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High Throughput Screening on Angiogenesis Inhibitor and Promoter of Medicinal Plants using a Protein Microarray Chip

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ABSTRACT : The effects of angiogenesis inhibitor from the extract libraries of Korean and Chinese medicinal plants were investigated using a protein microarray chip. Protein chip was constructed by immobilization of integrin $\alpha_5\beta_1$ on protein chip base plates and employed for screening active extracts that inhibit the integrin-fibronectin interaction from the extract libraries. The 100 extracts of medicinal plants were obtained from extract bank of National Institute of Crop Science, RDA. The 14 extracts among 100 extract libraries were shown efficient inhibition activity for the interaction between integrin-fibronectin. The medicinal plants of 14 extracts were *Vitex negundo* var. incisa (Lam.) C.B. Clarke, *Epimedium koreanum* Nakai, *Cedrela sinensis* A. Juss, *Ipomea aquatica* Forsk, *Schisandra chinensis* Baill, *Pulsatilla koreana* Nakai, *Paeonia lactiflora* Pall. var.hortensis Makino, *Oenothera odorata*, *Allium chinense*, *Allium victorialis* var. platyphyllum MAKINO, *Polygonatum odoratum* Druce var. pluriflorum Ohwi, *Hosta lancifolia*, *Agrimonia pilosa* L. var. japonica Nakai and *Potentilla chinensis* SER. The *Paeonia lactiflora*, *Oenothera odorata*, and *Agrimonia pilosa* from these 14 extracts libraries were shown strong inhibition activity of integrin $\alpha_5\beta_1$.

Key words : Angiogenesis, Extract Bank, ProteoChip, Protein Microarray Chip

INTRODUCTION

The discovery of new drug and functional foods from medicinal plants requires the use of diverse assays to assess the various effects of medicinal plants, as well as shortening of drug discovery time and cost. Recently, genome maps of human and some plants were reported by many scientists, and these revelations of genomic data provided the essential information of specific disease and gene function in the protein level. Protein microarray allows us to analyze not only protein-protein interactions (PPI) but also protein-small molecule interactions and protein-DNA interactions, providing important functional information for newly identified genes derived from the genome project (Lueking *et al.*, 1999, Joos *et al.*, 2000, and Lee *et al.*, 2004). It has been recently reported that DNA-protein (Bulyk *et al.*, 1999), protein-protein (Uetz *et al.*, 2000), receptor-ligand (Macbeath G, 1999), enzyme-substrate interactions (Arenkov *et al.*, 2000) and transfected cells expressing different cDNAs (Ziauddin *et al.*, 2001) were analyzed by microarray-based assays (Lee *et al.*, 2004).

The angiogenesis is first named by Hertig in 1935 and this mechanism, which all tumor growth is angiogenesis-dependent, was revealed by Folkman (Folkman J, 1971). Angiogenesis is the process by which new blood capillaries are formed from pre-existing blood vessels, occurs during development and tissue regeneration, wound healing, chronic inflammatory conditions, psoriasis, metastasis, age related macular degeneration, and in diabetic retinopathy (Sridhar *et al.*, 2005). The angiogenesis mechanism of coronary artery disease, stroke, myocardiac infraction, ulcer and delayed wound healing not yet probed.

This report describes the results of our screening of selected Korean and Chinese medicinal plants from extract bank of National Institute of Crop Science (NICS) on angiogenesis and anti-angiogenesis activities.

MATERIAL AND METHODS

Plant materials

The 100 plant materials were obtained from extract bank of National Institute of Crop Science, RDA and extracted with

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Table 1. The effects on angiogenesis inhibitor and promoter against the integrin $\alpha_5\beta_1$ receptor of 100 medicinal plants

No.	Plant	Used part	Extract Temp (℃)	Fluorescence intensity
1	*Juglans sinensis* Dode	Leaf	74	0.80
2	*Solanum phoeinocarpum Nakamura et Odashima*	Fruit	50	0.88
3	*Lycium chinense Mill*	Fruit	74	1.03
4	*Kummerowia striata (Thunb.) Schindl.*	Aerial part	50	1.11
5	*Carthamus tinctorius* L.	Flower	74	0.99
6	*Saussurea lappa* CLARKE	Root	50	1.55
7	*Eupatorium forturei* Turcz.	Leaf	74	0.99
8	*Inula helenium* L.	Aerial part	50	1.28
9	*Adenocaulon hinalaicum*	Aerial part	50	1.28
10	*Chrysanthemum indicum* L.	Bud	74	1.14
11	*Siegesbeckia orientalis L var glabrescens*	Aerial part	room temp	0.92
12	*Cirsium maackii Maxim*	Aerial part	74	0.92
13	*Carthamus tinctorius L.*	Aerial part	50	0.96
14	*Galium verum var. asiaticum*	Aerial part	50	1.12
15	*Mosla dianthera* Maxim	Leaf	74	1.24
16	*Elsholtzia splendens* NAKAI	Aerial part	50	1.12
17	*Xanthium strumarium Linne*	Aerial part	74	1.12
18	*Salvia plebeia*	Aerial part	50	0.96
19	*Gastrodia elata* Blume	Root	74	0.97
20	*Pyrola japonica klenze ex* Alefeld	Leaf, stem	74	1.04
21	*Euonymus alatus* Sieb	Branch	74	0.97
22	*Machilus thunbergii*	Leaf	74	0.96
23	*Ulmus arvifolia* JACQ	Bark	74	0.96
24	*Campsis grandiflora* (Thunb.) K. Loisel	Aerial part	50	0.88
25	*Acer mono* MAX	Leaf	74	0.84
26	*Commelina communis* Linne	Aerial part	74	0.98
27	*Euphorbia lathyris* L.	Aerial part	50	0.92
28	*Sedum erythrostictum* MIQ.	Aerial part	50	0.98
29	*Acanthopanax senticosus (Rupr. Et Max.) Harmskai*	Branch	74	0.92
30	*Dioscorea butatas* Decne	Root, stem	74	0.84
31	*Polygonum cuspidatum* Sieboki et Zuarinii	Leaf	74	0.80
32	*Pleuroterus multiflorus* Turcz	Root	74	0.92
33	*Vitex negundo var. incisa* (Lam.) C.B. Clarke	Leaf	74	0.68
34	*Epimedium koreanum* Nakai	Leaf, root	74	0.74
35	*Cedrela sinensis* A. Juss	Leaf	74	0.74
36	*Ipomea aquatica Forsk*	Root	50	0.77
37	*Dryopteris crassirhizoma* Nakai	Aerial part	74	0.85
38	*Schisandra chinensis* Baill	Fruit	74	0.76
39	*Hypericum ascyron* L.	Aerial part	74	1.08
40	*Pulsatilla koreana* Nakai	Root	74	0.76
41	*Paeonia lactiflora Pall. var.hortensis* Makino	Root	74	0.62
42	*Oenothera odorata*	Seed	50	0.54
43	*Luffa cylindrica* ROEM	Fruit	50	0.84
44	*Scilla scilloides (LIND.) DRUCE*	Aerial part	50	0.95
45	*Disporum sessile* D.DON	Aerial part	50	0.80
46	*Allum chinense*	Aerial part	74	0.70
47	*Allium victorialis var. platyphyllum* MAKINO	Aerial part	50	0.69
48	*Polygonatum odoratum Druce var. pluriflorum* Ohwi	Root	74	0.77
49	*Hosta lancifolia*	Root	50	0.77
50	*Hydrangea macrophylla Ser.*	Leaf	74	1.09

Table 1. continued

No.	Plant	Used part	Extract Temp (℃)	Fluorescence intensity
51	*Coix lachryma-jobi* var. mayuen (ROMAN.) STAPF	Fruit	50	1.48
52	*Coix lachryma-jobi*	Fruit	50	1.14
53	*Elaeagnus umbellata* Thunb.	Aerial part	74	1.27
54	*Belamcanda chinensis* DC	Stem	74	1.26
55	*Achyranthes japonica* Nakai	Root	74	1.51
56	*Broussounetia kazinoki Sieb. et Zucc*	Leaf	74	1.19
57	*Cyperus rotundus* Linne	Root	74	0.94
58	*Angelica utilis*	Aerial part	50	1.06
59	*Angelica acutiloba* Kitagawa	Aerial part	50	1.15
60	*Cnidium officinale* Makino	Root	74	0.87
61	*Bupleurum falcatum* L.	Root	74	1.03
62	*Torilis japonica* D. C.	Fruit	74	1.13
63	*Ledebouriella seseloides* Wolff	Root	74	1.10
64	*Conium maculatum*	Root	50	0.88
65	*Angelica gigas* Nakai	Root	74	1.05
66	*Ostericum koreanum* Kitagawa	Root	74	0.80
67	*Sium suave WALTER.*	Aerial part	50	1.36
68	*Anethum graveolens L.*	Root	50	1.21
69	*Pimpinella brachycarpa (KOM) NAKAI*	Aerial part	50	1.29
70	*Melandryum firmun*	Aerial part	50	1.30
71	*Saponaria vaccaria* L.	Aerial part	50	1.07
72	*Dianthus chinensis Linne*	Aerial part	50	1.04
73	*Silene armeria L.*	Aerial part	50	0.88
74	*Equisetum arvense* Linne	Aerial part	74	1.03
75	*Equisetum hiemale Linne*	Aerial part	50	1.07
76	*Portulaca oleracea* Linne	Aerial part	74	1.03
77	*Armoracia lapathifolia* Gilib	Aerial part	74	1.35
78	*Hibiscus mutabilis*	Aerial part	50	1.18
79	*Chelidonium majus var. asiaticum* (Hara) Owwi	Aerial part	50	1.08
80	*Citrus aurantium* L.	Fruit	74	1.20
81	*Ruta grabeolens* L.	Aerial part	50	0.86
82	*Phellaodendron amurense Rupr.*	Bark	74	0.93
83	*Polygala tenuifolia WILLDENOW*	Root	74	1.05
84	*Ginko biloba Linne*	Fruit	74	1.02
85	*Lonicera japonica* Thunb	Flower	74	0.82
86	*Corylus heterophylla var. thunbergii Bl.*	Leaf	74	0.88
87	*Duchesnea chrysantha*	Aerial part	50	0.99
88	*Crataegus pinnatifida* Bunge	Fruit	74	0.84
89	*Agrimonia pilosa L. var. japonica Nakai*	Stem	74	0.64
90	*Potentilla chinensis SER*	Aerial part	50	0.74
91	*Lithospermum erythrorhizon* S. et Z.	Aerial part	50	0.84
92	*Symphytum officinale L.*	Aerial part	74	0.86
93	*Rhododendron fauriae Franch var. rufescwns* Nakai	Aerial part	74	0.85
94	*Adenophora triphylla var. Japonica* HARA	Root	50	0.93
95	*Codonopsis pilosula* (FR.) NANNF	Aerial part	50	1.04
96	*Cornus officinalis* Sieb et Zucc	Fruit	74	0.81
97	*Canavalia gladiata* DC.	Fruit	50	1.08
98	*Cassia occidentalis* L.	Aerial part	74	1.55
99	*Lotus corniculatus* var. japonicus	Aerial part	50	1.48
100	*Verbascum thapsus*	Root	50	1.42

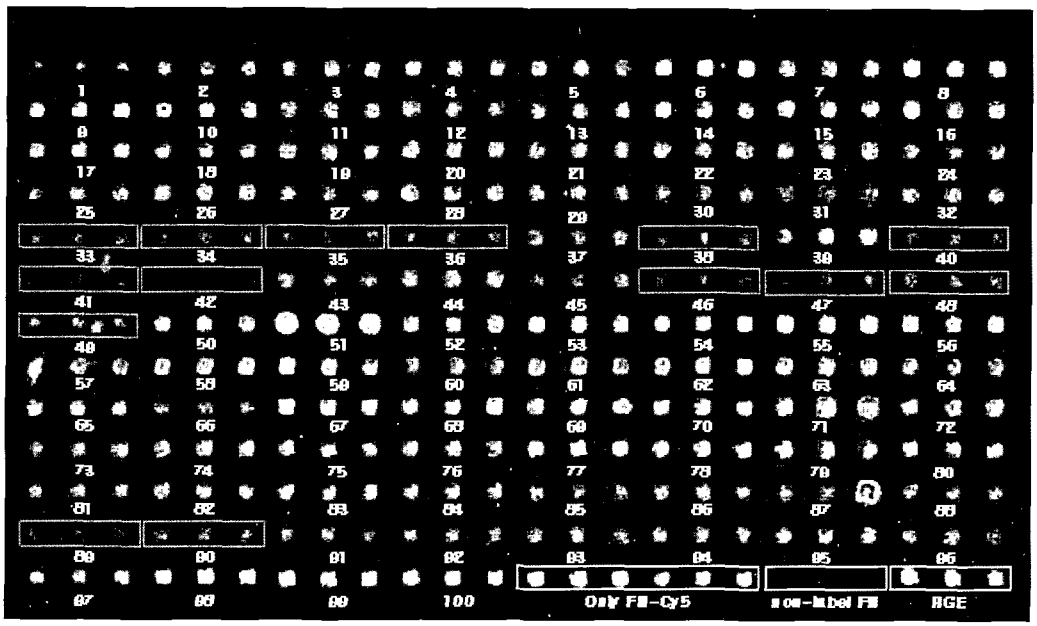

Fig. 1. The High throughput screening (HTS) of antagonistic materials against the integrin $\alpha_5\alpha_1$ receptor from the library of natural plant extracts including 100 medicinal plants by using the integrin microarray. The number means the order of medicinal plants.

99% methanol at room temp, 50 ℃ and 74 ℃. The used parts of plant materials are shown in Table 1.

Proteins and peptides

Target proteins used in this article are integrin-fibronectin systems. Integrin $\alpha_5\beta_1$, a member of integrin family and fibronectin, ECM proteins, were purchased from Chemicon International, Inc. (Temecula, CA).

Integrin microarrary printing

To fabricate protein microarrays, a high-precision contact printing robotic arrayer (CM-1000; Proteogen, Inc., Seoul, Korea) equipped with Stealth Micro-spotting Pins (SMP10; TeleChem, Sunnyvale, CA) was used for delivering nanoliter volumes of integrins onto ProLinker™-coated glass slides (ProteoChip; Proteogen, Inc., Seoul, Korea), yielding spots of about 300 to 320 ㎛ in diameter, respectively. Integrins $\alpha_5\beta_1$ (200 ㎍/mL) diluted with a phosphate-buffered saline (PBS) solution containing 10 mM β-octylthioglucopyranoside, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 20% glycerol to prevent evaporation of the nanodroplets were spotted and incubated at 37℃ for 3 h followed by washing with 0.5% PBST (PBS containing 0.5% Tween-20). The integrin microarray was stored at 4℃ until before use.

Preparation of mixture solution

Fibronectin was labeled with Cy5 fluorescent mono-reactive dyes (Amersham Pharmacia Biotech, Uppsala Sweden), according to the manufacturer's instructions (Sukhanov *et al.*, 2005). Mixture solutions for the competitive inhibition assay were prepared by mixing fibronectin (5 ㎍/mL) and natural plant extract (finally 50 ㎍/mL) diluted with a phosphate-buffered saline (PBS) solution containing 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 20% PEG to prevent evaporation of the nanodroplets. At the same time, 2.5% dimethyl sulfoxide (DMSO) was added into the mixture solution to increase the solubility of natural plant extract.

Competitive inhibition assay

Integrin microarrays were blocked with 3% BSA for 1 h and washed 3 times with 0.5% PBST. After washing with PBST, integrin microarrays were subsequently spotted with a mixture solution including each natural plant extract (finally 50 ㎍/mL) and integrin ligands, fibronectin (finally 5 ㎍/mL) labeled with a fluorescent probe at 37℃ with 80% humidity and incubated at the same condition for 1 h. Fluorescence intensities of the spots on the integrin chip were detected by a fluorescence laser scanner after washing 3 times with PBST. We used Cy5-labeled fibronectin only and RGE (GRGESP) as a positive control, and non-labeled fibronectin only as a negative control. Competitive inhibitors are determined by measuring relative fluorescence intensities between each mixture spot and control spot (Cy5 labeled fibronectin only).

Fluorescence scanning analysis

The ProteoChip arrays thus prepared for protein immobilization and protein-protein interaction were scanned in a ScanArray Lite model (GSI Lumonics, Inc., Ottawa, Canada) scanner using 532 ㎚ or 635 ㎚ lasers. The numerical values of bound proteins were determined by the fluorescence intensity of each spot on the array by software installed in the fluorescence scanner. The location of each analyte spot on the array was outlined using the gridding software ImaGene (BioDiscovery, Inc., El Segundo, CA).

$$\text{Fluorescence intensity} = \frac{\text{Fibronectin (5 } \mu g/mL) + \text{Natural Produck (5 } \mu g/mL)}{\text{Purify } \alpha_5\beta_1 \text{ (200 } \mu g/ml)}$$

RESULTS AND DISCUSSION

The results of angiogenesis inhibitor activity from the 100 extract libraries of Korean and Chinese medicinal plants using protein microarray chip were shown as Fig. 1 and Table 1. The 6 extracts, which is *Saussurea lappa* CLARKE, *Coix lachryma-jobi* var. mayuen (ROMAN.) STAPF, *Achyranthes japonica* Nakai, *Cassia occidentalis* L., *Lotus corniculatus* var. japonicus, and *Verbascum thapsus*, showed the strong anti-angiogenesis activity. On the other hand, the 14 extracts from 100 extract libraries showed the angiogenesis inhibition activity of integrin á5â1. The medicinal plants of 14 extracts were follow as; *Vitex negundo* var. incisa (Lam.) C.B. Clarke, *Epimedium koreanum* Nakai, *Cedrela sinensis* A. Juss, *Ipomea aquatica* Forsk, *Schisandra chinensis* Baill, *Pulsatilla koreana* Nakai, *Paeonia lactiflora* Pall. var.hortensis Makino, *Oenothera odorata*, *Allium chinense*, *Allium victorialis* var. platyphyllum MAKINO, *Polygonatum odoratum* Druce var. pluriflorum Ohwi, *Hosta lancifolia*, *Agrimonia pilosa* L. var. japonica Nakai and *Potentilla chinensis* SER. Among above 14 extracts libraries, *Paeonia lactiflora*, *Oenothera odorata*, and *Agrimonia pilosa* showed the strong angiogenesis inhibition activity of integrin $\alpha_5\beta_1$. Deoxypodophyllotoxin from *Pulsatilla koreana* in these 14 extracts libraries have reported as angiogenesis inhibitor (Kim *et al.*, 2002). The new drug and functional foods of anti-angiogenesis and angiogenesis will be expected from 6 candidate plants of anti-angiogenesis activity and 14 candidate plants of angiogenesis inhibitor activity.

Angiogenesis inhibitors are able to interfere with various steps of angiogenesis, on the other hand, angiogenesis promoters can stimulate angiogenesis occur basement destruction of blood vessels, proliferation and migration of endothelial cells (Wang *et al.*, 2004).

The angiogenesis inhibitors and promoters were reported from medicinal plants, woody plants, and fungi by many researchers so far. The genistein of diphenolic isoflavonoids and lignans (Fotsis *et al.*, 1993), two saponins from red ginseng, 20(*R*)- and 20(*S*)-ginsenoside Rg_3 (Mochizuki *et al.*, 1995), ginseng saponins and some related triterpenoid compounds (Shibata *et al.*, 2001), isoliquiritin from licorice root extract (Kobayashi *et al.*, 1995), shikonin from *Lithospermum erythrorhizon* (Hisa *et al.*, 1998), *Viscum album* coloratum extract (Yoon *et al.*, 1995), ether fraction of water soluble extract of *Populus nigra* leaves (Glinkowska *et al.*, 1997), soybean phytochemicals (Zhou *et al.*, 1999), *Chrysobalanus icaco* methanl extract (Alves De Paulo *et al.*, 2000), an extract of the fern *Polypodium leucotomos* (Gonzalez *et al.*, 2000), torilin from *Torilis japonica* (Kim *et al.*, 2000), *Cassia garrettiana* heartwood extract (Kimura *et al.*, 2000), *Agaricus blazei* extract (Takaku *et al.*, 2001), epigallocatechin gallate from green tea (Jung and Ellis, 2001), and resveratrol from grapes (Brakenhielm *et al.*, 2001) had anti-angiogenic activity in vitro or in vivo. But, saponin from Ginseng *Radix rubra* (Morisaki *et al.*, 1995), asiaticoside isolated from *Centella asiatica* (Shukla *et al.*, 1999), ginko-biloba extracts (Juarez *et al.*, 2000), β-sitosterol from *Aloe vera* (Choi *et al.*, 2002) enhanced angiogenesis in vivo. But the many time and costs in studies of these angiogenesis inhibitor and promoter were exhausted.

The High throughput screening by using protein microarray chip in new drug and functional foods discovery is possible to reduce the time and costs with nanoliter or nanogram level of protein and compound libraies for a spot. This report describes the results of our high throughput screening of 100 extract libraries of NICS on angiogenesis and anti-angiogenesis activities by using protein microarray chip.

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