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The *In Vitro* Inhibition of Human Neutrophil Elastase Activity by some Yemeni Medicinal Plants

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Abstract

15 extracts of different polarities (dichloromethane, methanol and aqueous extracts) from 5 Yemeni medicinal plants (*Aspilia helianthoides*, (Schumach. & Thonn.) Oliv. & Hiern subsp. *ciliate* (Schumach.) C.D. Adams leaves; *Ceropegia rupicola*, Defl. var. *rupicola* whole plant; *Kniphofia sumarae*, Defler whole plant; *Pavetta longiflora*, Vahl. subsp. *longiflora* leaves; and *Plectranthus* cf *barbatus* (Thulin & Gifri) leaves) were tested for their inhibitory effects against the enzymatic activity of human neutrophil elastase (HNE) (EC 3.4.21.37) in an in vitro human neutrophil elastase inhibition assay. 14 extracts at various concentrations were found able to inhibit the activity of HNE. Among the plants tested, *A. helianthoides* was the most active inhibitor of HNE. The dichloromethane extracts of all tested plants, exhibited more inhibitory effect on HNE activity than the methanolic and aqueous extracts. The dichloromethane extract of *A. helianthoides* showed the most active inhibitory effect on the HNE activity (IC_{50} = 0.4µg/ml). Within the HNE inhibitory active methanolic extracts, those of *A. helianthoides* and *P.* cf *barbatus* were the most active inhibitors with IC_{50} of about 3µg/ml. These results provide some scientific justification for the use of *A. helianthoides* in traditional medicine and indicate the presence of HNE inhibitory constituents in the active tested plants.

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Keywords

Aspilia helianthoides • *Kniphofia sumarae* • *Pavetta longiflora* • P*lectranthus cf barbatus* • Human neutrophil elastase inhibition

Introduction

Human neutrophil elastase (HNE) (EC 3.4.21.37), a member of the Chymotrypsin family of serine proteases (SI) is primarily located in the azurophil granules of polymorphonuclear leucocytes. Physiologically it is involved in the degradation of bacteria and immune complexes phagocytosed by the polymorphonuclear leucocytes [1]. HNE can degrade a wide variety of biomacromolecules that exhibit important biological functions. These include extracellular matrix proteins such as elastin, which plays a major structural function in the lungs, arteries, skin and ligaments, collagen types I, II, III, IV, VIII, IX and XI, fibrin, fibronectin, cartilage proteoglycans, cytokines, the platelet IIb/IIIa receptor and cadherins. HNE is also capable of degrading a variety of soluble proteins, including coagulation factors, immunoglobulins, complement, and many protease inhibitors [1, 2, 3]. The release of HNE, in response to several inflammatory stimuli, and its extracellular proteolytic activity are regulated and are normal beneficial functions of polymorphonuclear neutrophils [2]. The elastase action is modulated by endogenous multiple proteinase inhibitors including α_1-proteinase inhibitor (α_1-PI). Despite this balance between HNE and proteinase inhibitors activities, there are several human diseases in which a breakdown of this control mechanism is implicated in their pathology. Insufficient levels of protease inhibitors has been therefore suggested as a contributing factor in a number of diseases including, among others, acute lung injury [2, 4, 5,], cystic fibrosis [6], ischemic reperfusion injury [7], rheumatoid arthritis [8], atherosclerosis [9], psoriasis [10] , and malignant tumors [11, 12]. Moreover alpha-1 antitrypsin (α_1- proteinase inhibitor) deficiency, an inherited disease, affects the lung and the liver. It leads to chronic obstructive pulmonary disease, emphysema, and even lung cancer development and in the

liver it causes a benign neonatal hepatitis syndrome and, to some small percentage of adults, liver fibrosis with progression to cirrhosis and hepatocellular carcinoma. [2, 13, 14]. Hence, over the past 30 years, elastase has been the object of extensive experimental and clinical research to develop efficient elastase inhibitors. At present, a number of natural and synthetic elastase inhibitors have been used in the treatment of HNE associated diseases. Of the natural elastase inhibitors, three human plasma derived α_1- protease inhibitors (Aralast, Prolastin and Zemaira) are used to prevent the progression of emphysema associated with α_1- antitrypsin deficiency disorder [15–17). Among the currently used synthetic elastase inhibitors, Sivelestat is applied for the treatment of acute lung injury and acute respiratory distress syndrome ARDS [18, 19], Gabexate mesilate, Nafamostat mesilate and Ulinastatin are used in Japan for the treatment of severe pancreatitis [20]. Furthermore, in a number of in vivo and in vitro models, several others synthetic elastase inhibitors have been found to prevent the deleterious effects of elastase in a variety of diseases. Attempts to develop useful therapeutic elastase inhibitors thereof are in progress [7, 21–23].

Since the past decade, natural compounds and plant extracts have been the targets of research as a potential source to explore for efficient elastase inhibitors [24–28]. The aim of this study was therefore to investigate the effects of a variety of extracts from some Yemeni plants on the activity of human neutrophil elastase.

Results and discussion

The plants used in this study, with the exception of *Aspilia helianthoides* that was claimed to be useful for wound healing, are not used in Yemeni traditional medicine such as *Kniphofia sumarae*, locally named “Bünit”, or utilized for purposes other than inflammatory diseases such as *Ceropegia rupicola*, locally named “Bukira”, that is applied externally for skin diseases and *Pavetta longiflora* and *Plectranthus* cf *barbatus*, which are used as an anti-malarial agent and as a haemostatic respectively. However related species of all tested plants are used in other countries for the treatment of inflammations and found experimentally to

possess anti-inflammatory activity as well [29–33]. Investigation the effect of the tested plant extracts on the HNE activity is able to reveal if these plants contain HNE inhibitors and therefore also possess anti-inflammatory activity. Moreover no data regarding the biological activities of these plants have been published till now except a study about the antimicrobial activity of some Yemeni medicinal plants including *Plectranthus* cf *barbatus*, which was found to possess antimicrobial activity [34].

Of 15 extracts of different polarities (dichloromethane, methanolic and aqueous extracts) screened at concentrations ranging from 0.1 till 100 µg/ml for their inhibitory effect against the human neutrophil elastase activity, 14 extracts at various concentrations were found able to inhibit the activity of HNE (Table 1). The validation of this assay was tested by using the selective HNE inhibitor GW 311616A (Positive control, IC_{50} =0.04 µg/ml) [28]. Among the plants tested, *A. helianthoides* was the most active inhibitor of HNE (Table 1). All the tested extracts showed a variable degree of inhibitory effect on HNE. Consequently a number of polar and non-polar constituents seem to be involved in the inhibition of the enzyme. The dichloromethane extracts of all tested plants excluding *C. rupicola* exhibited more inhibitory effect on HNE activity than the methanolic and aqueous extracts (Table 1). Significant reduction in the activity of HNE ($P<0.01$-$P<0.02$) was observed at a concentration of 5µg/ml and by *A. helianthoides* even by 0.5µg/ml and above of all dichloromethane extracts of the tested plants except of *C. rupicola* (Figure 1). The dichloromethane extract of *A. helianthoides* was the most active inhibitor of HNE activity (IC_{50} = 0.4µg/ml) (Table 1). Among the HNE inhibitory active methanolic extracts, those of *A. helianthoides* and *P.* cf *barbatus* were the most active inhibitors with IC_{50} of about 3µg/ml (Table 1, Figure 2). Previous studies [25, 35] that have used the same in vitro HNE inhibition assay have indicated that among a number of phenolic compounds (flavonoids (as glycosides and aglyca), caffeic acid and its derivatives) including constituents of some medicinal plants used in the treatment of inflammation, hyperosid (IC_{50} = 0.15 µg/ml), was found to be the most active HNE inhibitors. Because phenolic compounds such as

flavonoids and caffeic acid esters occur throughout the plants, the observed HNE inhibitory effect of the tested extracts might be partially attributed to the phenolic compounds.

The HNE inhibitory effect of *A. helianthoides* observed in our study therefore provides some scientific validation for the use of this plant to treat wounds. The inhibitory effect on HNE and its magnitude demonstrated by the dichloromethane and methanolic extracts especially of *A. helianthoides*, *P.* cf *barbatus*, *K. sumarae*, and *P. longiflora* suggests that these plants could contain HNE inhibitory constituents that might be able to target the destructive and inflammatory actions of human neutrophil elastase. The dichloromethane extract of *A. helianthoides* is currently being further investigated as a promising candidate for the development of useful HNE inhibitors.

Tab. 1: Inhibition of the HNE activity by the tested extracts

Plant sample/extract	IC_{50} (µg/ml)
Aspilia helianthoides	
Dichloromethane extract	0.4
Methanolic extract	2.9
Aqueous extract	8.5
Ceropegia rupicola	
Dichloromethane extract	9.5
Methanolic extract	6.6
Aqueous extract	-
Kniphofia sumarae	
Dichloromethane extract	1.6
Methanolic extract	4.5
Aqueous extract	79.4
Pavetta longiflora	
Dichloromethane extract	3.1
Methanolic extract	14.7
Aqueous extract	55.0
Plectranthus* cf *barbatus	
Dichloromethane extract	1.6
Methanolic extract	2.5
Aqueous extract	10.0
As positive control: GW 311616A (IC_{50} = 0.04 µg/ml)	

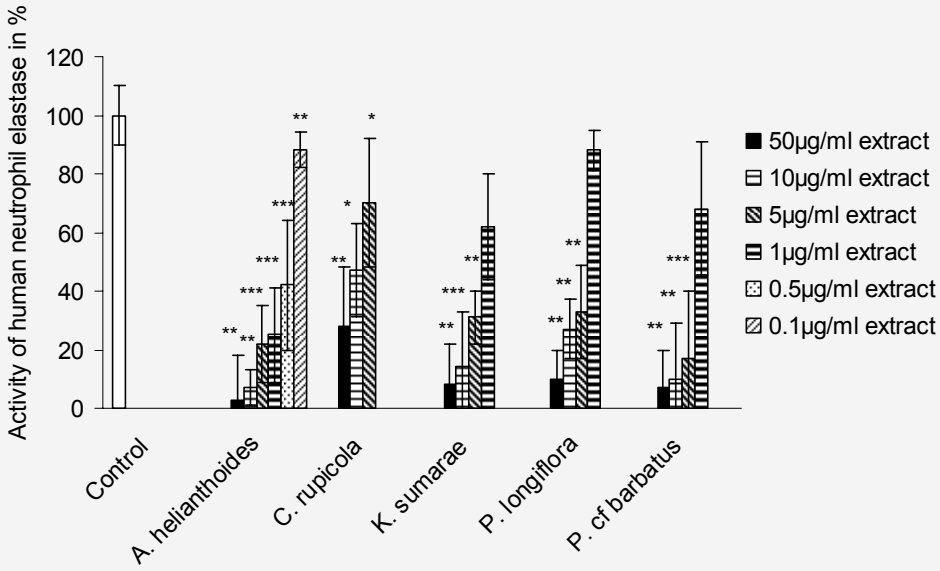

Figure 1: Inhibitory effect of the dichloromethane extracts of *Aspilia helianthoides, Ceropegia rupicla, Kniphofia sumarae, Pavetta longiflora*, and *Plectranthus* cf *barbatus* on the human neutrophil elastase activity

*** Significant at P < 0.01, ** Significant at P < 0.02, * Significant at P < 0.05

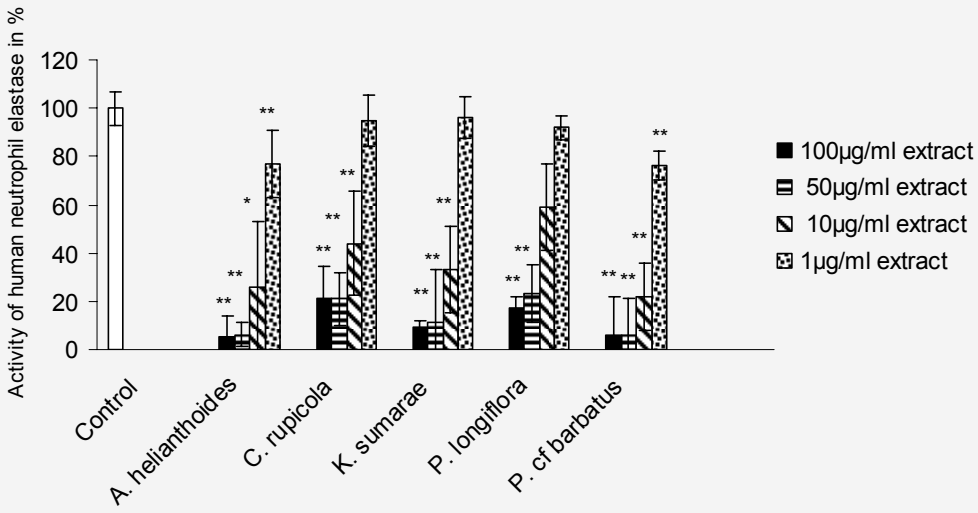

Figure 2: Inhibitory effect of the methanolic extracts of *Aspilia helianthoides, Ceropegia rupicola, Kniphofia sumarae, Pavetta longiflora* and *Plectranthus* cf *barbatus* on human neutrophil elastase activity

** Significant at P < 0.02, * Significant at P < 0.05

Experimental

Materials

Plants used in this study *Aspilia helianthoides* (Schumach. & Thonn.) Oliv. & Hiern subsp. *ciliate* (Schumach.) C.D. Adams, (Syn: *A. smithiana* Oliv. & Hiern; *A. abyssinica* (Sch.Bip.) Vatke; *A. dewevrei* O. Hoffm.; *A. ciliate* (Schumach.) Wild) (Asteraceae), leaves; *Ceropegia rupicola* Defl. var. *rupicola* (Asclepiadaceae), whole plant; *Kniphofia sumarae* Defler (Asphodelaceae), whole plant; *Pavetta longiflora* Vahl. subsp. *longiflora* (Syn: *P. arabica* Bremek; *P. deflersii* Bremek) (Rubiaceae), leaves; and *Plectranthus* cf *barbatus* (Thulin & Gifri) (Labiatae), leaves) [36, 37] were collected in Taiz province and Yaffe Heights, Lahj province, Republic of Yemen. The authentication of the plants was made under the supervision of Dr. A.W. Al-Khulaidi at the Agriculture Research Center in Dhamar-Yemen. The plant materials were shade dried at ambient temperature and then ground in a grinder.

The enzyme substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-Nitroanilid and the soybean Trypsin inhibitor were purchased from Sigma. Human neutrophil elastase and Tris-HCl- buffer were supplied by Serva.

Preparation of extracts

A series of subsamples (50g each) of each plant were extracted with a solvent (Dichloromethane, methanol and distilled water) by shaking for 3 hours at ambient laboratory temperature, and then in an ultrasonic bath for 20 min. The extraction was performed 3 times. The collected, filtered extracts were then concentrated to dryness. A series of concentrations (0.1, 0.5, 1, 5,10, 50, 100µg/ml) were prepared for the test from the dichloromethane and methanol extracts dissolved in dimethylsulfoxide (DMSO), and then diluted with the assay buffer and from the aqueous extract dissolved in distilled water and then diluted with the assay buffer.

Elastase assay

The determination of human neutrophil elastase activity was performed according to Löser [24]. Briefly, 125µl substrate solution (1.4mM N-MeO-Suc-Ala-Ala-Pro-Val-p-NA in Tris-HCl-buffer, pH 7.5) was added to 405 µl Tris-HCl-buffer, pH 7.5, with 50 µl extract solution. After the addition of 20 µl enzyme solution (approximately 3mU), the samples were vortexed and then incubated for 1 h. at 37 ºC. The reaction was stopped by addition of 500µl soybean trypsin inhibitor solution (0.2mg/ml Tris-HCl-buffer, pH 7.5). The samples were then vortexed and the absorbance was measured at 405nm. The remaining activity of HNE activity (as % of the control) was calculated in comparison to the control without inhibitor, considering the influence of the buffer, substrate, solvent and test extract. The inhibitory effect of the selective HNE inhibitor GW 311616A (IC_{50} =0.04 µg/ml) that was already tested by using the same assay [28] was considered as a positive control.

Statistics

The assay was performed two to three times with triplicate parallel samples. The remaining activity of the enzyme was calculated as percentage to the control without inhibitor, considering the influence of the buffer, DMSO, substrate and test extract. All values were expressed as mean ± standard deviations. Wilcoxon's U-test was used to test the significance. IC_{50} values were obtained from dose-effect curves by linear regression.

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