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Inhibition of neutrophil elastase activity by phenolic compounds from plants

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The influence of 40 phenolic compounds from plants were tested on the enzymatic activity of neutrophil elastase (EC 3.4.21.37). Among the flavonoids especially the compounds with a catecholic structure showed a strong inhibitory activity in the range of μmol/l, hyperosid was the strongest inhibitor with an IC_{50} of about 0.3 μmol/l. Lipophilic caffeic acid esters and phenolic compounds with catecholic structure elements in general inhibited the enzyme. Because of the specific role of neutrophil elastase in the inflammatory process its inhibition by phenolic natural compounds might be one explanation for the use of medicinal plants containing phenolic compounds in the treatment of inflammation.

1. Introduction

Neutrophil elastase (leukocyte elastase, EC 3.4.21.37) belongs to the chymotrypsin family of serine proteinases (S1), and is formed of a single peptide chain of 218 amino acid residues and four disulfide bridges. The pH optimum of the enzyme is close to neutrality, and the catalytic site is composed of three hydrogen-bonded amino acid residues, His57, Asp102 and Ser195, which form the so-called charge-relay system or catalytic triad. Peptide bond hydrolysis occurs in several steps. The initial reversible formation of an adsorption complex between the serine proteinase and its substrate is followed by a nucleophilic attack of the γ-oxygen of Ser195 on the peptide bond with subsequent formation of an acyl enzyme intermediate and release of the amino portion of the substrate. This covalent intermediate is hydrolysed in the deacylation step, which regenerates active enzyme and release the carboxy moiety of the substrate [1]. The human neutrophil elastase has been identified in 1968, the term elastase describes an enzyme capable of the proteolytic release of soluble peptides from insoluble elastin [2].

Unlike most proteinases, elastases are able to cleave fibrous elastin, an important extracellular matrix protein that has the unique property of elastic recoil, and plays a mechanical function in lungs, arteries, skin and ligaments. In addition to elastin, elastase cleaves many proteins with important biological functions, including collagen type I, II, III, IV, VIII, IX and XI, other matrix proteins including fibronectin, laminin, and cartilage proteoglycans. The major physiological function of neutrophil elastase is probably to digest bacteria and immune complexes phagocytosed by the polymorphonuclear leukocyte. It is likely that the enzyme also plays a role in leukocyte migration from blood to tissue and in apoptosis of polymorphonuclear leukocytes. Human neutrophil elastase seems to be included in the pathogenesis of different inflammatory diseases, like lung emphysema, cystic fibrosis, the adult respiratory distress syndrom (ARDS), rheumatoid arthritis and infectious diseases [3]. The indications of involvement of human neutrophil elastase in these different diseases had prompted therapeutic research based on elastase inhibitors. Plasma-derived α_1-proteinase inhibitor is presently used in patients with hereditary inhibitor deficiency [4]. Attempts have been made to use recombinant mucus proteinase inhibitor in cystic fibrosis [5]. Also, some synthetic elastase inhibitors that have been designed in the last two decades are very potent and orally active molecules, which might be used in medicine in the near future [6].

A broad screening of natural compounds as elastase inhibitors has not been published until now, nevertheless single reports about inhibitory activity of flavonoids and caffeic acid derivatives are published [7–9]. In this paper we would like to demonstrate the effect of a variety of naturally occurring phenolic compounds on human neutrophil elastase activity. Some of the substances tested are constituents of medicinal plants used in the treatment of inflammation or other diseases connected with the proteolytic activity of neutrophil elastase.

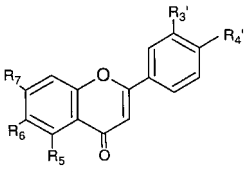
2. Investigations, results and discussion

Neutrophil elastase is released from neutrophils that migrate to sites of infection or other damaged tissues during the early stages of inflammation, and is consequently a useful marker of inflammation. Its concentration in polymorphonuclear leukocytes is very high (3 μg enzyme per 10^6 cells) [10].

Neutrophil elastase activates also other proteinases included in the breakdown of matrix proteins, e.g. procollagenase, prostromelysin and progelatinase [11], inactivates a number of endogenous proteinase inhibitors such as antithrombin [12], α_2-antiplasmin, C-inactivator, α_1-antichymotrypsin [13], and tissue inhibitor of metalloproteinases [14]. Under physiological conditions neutrophil elastase activity required after acute connective tissue injury, such as wounds, in the initial stage of tissue repair [15]. Only in a chronic inflammatory process the role of neutrophil elastase activity is changed to a pathological factor which promotes inflammation and inhibits the process of healing. Starting from investigations into inhibition studies of neutrophil elastase by extracts of *Solidago viraurea* L., a medicinal plant used for a long time in the treatment of inflammatory disorders, and the fact that besides saponins especially flavonoids with quercetin as aglykon and different caffeic acid esters are the main constituents of these extracts [8], we tested a variety of flavonoids and caffeic acid derivatives in an elastase assay using human isolated neutrophil elastase as enzyme source.

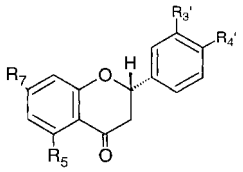
Tables 1, 2 and 3 show the results obtained with selected flavonoids in the human neutrophil elastase inhibitory assay. The most active inhibitor was hyperosid, the 3-*O*-galactosid of quercetin. The glycosidic part of quercetin-related flavonoids seems to be important for the inhibitory activity, because different glycosids of quercetin showed different IC_{50}-values. The glycosides hyperosid and isoquercitrin inhibited the enzyme stronger than the pure aglykon quercetin, and the glycosides rutin and quercitrin were less active than quercetin. Among the flavonoids

Table 1: Elastase inhibition by flavones



	IC_{50} (μM)	R5	R6	R7	R3′	R4′
Luteolin	12	OH	H	OH	OH	OH
Baicalein	2.2	OH	OH	OH	H	H
Chrysin	6.7	OH	H	OH	H	H
Diosmetin	83	OH	H	OH	OH	OCH_3

Table 3: Elastase inhibition by flavanones/glycosides



	IC_{50} (μM)	R5	R7	R3′	R4′
Naringenin	84	OH	OH	H	OH
Eriocitrin	>400	OH	O-rut	OH	OH

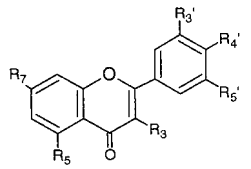
At the highest concentration of 400 μmol Eriocitrin tested, no inhibition higher than 20% has been measured

especially the compounds with a catecholic structure, i.e. two hydroxyl functions in 3′- and 4′-position showed a stronger inhibitory activity than flavonols with more hydroxyl functions and/or in other positions (kaempferol, myricetin, astragalin). The flavanon naringenin and the eriodyctiol glycosid eriocitrin had a very low inhibitory potency or were inactive in the assay demonstrating that the double bond between C-2 and C-3 in the chroman system enhances the inhibitory activity against neutrophil elastase. Also catechin as flavan-3-ol-derivative was inactive up to 400 μM in the elastase inhibition assay, despite a catecholic structure in the 2-phenyl moiety (data not shown). This assumption is enhanced by the effect of different flavons tested in the assay (chrysin, luteolin, baicalein). The methylation of hydroxyl function in flavons (diosmetin) decreased the inhibition activity.

The urinary metabolites of flavonoids [16], like 3-hydroxyphenylacetic acid and 4-methylcatechol (Table 4) did not influence the elastase activity up to concentrations of 400 μM, whereas 3,4-dihydroxyphenylacetic acid with the catechol structure inhibited the enzyme with an IC_{50} of 135 μM. The importance of this structure element is demonstrated by an IC_{50} value of 10.2 μM from nordihydroguaiaretic acid (**8**), a lignan with two dihydroxyphenyl functions in the molecule which IC_{50} is one magnitude of order lower than that of dihydroxyphenylacetic acid (Table 4).

Caffeic acid (**1**) and its esters were another group of compounds included in our investigations containing also the dihydroxyphenyl function. Table 4 shows caffeic acids and related natural compounds occurring in a variety of medicinal plants used for the treatment of inflammatory diseases. Caffeic acid itself inhibited the enzyme with a rather low activity ($IC_{50} = 93$ μM), isoferulic acid and ferulic acid with a methylation of one of the hydroxyl functions at the ring system were inactive up to a concentration of 1 mM, the same result was obtained with *p*-coumaric acid. The esterification of caffeic acid with other alcohols enhanced in some cases the inhibitory activity against neutrophil elastase. Examples for this are 3,5-dicaffeoylquinic acid (**7**), rosmarinic acid (**2**), caffeic acid phenethyl ester (**3**), caffeic acid bornyl ester (**5**) and *trans*-drimenyl caffeic acid ester (**4**). These esters showed IC_{50} values up to two orders of magnitude lower than caffeic acid itself. From these results we would like to hypothesize that the esterification with a lipohilic partner increases the inhibitory activity of caffeic acid esters. The fact that chlorogenic acid and some other caffeoyl esters showed no or rather low inhibitory activity, like 1,5-dicaffeoyl quinic acid (**6**) demonstrates that beside of the caffeic ester structure some other structural prerequisites must be existing for a strong inhibitory effect of caffeic acid derivatives. Other natural polyphenolic compounds like epiphyl-

Table 2: Elastase inhibition by flavonols/glykosides

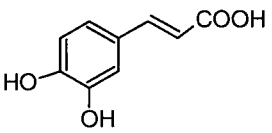


	IC_{50} (μM)	R3	R5	R7	R3′	R4′	R5′	
Quercetin	2.4	OH	OH	OH	OH	OH	H	
Hyperosid	0.3	*O*-galac	OH	OH	OH	OH	H	
Quercitrin	11.1	*O*-rham	OH	OH	OH	OH	H	
Rutin	9.8	*O*-rut	OH	OH	OH	OH	H	
Isoquercitrin	1.4	*O*-gluc	OH	OH	OH	OH	H	
Kaempferol	6.3	OH	OH	OH	H	OH	H	
Astragalin	304	OH	OH	OH	H	OH	H	
Myricetin	21.1	OH	OH	OH	OH	OH	OH	
Rhamnetin	18.3	OH	OH	OCH_3	OH	OH	H	
Morin	11.6	OH	OH	OH	H	OH	OH	2′OH

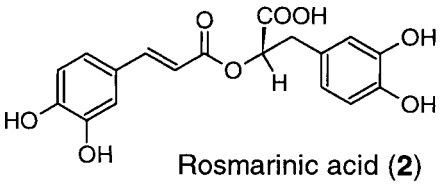
Table 4: Elastase inhibition by caffeic acid derivatives and other phenolic compounds

Compd.	IC_{50} (μM)
Caffeic acid	93.0
Isoferulic acid	>1000
Ferulic acid	>1000
p-Coumaric acid	>1000
Chlorogenic acid	>400
Rosmarinic acid	7.0
Caffeic acid phenethyl ester (CAPE)	37.0
trans-Drimenylcaffeic acid ester	0.2
Caffeic acid bornyl ester	1.6
(−)-Quinic acid	>400
3,5-Dicaffeoylquinic acid	0.2
1,5-Dicaffeoylquinic acid (Cynarin)	151
4-*O*-Caffeoylquinic acid	480
3-*O*-Caffeoylquinic acid	450
4-*O*-Caffeoyl-L-threonic acid	194
2-*O*-Caffeoyl-L-threonic acid	>400
2-*O*-Caffeoylmalic acid	33.7
Nordihydroguaiaretic acid	10.2
Epiphyllinic acid	2.2
Epiphyllinic acid-9,5″-6-shikimic acid ester	1.5
3,4-Dihydroxyphenylacetic acid	135
3-Hydroxyphenylacetic acid	>400
4-Methylcatechol	>400

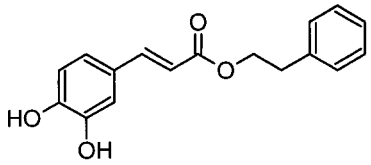
At the highest concentration of 1000 or 400 μM of substances tested, for isoferulic acid, *p*-coumaric acid, and ferulic acid as well as for chlorogenic acid, (−)-quinic acid, 2-*O*-caffeoyl-L-threonic acid, 3-hydroxyphenylacetic acid, and 4-methylcatechol no inhibition higher than 20% has been measured



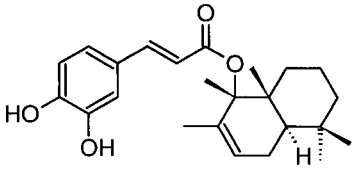
Caffeic acid (**1**)



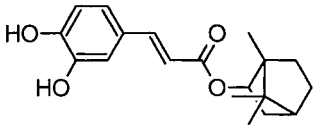
Rosmarinic acid (**2**)



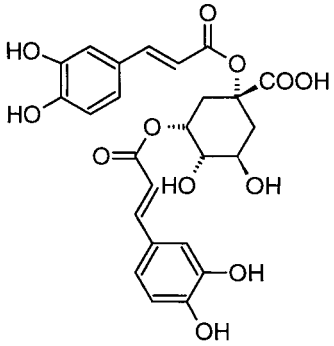
Caffeic acid phenethyl ester (**3**)



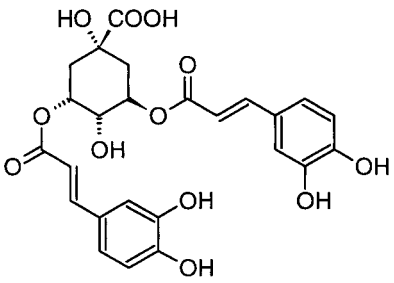
trans-Drimenylcaffeic acid ester (**4**)



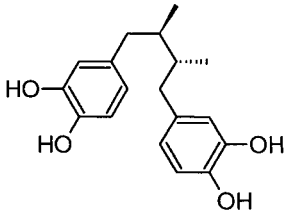
Caffeic acid bornyl ester (**5**)



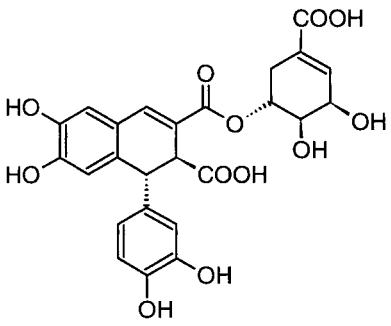
1,5-Dicaffeoylquinic acid (**6**)



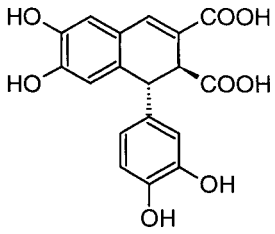
3,5-Dicaffeoylquinic acid (**7**)



Nordihydroguaiaretic acid (**8**)



Epiphyllinic acid-9,5``-6-shikimic acid ester (**10**)



Epiphyllinic acid (**9**)

linic acid (**9**) or its shikimic acid ester (**10**) also inhibited the enzyme with low IC_{50}, demonstrating the influence of catecholic structures on inhibitory activity.

Because of the importance of neutrophil elastase as an antibacterial protein connected with the enzymatic degradation of bacterial membrane proteins [17], the treatment of inflammation induced by bacterial infection with inhibitors of neutrophil elastase is not useful. On the other hand a broad spectrum of non-infectious inflammatory diseases might be successfully treated by medicinal plants or herbal preparations containing flavonoids and/or caffeic acid esters which are inhibitors of neutrophil elastase. Especially the treatment of inflamed wounds on the surface of skin or mucous membranes with preparations containing active flavonoids or caffeic acid derivatives could be positively influenced. The inhibition of elastase activity is connected with a decrease of matrix protein degradation and results in the attenuation of the progression of the inflammatory process.

The bioavailability of different flavonoids is still controversially discussed, but caffeic acid is absorbed after oral administration [18, 19]. Also esters of caffeic acid are ab-

sorbed after oral application, in plasma only caffeic acid could be detected [20]. So far caffeic acid esters could act as prodrugs metabolised to caffeic acid in the body which might act as inhibitor of neutrophil elastase activity.
The results may help to answer the question if selected phenolic natural products have the potency to participate in a proposed anti-inflammatory effect of medicinal plants. The presence of a phenolic compound able to inhibit the neutrophil elastase activity supports this assumption.

3. Experimental

3.1. Materials

Flavonoids, caffeic acid, caffeic acid phenethylester, chlorogenic acid, nordihydroguaiaretic acid and rosmarinic acid were purchased from Carl Roth GmbH or Sigma Chemical Co., 3,4-dihydroxyphenylic acid, 3-hydroxyphenylic acid and 4-methylcatechol were provided by Prof. P. G. Pietta, Milan (Italy). Isoferulic acid, ferulic acid, *p*-coumaric acid, 4-*O*- and 3-*O*-caffeoylquinic acid, 4-*O*- and 2-*O*-caffeoyl-L-threonic acid, 2-O-caffeoylmaleic acid, and 1,5-dicaffeolyquinic acid (cynarin) were supplied by Prof. A. Nahrstedt, Münster (Germany), 3,5-dicaffeolyquinic acid was provided by PD Dr. Bader, Berlin (Germany), bornylcaffeate was gifted by Prof. I. Merfort, Freiburg (Germany) and *trans*-drimenyl caffeic acid ester was supplied by Prof. H. Becker, Saarbrücken (Germany). All compounds isolated had a purity of more than 90%, proved by TLC or HPLC.
The enzyme substrate MeO-Suc-Ala-Ala-Pro-Val-pNA was purchased by Sigma, human neutrophil elastase was supplied by ICN.
All test compounds were dissolved in DMSO (10 mM stock solution) an then diluted with assay buffer. The influence of DMSO on enzyme activity was considered in controls.

3.2. Elastase assay

The determination of neutrophil elastase activity was performed with human leukocyte elastase [9]. Briefly, 250 µl substrate solution (700 µM MeO-Suc-Ala-Ala-Pro-Val-pNA in Tris-HCl-buffer, pH 7.5) were mixed with 100 µl test solution (test substances solubilized in Tris-HCl-buffer, pH 7.5) and vortexed. After the addition of 250 µl enzyme solution (approximately 0.5 mU) the samples were incubated for 1 h at 37 °C. The reaction was stopped by addition of 500 µl soybean trypsin inhibitor solution (2 mg/ml Tris-HCl-buffer, pH 7.5) and placed in an ice bath. After vortexing the absorbance was read at 405 nm.

3.3. Statistics

All assays were performed at least three times with duplicate samples. Inhibition rates were calculated in percent to controls without inhibitors. IC_{50} values were determined from dose-effect-curves by linear regression.

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