by pancreatic elastase. *Exp Molec Path,* 42, 44 - 9.
13.*Martin-Tanguy J, Guillaume J, Kossa A.* 1977, Condensed tannins of horse bean seeds: Chemical structure and apparent effects on poultry. *J Sci Food Agric,* 28, 757 - 65.
14.*Mitaru BN, Blair R, Bell JM, Reichert RD.* 1982, Tannin and fibre contents of rapeseed and canola hulls. *Can J Anim Sci,* 62, 661 - 3.
15.*Miyazaki S.*1984. Biological activities of partially purified elastase produced by Flavobacterium meningosepticum. *Microbiol Immunol,* 28, 1083 - 92.
16.*Oh HI, Hoff JE, Armstrong GS, Haff LA.* 1980, Hydrophobic interaction in tannin-protein complexes. *J Agric Food Chem* 28, 394 - 8.
17.*Oikarinen A, Kustila U, Witto J.* 1986, Characterization of elastase-like enzymes in various blistering diseases. *Acta Derm Vener (Stockholm),* 66, 1 - 5.
18.*Rosival I, Šulík E, Šlesárová L, Korének M, Blahovec J.* 1993, The determination of chymotrypsin activity in animal stools. *Folia vet,* 37, 1-2, 19 - 22.
19.*Shah A, Panchal N, Agarval AK.* 2001, Concomitant allergic bronchopulmonary aspergillosis and allergic Aspergillus sinusitis: a review of an uncommon association. *Clin Exp Allergy,* 31, 1896 - 905.
20.*Šutiak V.* 1997, The observation of the effect of selected remedies on some manifestations of skin inflammations in mice. In: Šutiak V., Berecky I., Neuschl J., Lopuchovsky J. A handbook of prescriptions and practical pharmacological exercises. *Košice: Datahelp* 1997, 113 - 4.
21.*Šutiak V, Gustin P, Delaunois A, Ansay M.* 1994, Protective effect of chloramphenicol against parathion-induced pulmonary oedema in rabbits. Influence of sex. *J Vet Pharmacol Therap,* 17, 77 - 9.
22. *Šutiak V.* 2001, Preface. In: Šutiak V, Skalka Jsr, Skalka Jjr, Šutiaková I, editors, A Handbook of Veterinary Phytotherapy. Košice: Vienala 8 - 9.
23.*Šutiaková I,Šutiak, V.* 1991a, Recent knowledge about enzymes belonging to serine proteinases. *Biochem Clin Bohemoslov,* 20, 2, 103 - 10.
24.*Šutiaková I, Šutiak V.* 1991b, Synthetic inhibitors of some serine proteinases. *Bioch Clin Bohemoslov,* 20, 3, 193 - 202.
25.*Tsao TCH, Yuang YCH, Lan RS, Shieh WB, Lee, CHM.* 1990. Respiratory failure from acute organophosphate and carbamate poisoning. *Chest,* 98, 631 - 6.
26.*Wretlind B, Pavlovskis OR.* 1983, Pseudomonas aeruginosa elastase and its role in pseudomonas infections. *Inf Dis,* 5, 998 - 1003.

INDUKCIJA INFLAMACIJE NA REPU MIŠA SA PROTEAZAMA IZ *ASPERGILLUS ORYZAE* I INHIBITORNI EFEKTI SINTETSKIH I PRIRODNIH SUPSTANCI *IN VITRO*

ŠUTIAK V, KORENEK M, ŠUTIAKOVA IRENA, KORENEKOVA BEATA, SOBEKOVA ANNA, VAŠKO L i NEUSCHL J

SADRŽAJ

U ovom radu su prikazani rezultati izazivanja inflamatornog procesa injekcijom različitih doza proteaza dobijenih iz *Aspergillus oryzae* u rep miša. Inflamacija na repu je procenjivana volumetrijski u toku tri dana a kliničke manifestacije su bile uočljive 30 dana. Razvoj inflamatornog procesa je bio u korelaciji sa dozom korišćenog enzima. Kada je ovaj enzim prerthodno inkubiran sa sintetičkim hromogenim supstratom Suc-(Gly)2-Phe-NAn u Tris/HCl puferu, zapaženo je da su sve tri komponente (1 = (Ala)2-Leu-NH-EtPh, 2 = Suc-(Ala)2-Pro-NH-EtPh i 3 = Glt-(Ala)2-Pro-NH-EtPh) ispoljavale inhibitorne efekte i smanjivale stepen inflamacije. Slični efekti su dobijeni i sa prirodnim preparatima tanina (Tanin prah., Tanifarm rastvor a.u.v., Farmatan kapsule. a.u.v., i Pycnogel tablete.)