A Neutrophil Multitarget Functional Bioassay to Detect Anti-inflammatory Natural Products

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A multitarget functional bioassay was optimized as a method for detecting substances interacting with the inflammatory process of activated neutrophil granulocytes, mainly to release elastase detected by *p*-nitroanilide (pNA) formation. Using this bioassay, 100 fractionated extracts of 96 plants were screened, with results presented in a manner that links recorded biological activity to phylogenetic information. The plants were selected to represent a major part of the angiosperms, with emphasis on medicinal plants, Swedish anti-inflammatory plants, and plants known to contain peptides. Of the tested extracts, 41% inhibited pNA formation more than 60%, and 3% stimulated formation. The extract of *Digitalis purpurea* enhanced pNA formation, and digitoxin, the active compound, was isolated and identified. Plant extracts that exhibited potent nonselective inhibition (>80% inhibition) were evaluated further for direct inhibition of isolated elastase and trypsin enzyme. The inhibitory effect of most tested extracts on the isolated enzyme elastase was similar to that of PAF- and fMLP-induced pNA formation. Compared to trypsin, inhibition of elastase by extracts of *Rubus idaeus* and *Tabernaemontana dichotoma* was significantly higher (80% and 99%, respectively). Inhibition of trypsin by the extract of *Reseda luteola* was high (97%). Orders such as Lamiales and Brassicales were shown to include a comparably high proportion of plants with inhibitory extracts.

The neutrophil granulocyte is a central component of the inflammatory process, having the ability to migrate to the inflammation site and to release toxic products such as proteolytic enzymes,[1] reactive oxygen species,[2−4] and cationic proteins[5] (e.g., defensins[6,7] and cathelicidins[8]) capable of killing invading pathogens. The targets of the neutrophil include bacteria, fungi, viruses, virally infected cells, and tumor cells. Neutrophil functions are initiated by cell surface receptors of chemoattractants, such as the bacterial product *N*-formyl methionyl-leucyl-phenylalanine (fMLP);[9−13] secreted products of stimulated phospholipid metabolism, such as the platelet activating factor (PAF),[14,15] and leukotriene B_4 (LTB_4);[16] and immunomodulatory molecules, such as the cytokine interleukin 8.[17,18] Neutrophils contain azurophilic, specific, and gelatinase granules and secretory vesicles containing various degrading enzymes. The exocytosis of granules and vesicles cause not only a release of lytic enzymes but, concurrently, increased cell response by production of reactive oxygen intermediates and chemotactic stimulation. These activities lead to the destruction of invading pathogens.[19,20]

Human neutrophil elastase (HNE; IEC 3.4.21.37) is a major secreted product of activated neutrophils and a major contributor to destruction of tissue in chronic inflammatory diseases such as lung emphysema, glomerulonephritis, arthritis, and rheumatoid arthritis.[21,22] HNE therefore appears to be a possible target for therapy of chronic inflammatory diseases.

The bioassay used in this study is a modification of an assay originally developed by Dewald et al.,[23] and later modified and adapted by Tuominen et al.,[24] to detect PAF-antagonists. In that assay, neutrophils were incubated with PAF, the antagonist under evaluation, and a chromogenic substrate SAAVNA (*N*-succinyl-L-alanyl-L-alanyl-L-valine-L-*p*-nitroanilide) for released elastase. Elastase proteolytically cleaves SAAVNA, which leads to formation of a colored product, *p*-nitroanilide (pNA), that can be quantified photometrically.[25,26] Making use of the complex biological processes occurring in and around the neutrophil allowed us to use the assay as a multitarget functional bioassay.[27]

A multitarget functional bioassay, the observed effects of which cannot be attributed directly to a specific mode of action, encompasses assays on whole animals, isolated organs, and intact cells. For example, an observed relaxation of the isolated guinea pig ileum can be due to mechanisms involving cell membrane receptors, second messengers, or ion channels, or due to other mechanisms. Single target bioassays, on the other hand, are typically tests run on an isolated enzyme or a receptor (receptor binding). For such assays, which are highly specific, the observed effect can be directly attributed to a specific mechanism, such as enzyme inhibition or receptor affinity.

Detection of a colored product formed by elastase released from a chemoattractant-stimulated neutrophil can be described as a multitarget functional bioassay, where both known and previously unknown potential drug targets are present. An observed inhibition of formation of pNA can, for example, be due to elastase inhibition, cytotoxic effects on the cell, or affinity for a receptor on the neutrophil (Figure 1). By the same method, chemoattractant activity of a test compound is also possible, seen as increased amount of pNA formed. In this study, we evaluated the originally described assay conditions with the aim of optimizing the bioassay for multitarget functional screening of natural products.

Using prefractionated plant extracts instead of crude extracts presumably facilitates finding bioactive compounds present in small amounts within the plant, and can be used to include or exclude activity from certain classes of substances. Common tannins, alkaloids, and flavonoids are plant substances well known for exhibiting biological activity in several assays[28−32] and can therefore obscure

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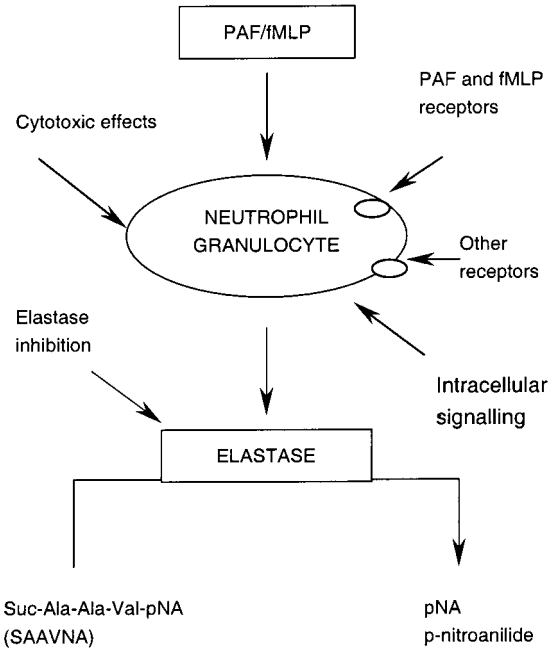

Figure 1. Schematic picture of the neutrophil viewed as a multitarget functional bioassay with various possible modes of action for test substances. Both inhibiting and stimulating effects can be detected.

observations of new compounds in assays of crude extracts. A fractionation protocol, earlier developed to detect biologically active polypeptides in plants,[33] was used in our study to screen 100 fractionated plant extracts for functional interactions with the chemoattractant-challenged human neutrophil.

In addition, the study includes an introductory classification of the fractionated plant materials, sorting them into mechanistic classes that can facilitate prioritizing the extracts for future studies. Figure 2 provides an outline of the classification procedure. The 100 fractionated plant extracts were first tested for inhibition of PAF- and fMLP-induced pNA formation and for ability to induce elastase release in the absence of challenge with PAF or fMLP. Inhibitory extracts were classified as being either selective inhibitors (of PAF or fMLP) or nonselective inhibitors (of pNA formation).

Plant extracts that were potent nonselective inhibitors of pNA formation were further evaluated for direct inhibitory effect on isolated elastase enzyme. To evaluate selectivity of elastase inhibition, extracts were also tested for ability to inhibit the enzyme trypsin. In addition, each fractionated plant extract was tested for hemolytic activity to determine whether it could damage cells.

Results and Discussion

In experiments the agent cytochalasin B was used, which, together with stimulation by inducers such as PAF and fMLP, converts neutrophils from phagocytic to secretory cells. Cytochalasin B enhances phagocyte secretion of lysosomal enzymes, which promote disaggregation of the intracellular actin network, and in turn, this promotes secretion by facilitating fusion of secretory granules with cell membranes.[34] When polymorphonuclear leukocytes (PMNs), subsequently challenged with PAF or fMLP, were exposed to cytochalasin B in various concentrations, ranging from 0 to 20 μg/mL, formation of pNA was markedly increased and then decreased in a concentration-dependent manner (Figure 3a). Formation of pNA in PAF- and fMLP-induced PMNs required cytochalasin B. The initial concentrations of PAF and fMLP were 0.3 and 0.01 μM. The optimal concentration of cytochalasin B for priming was 5 μg/mL, which induced a 5-fold increase in formation of pNA, compared to PMNs incubated with PAF or fMLP alone.

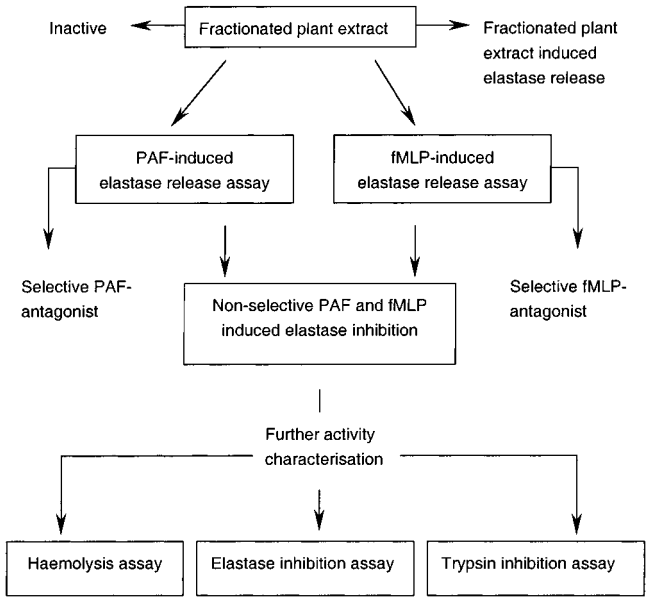

Figure 2. Schematic representation of the evaluation of the activity of the fractionated plant extracts, which were first tested for the ability to induce elastase release in the absence of PAF or fMLP, and then for inhibiting PAF- and fMLP-induced pNA formation. Inhibiting extracts were catagorized as being selective or nonselective inhibitors of PAF or fMLP. The plant extracts that showed a high nonselective inhibition of pNA formation (>80% inhibition) were further evaluated for direct inhibitory effect on the isolated enzyme elastase. To roughly evaluate selectivity for elastase inhibition, the extracts were also tested for ability to inhibit the enzyme trypsin. In addition, the fractionated plant extracts were tested for hemolytic activity.

Amount of formation of pNA depended on SAAVNA concentration (Figure 3b). To ensure that the formation of pNA was not limited by substrate concentration, increasing SAAVNA concentrations were added until pNA formation reached a maximum. Concentrations of cytochalasin B and PAF/fMLP were kept constant. For 10 μM PAF and fMLP, pNA reached a maximum at a concentration of 1.6 mM of SAAVNA. At higher concentrations, pNA formation decreased.

To evaluate the selectivity of inhibitors of the PAF- or fMLP-induced exocytosis, dose titrations of both agents were done. Concentration−response curves for the inducers PAF and fMLP were recorded. The PMNs were incubated with cytochalasin B and subsequently exposed to the chemoattractants (PAF or fMLP) at concentrations ranging from 0.0001 to 1000 μM. The two inducers were tested in parallel using PMNs from the same preparation. Then the amount of pNA was determined, as a measure of amount of released extracellular elastase. Figure 3c shows the concentration−response relationships for PAF- and fMLP-induced elastase release. Equal biological responses for PAF and fMLP, reaching approximately 80% of maximum, were obtained at a concentration of 0.1 μM (Figure 3c), which was chosen as assay condition.

Because the concentrations of PAF and fMLP were then reduced to 0.1 μM to give equal biological responses, the concentration of SAAVNA was reevaluated. Increasing concentrations of SAAVNA were added until pNA formation reached the maximum (0.8 mM SAAVNA).

To further validate the optimized assay, one additional agonist, interleukin-8 (IL-8), and one additional indicator

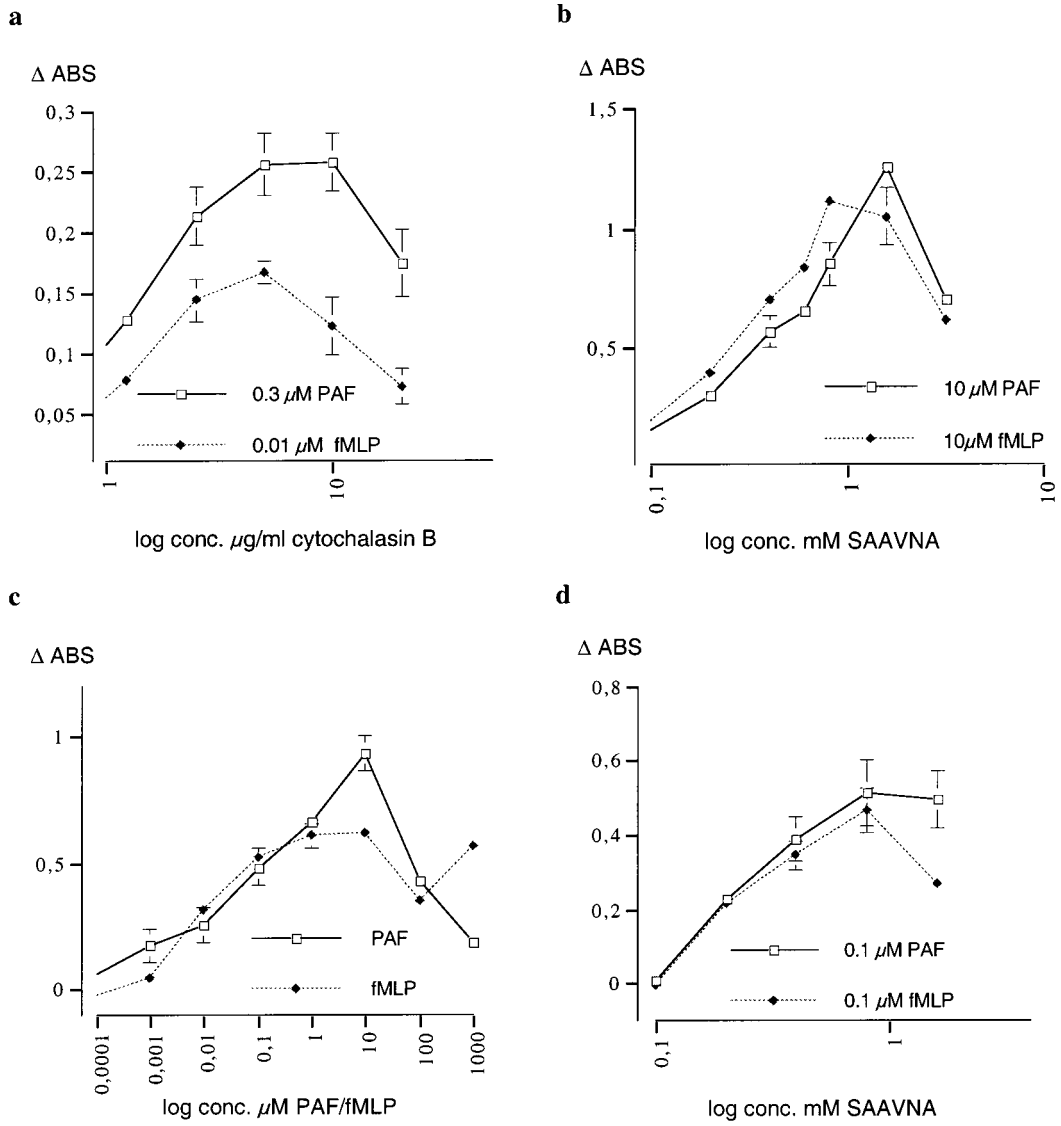

Figure 3. Relationships between concentration and response (influence on pNA formation) measured by absorbance (ABS). (a) Formation of pNA at several concentrations of cytochalasin B. No effects of the chemotactic peptide fMLP or PAF on the pNA formation were observed in the absence of cytochalasin B. (b) Formation of pNA at several concentrations of SAAVNA, with maximum formation reached at at 1.6 mM. A concentration of 10 μM for PAF and fMLP was used to induce exocytosis. (c) PAF- and fMLP-induced concentration-dependent responses by release of elastase. Biological responses of similar magnitudes were obtained for both PAF and fMLP at concentration 0.1 μM. (d) Increasing concentrations of SAAVNA were added until formation of pNA reached a maximum. The concentration 0.8 mM was chosen as the assay condition. All the points represent mean values ± SEM from three independent experiments run in duplicate.

of exocytosis, generation of reactive oxygen species, were evaluated under the test conditions described. Figure 4 shows IL-8-induced elastase release. The same concentration as of PAF and fMLP (i.e., 0.1 μM) can be used to induce, by IL-8, elastase release from PMNs. For measuring generation of the superoxide anion O^{2-} in PAF- or fMLP-activated neutrophils, cytochrome C reduction methodology was used.[35] As seen in Figure 5, fMLP induced a higher increase in the production of O^{2-} than did PAF. This agrees with the literature, where PAF is reported to only weakly stimulate superoxide generation of neutrophil granulocytes (in concentrations below 10 μM), while fMLP is reported to be more potent (EC_{50}: 48 nM).[36]

Because testing of multiple concentrations of plant extracts in a full-scale screening, without access to robotics, is a cumbersome and time-consuming task, a suitable single concentration for extract testing was sought. Twenty-three randomly selected plant extracts were prescreened at 10 and 100 μg/mL (data not shown). At an exposure concentration of 100 μg/mL, inhibition for seven of these plant extracts was more than 60%. At 10 μg/mL, on the other hand, none of the compounds induced significant inhibition. From this initial study, a final concentration of 100 μg/mL was chosen for the full-scale screening. This provided a safety margin against overlooking active compounds occurring at low concentrations in the extracts. Working at this concentration, the assay could also be used for a preliminary ranking of the potency of the extracts, but concentration−response experiments would then be needed for more precise information about potency.

For evaluation of reference compounds in independent experiments, repeatability and reproducibility of the assay was good, and different modes of action could clearly be detected. When added to cytochalasin B-treated PMNs, the PAF-antagonist ginkgolide BN 52021[37] exhibited a selective inhibitory effect on PAF, as expected, and the IC_{50} value (Table 1) was 72 μg/mL, which is consistent with previously reported values for inhibition of PAF by the ginkgolide BN 52021.[23] At the highest tested concentration (100 μg/mL), ginkgolide was unable to inhibit the fMLP inducer. The elastase inhibitor α_1-antitrypsin[38] [IC_{50}: 10 μg/mL (PAF), 11 μg/mL (fMLP)] showed a nonselective inhibitory effect.

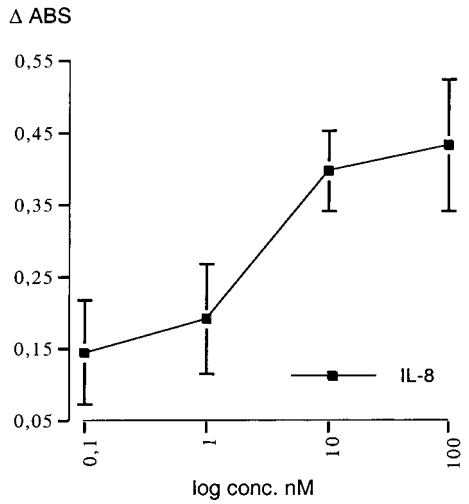

Figure 4. Amount of pNA formation (i.e., absorbance, ABS) at different concentrations of the inducer IL-8. Each point represents the mean value ± SEM from two to three independent experiments run in duplicate.

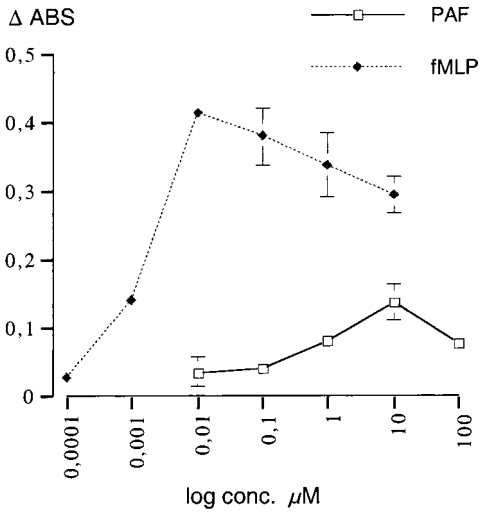

Figure 5. Superoxide production by PMNs when stimulated by the addition of PAF (□) and fMLP (◆). Each point is the mean absorbance value ± SEM of three independent experiments run in duplicate.

Table 1. Reference Substances Tested in the Optimized Assay

substance	PAF IC_{50} μg/mL	*n*	fMLP IC_{50} μg/mL	*n*
α_1-antitrypsin	10	9	11	9
trypsin-inhibitor	10	9	8	10
BN 52021	72	12		11

After optimization, the assay operated successfully for 100 plant extracts from 96 species, representing 46 families of 26 orders, where both PAF and fMLP were used as exocytosis inducers. For a primary selection of plants, the following protocol was used: sample widely; sample plants known to produce peptides; sample Swedish antiinflammatory plants; and include a number of traditionally used medicinal plants. This resulted in a set of 96 taxa, listed in Table 2. We followed classifications suggested by the Angiosperm Phylogeny Group.[39−41] Of the 40 recognized orders of higher plants, 23 are represented among the tested samples (Figure 6).

Of the 100 tested extracts, 41% inhibited pNA formation more than 60%; 18% nonselectively inhibited pNA formation less than 30%; and 3% stimulated formation of pNA, without the challenge of PAF or fMLP. In the order

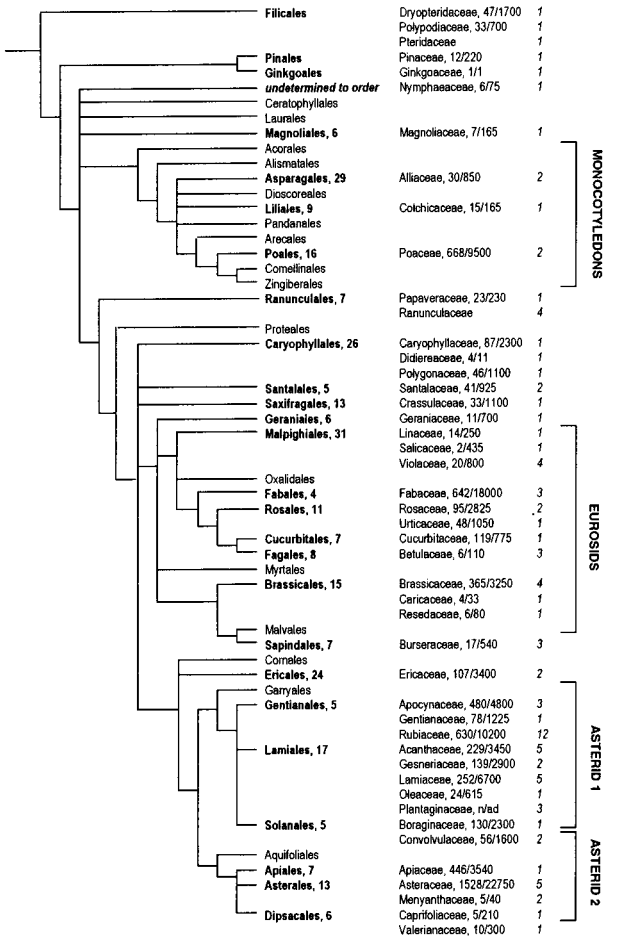

Figure 6. Systematic position and distribution of taxa included in the study. Phylogenetic relationships according to APG (1998)[39] and NCBI/GenBank taxonomy section. Numbers following ordinal names indicate approximate number of included families, numbers following familial names indicate approximate number of genera and species, respectively. Ordinal names in bold type face indicate orders represented in the study.

Malpighiales, 4 out of 7 tested extracts inhibited pNA formation less than 30%, whereas in the family Violaceae, 3 of 4 tested species inhibited formation less than 30%, and one, *Viola patrinii*, stimulated formation (Table 2). In other orders, such as Lamiales and Brassicales, a comparably high proportion of the tested species showed prominent inhibitory activity. For Lamiales, 11 out of 17 tested extracts inhibited pNA formation more than 60%. The fractionated plant extract of *Nymphoides peltata* of Menyanthaceae inhibited pNA formation of PAF-induced elastase release 36 ± 14%, and fMLP-induced elastase release 90 ± 3%, which may indicate selective inhibition by fMLP receptors. However, since all the fractionated plant extracts contain a mixture of substances, more than one active compound is possible, and therefore the presence of selective inhibitors in fractions cannot be ruled out, which can influence and modify nonselective inhibition.

The fractionated plant extracts that nonselectively inhibited pNA formation more than 80% were tested for ability to inhibit, in a single target bioassay, the isolated enzyme elastase. For comparison, other plant extracts were included as negative controls. The fractionated plant extracts inhibited the isolated elastase in the same concentration range as they inhibited pNA formation (Table 2). *Mitrostigma axillare* and *Justitia adathoda* inhibited pNA formation of PAF-induced elastase release 93 ± 1% and 84 ± 2%, and fMLP-induced elastase release 93 ± 0%

Table 2. Activities of the Fractionated Plant Extracts at a Concentration of 100 μg/mL, Listed According to Their Systematics Position[a]

PHYLUM / CLASS/SUBCLASS / Order, number of families in order / Family, 'approximate number of genera/species' / *Genus & species*	PAF %inhib.	n	fMLP %inhib.	n	Elastase %inhib.	n	Trypsin %inhib.	n	Haemolytic
PTERIDOPHYTA									
FILICOPSIDA									
Filicales									
Dryopteridaceae, 47/1700									
Dryopteris filis-max	60±9	4	61±4	4	nd		45	2	nd
Polypodiaceae, 33/700									
Polypodium vulgare	98±1	8	98±1	8	nd		nd		nd
Pteridaceae									
Pteridium aquilinum	75±5	4	84±6	4	nd		nd		No
CONIFEROPHYTA									
CONIFEROPSIDA									
Pinales									
Pinaceae, 12/220									
Picea abies	52±6	4	52±5	4	69±1	4	nd		No
GINKGOPHYTA									
GINKGOOPSIDA									
Ginkgoales									
Ginkgoaceae, 1/1									
Ginkgo biloba	56±3	3	59±3	3	nd		nd		No
MAGNOLIOPHYTA									
ANGIOSPERM ROOT									
undetermined to order									
Nymphaeaceae, 6/75									
Nymphaea alba	75±2	3	76±0	4	79	2	nd		No
Magnoliales, 6									
Magnoliaceae, 7/165									
Magnolia kobus	41±14	3	21±5	3	nd		nd		No
MONOCOTYLEDONS									
Asparagales, 29									
Alliaceae, 30/850									
Allium sativum	4±10	4	8±14	4	nd		9	2	nd
Aloe lateritia	79±3	4	80±2	4	nd		nd		nd
Liliales, 9									
Colchicaceae, 15/165									
Colchicum autumnale	85±3	4	73±9	4	82±3	4	44	2	No
Poales, 16									
Poaceae, 668/9500									
Elymus repens	14±10	4	18±12	4	nd		nd		No
Poa pratensis	0±8	4	-3±4	4	nd		nd		No
EUDICOTYLEDONS									
Ranunculales, 7									
Papaveraceae, 23/230									
Fumaria officinalis	4±17	4	17±18	4	nd		22	2	No
Ranunculaceae									
Actaea alba	58±4	4	59±5	4	nd		nd		No
Anemone canadensis	57±15	4	60±12	4	59±3	4	59	2	No
Anemone nemorosa	98±4	4	98±5	4	nd		nd		nd
Helleborus cyclophyllus	25±4	4	13±12	4	nd		nd		No
Caryophyllales, 26									
Caryophyllaceae, 87/2300									
Silene rubrum	65±11	4	82±5	4	80±2	4	31	2	No
Didiereaceae, 4/11									
Alluaudia humbertii	32±6	4	39±3	4	55±3	4	50	2	No
Polygonaceae, 46/1100									
Rheum x *hybridum* (Viktoria)	51	2	34	2	nd		nd		No
Santalales, 5									
Santalaceae, 41/925									
Phoradendron tomentosum[c]	48±5	6	38±7	6	nd		nd		nd
Viscum heyneanum	99±1	4	87±5	4	99±0	4	51	2	No
Saxifragales, 13									
Crassulaceae, 33/1100									
Sedum rosea	95±0	4	89±1	4	88±2	4	nd		No
ROSIDS									
Geraniales, 6									
Geraniaceae, 11/700									
Geranium silvaticum	67±1	4	66±3	4	77±3	4	nd		No
Malpighiales, 31									
Linaceae, 14/250									
Linum austriacum	0±4	4	11±5	4	nd		nd		No
Salicaceae, 2/435									
Populus tremula	77±3	4	74±5	4	82±1	3	51		No
Violaceae, 20/800									
Viola arvensis	12±8	6	16±7	7	4		nd		No
Viola cornuta	16±10	4	26±6	4	nd		nd		No
Viola patrinii fruit	[b]		[b]		nd		55	2	No
Viola patrinii herba	[b]		[b]		nd		-19	2	Yes
Viola tricolor	4±11	4	9±7	4	nd		nd		No
Fabales, 4									
Fabaceae, 642/18000									
Caesalpinia benthamianum	55±4	4	58±6	4	nd		40	2	nd
Cytisis scoparius	23±17	4	41±8	4	nd		nd		No
Trifolium pratense	47±5	5	60±1	3	65±1	4	46	2	No
Rosales, 11									
Rosaceae, 95/2825									
Rubus idaeus	71±8	6	57±8	4	80±1	3	8	2	No
Sorbus aucuparia wood	19±4	4	21±3	4	nd		nd		No
Sorbus aucuparia bark	48±7	4	41±9	4	nd		45	2	No
Sorbus aucuparia leaf	29±6	4	28±8	4	nd		nd		No
Urticaceae, 48/1050									
Urtica dioica	74±3	4	75±3	4	88±1	4	50	2	No
Cucurbitales, 7									
Cucurbitaceae, 119/775									
Momordica charantia	75±4	4	64±10	4	85±1	4	64	2	No
Fagales, 8									
Betulaceae, 6/110									
Alnus glutinosa	82±6	4	68±4	4	69±1	4	22	2	Yes
Betula verrucosa	57±12	4	62±8	4	nd		39	2	Yes
Corylus avellana	79±4	4	56±3	4	62±6	4	nd		nd

PHYLUM CLASS/SUBCLASS **Order, number of families in order** Family, 'approximate number of genera/species' *Genus & species*	**PAF** %inhib.	n	**fMLP** %inhib.	n	**Elastase** %inhib.	n	**Trypsin** %inhib.	n	**Haemolytic**
Brassicales, 15									
Brassicaceae, 365/3250									
Brassica rapa	68±2	4	62±7	4	nd		nd		No
Bunias orientalis	67±9	4	64±8	4	nd		nd		No
Crambe maritima	84	2	90	2	nd		nd		No
Isatis tinctoria	65±16	4	47±11	4	nd		51		No
Caricaceae, 4/33									
Carica papaya	66±3	4	64±5	4	nd		nd		nd
Resedaceae, 6/80									
Reseda luteola	68±7	4	70±3	4	82±1		97		No
Sapindales, 7									
Burseraceae, 17/540									
Boswellia frereana	64±9	4	74	2	nd		nd		nd
Commiphora guidottii	78±7	4	73±3	4	59±8	4	nd		nd
Commiphora myrrha	70±4	4	72±7	4	nd		nd		nd
ASTERIDS									
Ericales, 24									
Ericaceae, 107/3400									
Arctostaphylus uva-ursi	20±13	4	26±8	4	nd		30	2	No
Erica carnea	32±7	4	37±3	4	nd		nd		No
EUASTERIDS I									
Gentianales, 5									
Apocynaceae, 480/4800									
Asclepias tuberosa	33±3	4	43±13	4	nd		24	2	No
Tabernaemontana dichotoma	95±0	4	97±1	4	99±0	4	11	2	No
Vinca minor	45±10	4	59±14	4	nd		44	2	No
Gentianaceae, 78/1225									
Gentiana punctata	57±3	4	56±5	4	nd		51	2	No
Rubiaceae, 630/10200									
Cinchonoideae									
Cinchona pubescens	50	2	37	2	nd		nd		No
Ixoroideae									
Coffea arabica	80±4	4	84±3	4	90±1	4	nd		No
Mitriostigma axillare	93±1	4	93±0	4	93±1	4	41	2	No
Rubioideae									
Asperula tinctoria	30±20	4	15±9	4	nd		nd		No
Galium album	64±4	4	66±3	4	nd		24	2	Yes
Galium aparine	27±10	4	42±20	3	nd		nd		No
Galium boreale	55±6	3	31±14	4	nd		nd		No
Galium odoratum	43±16	4	64±19	3	nd		nd		No
Galium saxatile	58	2	51	2	nd		42	2	No
Galium verum	44±8	4	39±8	4	nd		40	2	No
Oldenlandia corymbosa	47±5	4	44±4	4	nd		36	2	nd
Rubia tinctorum	68±17	4	70±21	3	nd		nd		No
Lamiales, 17									
Acanthaceae, 229/3450									
Andrographis paniculata	82±3	4	79±4	4	73±4	4	57	2	No
Aphelandra flava	72±4	4	77±2		82	2	nd		No
Eranthemum albiflorum	97±1	4	96±0	4	98	2	nd		nd
Justitia adathoda	84±2	4	80±3	4	84±1	4	51	2	No
Justitia betonica	50±15	4	51±8	4	nd		nd		No

PHYLUM CLASS/SUBCLASS **Order, number of families in order** Family, 'approximate number of genera/species' *Genus & species*		**PAF** %inhib.	n	**fMLP** %inhib.	n	**Elastase** %inhib.	n	**Trypsin** %inhib.	n	**Haemolytic**
Gesneriaceae, 139/2900										
Haberlea rhodopensis		64	2	66	2	nd		nd		
Saintpaulia ionantha	leaf	78±2	4	85±3	4	71±2	4	50	2	Yes
Saintpaulia ionantha	flower	76±8	4	71±1	4	74±3	4	55	2	No
Lamiaceae, 252/6700										
Lamium album		29±6	4	30±8	4	nd		46	2	No
Leonurus cardiaca		74±5	4	71±3	4	nd		nd		No
Lycopus virginica		69±3	3	73	2	83	2	nd		No
Stachys byzantina		66±2	4	57±8	4	nd		nd		No
Teucrium lucidum		83±2	4	79±1	4	85±2	4	nd		No
Oleaceae, 24/615										
Syringa vulgaris		27±19	4	16±12	4	nd		50	2	No
Plantaginaceae, n/a[d]										
Digitalis lanata		[b]		[b]						
Digitalis purpurea		[b]		[b]						
Plantago major		85±3	4	82±3	4	nd		nd		No
Solanales, 5										
Boraginaceae, 130/2300										
Symphytum asperum		12±12	4	2±8	4	nd		nd		No
Convolvulaceae, 56/1600										
Convolvulus arvensis		67±3	4	70±5	4	77±1	4	51	2	No
Ipomoea quamoclit		45±10	4	43±8	4	nd		nd		No
EUASTERIDS II										
Apiales, 7										
Apiaceae, 446/3540										
Anthriscus silvestris		58±1	3	42±11	4	nd		nd		No
Asterales, 13										
Asteraceae, 1528/22750										
Achillea millefolium		45±5	4	26±4	4	37±11	4	32	2	Yes
Arnica mollis		32±16	4	18±5	4	nd		38	2	No
Echinaceae purpurea		7	2	12	2	nd		nd		nd
Matricaria inodora		27±8	4	34±11	4	nd		nd		No
Tussilago farfara		38	2	31	1	nd		nd		nd
Menyanthaceae, 5/40										
Menyanthes trifoliata		32±7	4	63±16	4	nd		42	2	No
Nymphoides peltata		36±14	4	90±3	4	43	2	nd		Yes
Dipsacales, 6										
Caprifoliaceae, 5/210										
Lonicera chrysanta	leaf	24±6	4	24±3	4	nd		43	2	No
Valerianaceae, 10/300										
Valeriana officinalis		72±4	4	71±4	4	79±1	4	31	2	No

[a] Organisms sampled for screening, listed according to their systematics position. Higher plants are arranged according to APG (1998);[39] lower plants follow taxonomy sections of NCBI/GenBank. Scientific names are according to *Index Kewensis*; number of genera and species is from Mabberley (1997).[54] [b] These fractions stimulated pNA formation, and were tested in 10–20 independent experiments run in duplicate. [c] This fraction, (Fraction BV) was isolated as described by Thunberg (1983).[55] [d] Recently recircumscribed.

nd: not determined.

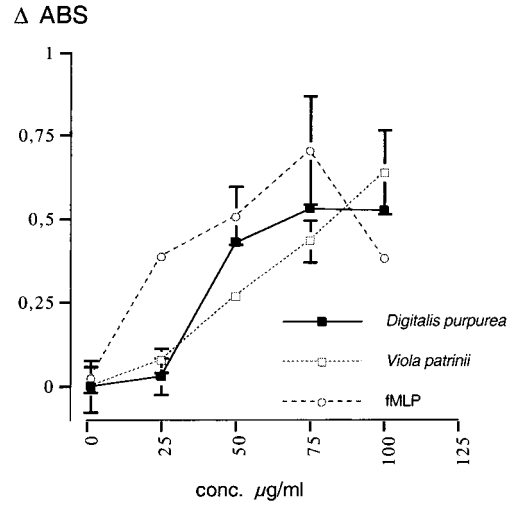

Figure 7. Amount of pNA formation induced by fractions from *Digitalis purpurea* (■) and *Viola patrinii* (□) compared to that of fMLP (○) stimulated neutrophils. Each point is the mean absorbance value ± SEM from three independent experiments.

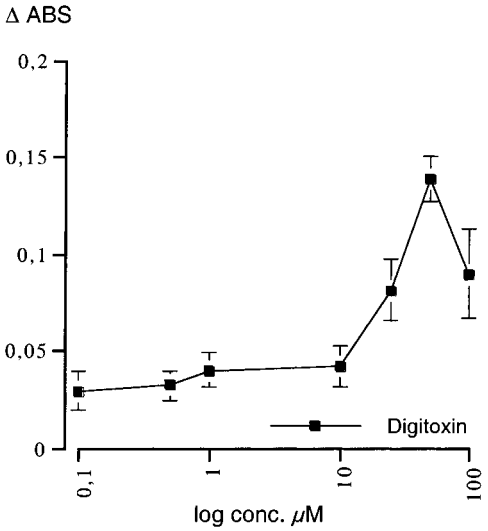

Figure 8. Amount of pNA formation from neutrophils treated by different concentration of the isolated digitoxin. Each point is the mean absorbance value ± SEM of three independent experiments.

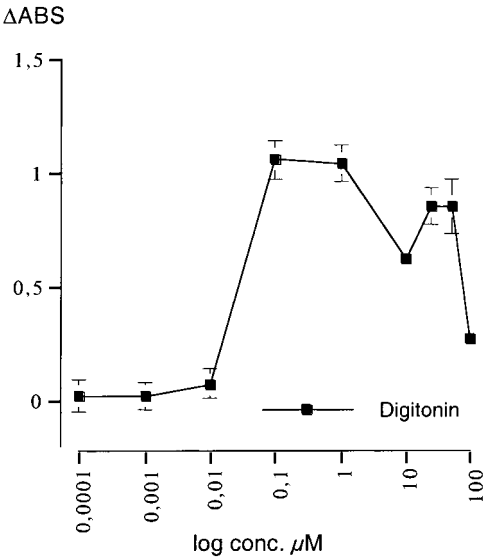

Figure 9. Amount of pNA formation from neutrophils in the response to digitonin in different concentrations. Each point is the mean absorbance value ± SEM for six experiments run in duplicate.

and 80 ± 3% (Table 2). These extracts were shown to inhibit the isolated enzyme elastase 93 ± 1% and 84 ± 1%, which indicates that the observed effects in the whole cell assay are probably due to inhibition of isolated enzyme elastase.

Some of the plant materials were also tested against the enzyme trypsin to give an indication of the enzyme selectivity of the observed inhibition of elastase. Inhibition of trypsin by extracts of *Reseda luteola* was high (97%). The extracts of *Rubus idaeus* and *Tabernaemontana dichotoma* inhibited elastase (80% and 99%) significantly more than they inhibited trypsin (8% and 11%). These results suggest that some of the extracts studied might contain selective protease inhibitors.

Examination of the fractionated plant extracts of *Digitalis lanata*, *Digitalis purpurea*, and *Viola patrinii* revealed an unusual and unexpected effect on the formation of pNA. The extracts from these plants significantly increased formation of pNA, in a concentration-dependent manner (Figure 7), independent of challenge with PAF or fMLP. The formation of pNA was dependent in these cases on the presence of cytochalasin B and was not inhibited by the PAF-antagonist ginkgolide BN 52021; but the presence of α_1-antitrypsin decreased the amount of pNA formed.

The cardiac glycoside digitoxin was isolated in a bioassay-guided fractionation procedure as the most potent compound in *D. purpurea* for enhancing pNA formation. The substance was identified by spectroscopic methods. The ^{1}H NMR spectrum (recorded at 600 MHz) and mass spectrum (obtained by the electrospray ionization technique) was in agreement with those of an authentic sample of digitoxin (Sigma). Digitoxin's concentration−response for enhancement of pNA formation is shown in Figure 8. In the optimized assay, the maximal pNA-formation enhancement by isolated digitoxin was lower than that of the inducers PAF, fMLP, and the original extract of *D. purpurea*. The substantial enhancement by the fractionated extract was possibly a synergistic effect involving more than one cardiac glycoside.

The saponin digitonin, a detergent from *D. purpurea* known to lyse plasma membranes and granules of neutrophils,[42] was earlier shown to induce both lysosomal enzyme release[43] and superoxide release from neutrophils.[44,45] Digitonin could, however, not be detected in the fractionated plant extract of *D. purpurea* by TLC or HPLC. Elastase is present in the neutrophils mostly in the azurophil or primary granules.[46,47] Digitonin's pNA-formation enhancement was dose-dependent (Figure 9) with two different maxima. This is likely the result of the two different granules containing elastase. Our findings are very similar to those of Garcia et al. (1985), who obtained a dual distribution of elastase in neutrophils.[48]

To compare the effects of digitoxin and digitonin, parallel samples of PMN were pretreated with either digitoxin or digitonin in several concentrations and then assayed for PAF- or fMLP-induced elastase release (data not shown). Interestingly, pretreatment of PMNs with digitonin resulted in a failure of the PMNs to respond and in increased pNA formation when PAF or fMLP was used as inducer. By contrast, PMNs pretreated with digitoxin were still able to increase pNA formation after challenge with the inducers PAF or fMLP. The release of elastase by digitoxin in our study (Figure 8) was unlike that by digitonin (Figure 9), which indicates that digitoxin has a mechanism of action other than that of digitonin. Whether the release of elastase by digitoxin is an agonistic receptor-mediated effect or a direct cytotoxic effect is unknown.

A lysis test on erythrocytes was appended to the neutrophil assay to indicate whether an inhibitory effect of the plant extracts at a concentration of 100 μg/mL would depend on a general damage of the cells, which may affect the release of elastase from the neutrophil. At extract concentration 100 μg/mL, hemolytic activity occurred in seven of the extracts tested (Table 2).

With increased acceptance that the distribution of chemical substances and substance classes in plants is not purely random, a logical consequence is that, to efficiently explore the chemical diversity of plants, one must also explore the biological and evolutionary diversity. Thus, from a phylogenetic perspective, selecting and evaluating results from a phytochemical screening allows diversity coverage to be estimated. Furthermore, the results will be most useful if sampling is expanded in order, for example, to select groups of plants with comparably good potential for the discovery of additional active substances. Eventually, with an increasing amount of information available for a particular assay, it can also be possible to make preliminary identifications of different modes of action. This would then be indirectly derived from the plausibility of occurrences of similar compounds in more or less closely related plants. However, environmental factors (e.g., presence/absence of predators, pathogens, or nutrients) should also be considered as a possible impact for the expression of these compounds.[49]

In conclusion, this work describes a bioassay adapted to detect substances or plant extracts that affect the cascade of cellular events of the challenged human neutrophil leading to an increased or decreased activity of the downstream target enzyme elastase. The effects of approximately 100 fractionated plant extracts of diverse phylogenetic origin were evaluated in this assay, and some secondary assays were appended to add complementary information on the mechanism of action of the agents tested.

Experimental Section

Plant Materials. The plant materials were mainly collected in Uppland, Sweden, and in the Uppsala University Botanical Garden. Voucher specimens have been deposited at the Uppsala University Herbarium (UPS). The sampled plants include 46 out of 464 families from 23 of the 40 orders of angiosperms recognized in the APG system[39] and, additionally, four families of Coniferophytes and Pteridophytes.

Fractionation of Plant Extracts. The 100 fractionated plant extracts from 96 plant species were obtained according to a previously described protocol.[33] Briefly, the dried, powdered plant material (4.0 g) was extracted with 4 × 75 mL of CH_2Cl_2. The CH_2Cl_2-soluble extractives were discarded. The main extraction with 3 × 75 mL of 50% EtOH was carried out analogously. Tannins from the evaporated 50% EtOH extract were removed by filtration through acidified polyamide 6S (Riedel-de Haen, Seelze, Germany) in a column. The column was eluted with 2% HOAc, followed by 50% EtOH, and 2% HOAc. The collected eluates were combined. The tannin-free extract was injected onto a size-exclusion column packed with Sephadex G-10 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). A mobile phase of EtOH (50%)/HOAc (2%)/NaCl 0.2 M was used to elute the high molecular fraction (>700 Da), which subsequently was applied to a C_{18} Isolute SPE (solid-phase extraction) column (5 g/20 mL; Sorbent AB, Sollentuna, Sweden). The column was washed with water, followed by elution with 20% EtOH and by 50%, 80% EtOH in NH_4HCO_3 buffer (50 mM). The combined eluates were evaporated in vacuo and lyophilized to yield the fraction used in this test.

Material. The following stock solutions were used for preparing the test solutions of cytochalasin B, SAAVNA, fMLP, and PAF: Cytochalasin B (Bachem Feinchemikalien AG, Switzerland), 5 mg/mL in 20% DMSO (0.965 mg/mL); the elastase substrate SAAVNA (Bachem Feinchemikalien AG, Switzerland), 300 mM in DMSO; fMLP (Sigma), 10 mM in EtOH and phosphate buffered saline (PBS) containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$ (SVA, Uppsala, Sweden), and 2.5% bovine serum albumin (BSA); PAF (Sigma), 10 mM in EtOH and PBS containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$, and 2.5% BSA. The trypsin substrate was BAPNA (=*N*-benzoyl-DL-arginine *p*-nitroanilide) (Sigma) solution (0.01 M BAPNA in 0.05 M TRIS-HCl adjusted to pH 7.5). The PAF-antagonist BN 52021 was kindly provided by Dr. P. Braquet, Institut Henri Beaufort, Le Plessis Robinson, France.

Neutrophil Isolation. PMNs were isolated from blood of healthy volunteers. Heparinized blood samples were mixed with 10% dextran-T-500 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in 0.9% NaCl for erythrocyte sedimentation. The cells from the leukocyte upper layer were resuspended in Ca^{2+}- and Mg^{2+}-free PBS, placed on Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and centrifuged at 500*g* for 30 min at 20 °C. After removal of the supernatant, the cell pellet was treated with one volume of ice-cold sterile water for 21 s, followed by the addition of 9 volumes of Ca^{2+}- and Mg^{2+}-free PBS (SVA, Uppsala, Sweden) in order to lyse the remaining red blood cells. After centrifugation (500*g* for 10 min, 4 °C), the PMNs were resuspended at a concentration of (10−30) × 10^6 cells/mL in PBS containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$.

Elastase Release Assay. The test tubes without the sample, but with an inducer (PAF or fMLP), gave the maximum release of elastase at the assay conditions. The test tubes without the sample and the inducers represented the spontaneous release of elastase. We added 10 μL of the test solution, or 10% EtOH, to 750 μL of the incubation medium containing SAAVNA in PBS containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$, and 2.5% bovine serum albumin (BSA).

To each test tube, cytochalasin B solution (5 μL) was added, followed by 100 μL of the PMN suspension. The test tubes were incubated for 5 min at 37 °C. The reaction was started by adding 100 μL of fMLP, PAF, or IL-8. To allow a direct comparison, samples were tested in parallel for both PAF- and fMLP-induced exocytosis, using PMN from the same preparation. Also, test tubes without the addition of PAF/fMLP were run in parallel.

After incubation at 37 °C for 10 min, the reaction was stopped by adding 250 μL of 2% citric acid, and then the tubes were centrifuged at 500*g* for 10 min. The absorbance of each sample (volume 1215 μL) was recorded 405 nm using a Shimadzu UV−vis double-beam recording spectrophotometer. All samples were run in duplicate. The absorbance of the corresponding background tube (without inducer) was subtracted from that of the sample. The percentage inhibition of fMLP or PAF formation of pNA was, as a percentage, the relative decrease in absorbance compared to that of fMLP or PAF alone (100%), using the formula

$$\% \text{ Inhibition} = \{1 - [(ABS_{(S+I)} - ABS_{(S+B)})/(ABS_{(I)} - ABS_{(B)})]\} \times 100 \quad (1)$$

where, in the subscripts, S is the sample; I, the inducer; and B, the buffer.

In our experience, the effect of DMSO, EtOH, or MeOH—giving a final concentration of 0.1%—had no significant effect on the PMN (data not shown) and did not appear to compromise the integrity of the results from the assay. Also, the use of alcohol decreases the risk of nonspecific binding of compounds (in the fraction) to the walls of glass and plastic.[50]

Superoxide Production. PMNs were isolated as described above. The assay used for measurement of the superoxide production by PMNs is a slight modification of the assay described by Jones and Hancock (1994).[51] Test tubes containing 400 μL of 100 μM cytochrome C (Sigma) in PBS (Ca^{2+} and Mg^{2+}) solution were prewarmed at 37 °C in a water bath. The cell suspension (50 μL) was added to all the test tubes, and to one of each pair was added 10 μL of the superoxide dismutase

(SOD, Sigma) in PBS solution to give a final concentration of 100 μg/mL. After incubation for 5 min at 37 °C, 0.1 μM of PAF, fMLP, or the test substance was added to all the test tubes. After 20 min incubation (37 °C) in a shaking water bath, the reaction was stopped by 5 min centrifugation (500*g*, 4 °C). The amount of the cytochrome C reduction was measured by recording the absorbance at 550 nm.

Inhibition Assay of the Isolated Elastase. The PMNs were isolated as described above. The cells were diluted with PBS (having Ca^{2+} and Mg^{2+}) and 2.5% BSA to a concentration of (1.5−5) × 10^6 cells/mL. The cells were activated by the addition of cytochalasin B to a final concentration of 5 μg/mL and then incubated for 10 min at 37 °C. Addition of PAF to a final concentration of 0.1 μM initiated the release of elastase from the PMNs. After incubation (10 min), the exocytosis was stopped by centrifugation of the solution at 500*g* for 10 min at 20 °C. After decanting the supernatant, the cell pellet consisting of the PMNs was discarded. The elastase-containing supernatant (750 μL) was added to the test tubes containing 10 μL of either the test solution or 10% EtOH. The background reference samples were inactivated by adding 250 μL of citric acid (2%). The reaction was initiated with the addition of 200 μL of SAAVNA solution to a final concentration of 0.8 mM. After incubation at 37 °C for 30 min, the reaction was stopped by adding 250 μL of citric acid (2%). The test tubes were centrifuged at 500*g* for 10 min. All samples were run in duplicate. The absorbance of each sample was measured at 405 nm, and the inhibition of elastase was calculated as described above.

Inhibition Assay of Trypsin. In the method used, described by Shibata et al. (1986),[52] a trypsin-containing solution is incubated with the test solution and BAPNA. The reaction of trypsin with its substrate leads to formation of a colored product, pNA, which can be quantified by UV measurement.

Trypsin was dissolved in 0.001 M HCl and 0.02 M $CaCl_2$ to a concentration of 200 μg/mL. This trypsin-containing solution (50 μL) was added to test tubes containing 100 μL of test solution or EtOH (0.1%). After incubation at 37 °C for 10 min, 500 μL of 0.01 M BAPNA solution (0.01 M BAPNA in 0.05M TRIS-HCl adjusted to pH 7.5) was added. The test tubes were incubated at 37 °C for 10 min. After the incubation, the reaction was stopped by adding 100 μL of 30% HOAc. The test tubes were centrifuged at 500*g* for 2 min. All samples were run in duplicate, and the absorbance of each sample was measured at 405 nm. The inhibition of trypsin was calculated as described above.

Hemolytic Test. Hessinger and Lenhoff (1973)[53] describe this test. Human erythrocytes, obtained from the blood of healthy volunteers, were separated from PMNs by sedimentation in a 10% solution of Dextran T-500 in 0.9% NaCl. The mixture of erythrocytes and dextran was centrifuged for 30 min (500*g*, 20 °C); and the supernatant, discarded. The remaining erythrocytes were washed with 50 mL of Krebs-Henseleit solution and centrifuged for 10 min (500*g*, 4 °C). The erythrocyte concentration was adjusted such that the absorbance for total hemolysis, by the addition of a saponin from Quillaja bark (Sigma) (*Quillaja saponaria* Molina), was between 1.2 and 1.4 at 570 nm. To each test tube, 100 μL of the test substance in Krebs-Henseleit solution and 900 μL of the erythrocyte test solution were added. After incubation for 15 min at 37 °C, the reaction was terminated by centrifugation for 10 min at 1000*g* and 20 °C. The absorbance of the samples was compared with a sample that was without blood (as a blank), with saponin for total hemolysis, and with water for no hemolysis. The assays were run in duplicate. The fractionated plant extracts were tested at a concentration of 100 μg/mL.

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References and Notes

(1) Travis, J. *Am. J. Med.* **1988**, *84*, 37−42.
(2) Nathan, C. F.; Arrick, B. A.; Murray, H. W.; DeSantis, N. M.; Cohn, Z. A. *J. Exp. Med.* **1981**, *153*, 766−782.
(3) Weiss, S. J. *J. Biol. Chem.* **1980**, *255*, 9912−9917.
(4) Babior, B. M. *Blood* **1984**, *64*, 959−966.
(5) Clark, R. A.; Olsson, I.; Klebanoff, S. J. *J. Cell Biol.* **1976**, *70*, 719−723.
(6) Ganz, T.; Lehrer, R. I. *Curr. Opin. Immunol.* **1994**, *6*, 584−589.
(7) Chertov, O.; Yang, D.; Howard, O. M. Z.; Oppenheim, J. J. *Immunol. Rev.* **2000**, *177*, 68−78.
(8) Zanetti, M.; Gennaro, R.; Romeo, D. *FEBS Lett.* **1995**, *374*, 1−5.
(9) Schiffmann, E.; Corcoran, B. A.; Wahl, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 1059−1062.
(10) Schiffmann, E.; Showell, H. V.; Corcoran, B. A.; Ward, P. A.; Smith, E.; Becker, E. L. *J. Immunol.* **1975**, *114*, 1831−1837.
(11) Williams, L. T.; Snyderman, R.; Pike, M. C.; Lefkowitz, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1204−1208.
(12) Marascot, W. A.; Phan, S. H.; Krutzsch, H.; Showell, H. J.; Feltner, D. E.; Nairn, R.; Becker, E. L.; Ward, P. A. *J. Biol. Chem.* **1984**, *259*, 5430−5439.
(13) Nardin, E. D.; Radel, S. J.; Genco, R. J. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 84−89.
(14) Baggiolini, M.; Dewald, B. *Pharmacol. Res. Commun.* **1986**, *18*, 51−59.
(15) Dewald, B.; Baggiolini, M. *Biochim. Biophys. Acta* **1986**, *888*, 42−48.
(16) Goldman, D. W.; Goetzl, E. J. *J. Immunol.* **1982**, *129*, 1600−1604.
(17) Proost, P.; Wuyts, A.; vanDamme, J. *Int. J. Clin. Lab. Res.* **1996**, *26*, 211−223.
(18) Curfs, J. H.; Meis, J. F. G. M.; Hoogkamp-Korstanje, J. A. *Clin. Microbiol. Rev.* **1997**, *10*, 742−780.
(19) Dahlgren, C.; Karlsson, A. *J. Immunol. Methods* **1999**, *232*, 3−14.
(20) Gullberg, U.; Bengtsson, N.; Bülow, E.; Garwicz, D.; Lindmark, A.; Olsson, I. *J. Immunol. Methods* **1999**, *232*, 201−210.
(21) Bolognesi, M.; Djinovic-Carugo, K.; Ascenzi, P. *Monaldi Arch. Chest Dis.* **1994**, *49*, 144−149.
(22) Dallegri, F.; Ottonello, L. *Inflamm. Res.* **1997**, *46*, 382−391.
(23) Dewald, B.; Baggiolini, M. *Biochem. Pharmacol.* **1987**, *36*, 2505−2510.
(24) Tuominen, M.; Bohlin, L.; Rolfsen, W. *Planta Med.* **1992**, *58*, 306−310.
(25) Bieth, J.; Wermuth, C. G. *Biochem. Biophys. Res. Commun.* **1973**, *53*, 383−390.
(26) Wenzel, H. R.; Engelbrecht, S.; Reich, H.; Mondry, W.; Tschesche, H. *Hoppe Seylers Z. Physiol. Chem.* **1980**, *361*, 1413−1416.
(27) Claeson, P.; Bohlin, L. *Trends Biotechnol.* **1997**, *15*, 245−248.
(28) Geahlen, R. L.; Koonchanok, N. M.; McLaughlin, J. L.; Pratt, D. E. *J. Nat. Prod.* **1989**, *52*, 982−986.
(29) Kashiwada, Y.; Nonaka, G.; Nishioka, I.; Lee, K. J.; Bori, I.; Fukushima, Y.; Bastow, K. F.; Lee, K. H. *J. Pharm. Sci.* **1993**, *82*, 487−492.
(30) Konishi, K.; Adachi, H.; Ishigaki, N.; Kanamura, Y.; Adachi, I.; Tanaka, T.; Nishioka, I.; Nonaka, G.; Horikoshi, I. *Biol. Pharm. Bull.* **1993**, *16*, 716−718.
(31) Crown, J.; O'Leary, M. *Lancet* **2000**, *355*, 1176−1178.
(32) Xu, H. X.; Wan, M.; Dong, H.; But, P. P.; Foo, L. Y. *Biol. Pharm. Bull.* **2000**, *23*, 1072−1076.
(33) Claeson, P.; Göransson, U.; Johansson, S.; Luijendijk, T.; Bohlin, L. *J. Nat. Prod.* **1998**, *61*, 77−81.
(34) Southwick, F. S.; Stossel, T. P. *Semin. Hematol.* **1983**, *20*, 305−321.
(35) Azzi, A.; Montecucco, C.; Richter, C. *Biochem. Biophys. Res. Commun.* **1975**, *65*, 597−603.
(36) Turner, N. C.; Wood, L. J.; Foster, M.; Gueremy, T. *Eur. Respir. J.* **1994**, *7*, 934−940.
(37) Földes-Filep, E.; Braquet, P.; Filep, J. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 1412−1417.
(38) Virca, G. D.; Schnebli, H. P. *Schweiz Med. Wochenschr.* **1984**, *114*, 895−898.
(39) Bremer, K.; Chase, M. W.; Stevens, P. F.; Anderber, A. A.; Backlund, A.; Bremer, B.; Briggs, B. G.; Endress, P. K.; Fay, M. F.; Goldblatt, P.; Gustafsson, M. H. G.; Hoot, S. B.; Judd, W. S.; Källersjö, M.; Kellogg, E. A.; Kron, K. A.; Les, D. H.; Morton, C. M.; Nickrent, D. L.; Olmstead, R. G.; Price, R. A.; Quinn, C. J.; Rodman, J. E.; Rudall, P. J.; Savolainen, V.; Soltis, D. E.; Soltis, P. S.; Sytsma, K. J.; Thulin, M. *Ann. Missouri Bot. Garden* **1998**, *85*, 531−553.
(40) Soltis, P. S.; Chase, M. W.; Mort, M. E.; Albach, D. C.; Zanis, M.; Savolainen, V.; Hahn, H. W.; Hoot, S. B.; Fay, M. F.; Axtell, M.; Swensen, S. M.; Nixon, K. C.; Farris, J. S. *Bot. J. Linn. Soc.* **2000**, *133*, 381−461.
(41) Savolainen, V.; Fay, M. F.; Albach, D. C.; Backlund, A.; Bank, M.; Cameron, K. M.; Johnson, S. A.; Lledó; Pintaud, J. C.; Powell, M.; Powell, M. C.; Sheahan, M. C.; Soltis, D. E.; Soltis, P. S.; Weston, P.; Whitten, W. M.; Wurdack, K. J.; Chase, M. W. *Kew Bull.* **2000**, *55*, 257−309.
(42) Styrt, B.; Johnson, P. C.; Klempner, M. S. *Tissue Cell* **1985**, *17*, 793−800.
(43) Smolen, J. E.; Stoehr, S. J.; Boxer, L. A. *Biochim. Biophys. Acta* **1986**, *886*, 1−17.
(44) Cohen, H. J.; Chovaniec, M. E. *J. Clin. Invest.* **1978**, *61*, 1081−1087.
(45) Spisani, S.; Marangoni, C.; Traniello, S. *Biochem. Int.* **1984**, *9*, 1−8.
(46) Dewald, B.; Rindler-Ludwig, R.; Bretz, U.; Baggiolini, M. *J. Exp. Med.* **1975**, *141*, 709−723.
(47) Clark, J. M.; Vaughan, D. W.; Aiken, B. M.; Kagan, H. M. *J. Cell Biol.* **1980**, *84*, 102−119.

(48) Garcia, R. C.; Peterson, C. G. B.; Segal, A. W.; Venge, P. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 1130−1136.
(49) Agrawal, A. A.; Tuzun, S.; Bent, E. *Induced plant defenses against pathogens and herbivores*; APS Press: St. Paul, MN, 1999.
(50) Molin, L. *Acta Pharmacol. Toxicol.* **1986**, *59*, 1−62.
(51) Jones, O. T. G.; Hancock, J. T. *Methods Enzymol.* **1994**, *233*, 222−229.
(52) Shibata, H.; Hara, S.; Ikenaka, T.; Abe, J. *J. Biochem.* **1986**, *99*, 1147−1155.
(53) Hessinger, D. A.; Lenhoff, H. M. *Arch. Biochem. Biophys.* **1973**, *159*, 629−638.
(54) Mabberley, D. J. *The plant-book*; Cambridge University Press: Cambridge, 1997.
(55) Thunberg, E. *Acta Pharm. Suec.* **1983**, *20*, 115−122.

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