—Reviews—

Search for Constituents with Neurotrophic Factor-Potentiating Activity from the Medicinal Plants of Paraguay and Thailand

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20 medicinal plants of Paraguay and 3 medicinal plants of Thailand were examined on nerve growth factor (NGF)-potentiating activities in PC12D cells. The trail results demonstrated that the methanol extracts of four plants, *Verbena littoralis*, *Scoparia dulcis*, *Artemisia absinthium* and *Garcinia xanthochymus*, markedly enhanced the neurite outgrowth induced by NGF from PC12D cells. Furthermore, utilizing the bioactivity-guided separation we successfully isolated 32, 4 and 5 constituents from *V. littoralis*, *S. dulcis* and *G. xanthochymus*, respectively, including nine iridoid and iridoid glucosides (**1**—**9**), two dihydrochalcone dimers (**10** and **11**), two flavonoids and three flavonoid glycosides (**12**—**16**), two sterols (**17** and **18**), ten triterpenoids (**19**—**28**), five xanthones (**29**—**33**), one naphthoquinone (**34**), one benzenepropanamide (**35**), four phenylethanoid glycosides (**36**—**39**) and two other compounds (**40** and **41**). Among which, 15 compounds (**1**—**4**, **10**—**11**, **14**—**18**, **29**—**31** and **34**) were new natural products. The results of pharmacological trails verified that littoralisone (**1**), gelsemiol (**5**), 7a-hydroxysemperoside aglucone (**6**), verbenachalcone (**10**), littorachalcone (**11**), stigmast-5-ene 3β,7α,22α-triol (**18**), ursolic acid (**19**), 3β-hydroxyurs-11-en-28,13β-olide (**24**), oleanolic acid (**25**), 2α,3β-dihydroxyolean-12-en-28-oic acid (**26**), 1,4,5,6-tetrahydroxy-7,8-di(3-methylbut-2-enyl) xanthone (**29**), 1,2,6-trihydroxy-5-methoxy-7-(3-methylbut-2-enyl) xanthone (**30**), 1,3,5,6-tetrahydroxy-4,7,8-tri(3-methyl-2-butenyl) xanthone (**31**), 12b-hydroxy-des-D-garcigerrin A (**32**), garciniaxanthone E (**33**) and (4*R*)-4,9-dihydroxy-8-methoxy-α-lapachone (**34**) elicited marked enhancement of NGF-mediated neurite outgrowth in PC12D cells. These substances may contribute to the basic study and the medicinal development for the neurodegenerative disorder.

Key words——constituent potentiating NGF's action; neurodegenerative disorder; PC12D cell; *Verbena littoralis*; *Scoparia dulcis*; *Garcinia xanthochymus*

INTRODUCTION

The use of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and glial cell line-derived neurotrophic factor (GDNF), as therapeutic agents for neurodegenerative disorders is a just approach to maintain neuronal function.[1—3] However, many of them including NGF are polypeptides of large molecule, can not cross the blood-brain barrier and are easily metabolized by peptidases when administered peripherally. A useful strategy of addressing the drug delivery problem is to administer drugs which are the low molecular substances possessing the characteristic that either enhance the action of or increase the expression of neuritogenic factors in the central nervous system.[4]

Medicinal plants are the world's oldest known health care products. Their importance is growing instead of diminishing. Not only is traditional medicine enjoying an upsurge in popularity in urban settings around the world, but medicinal herbs are increasingly the focus for phytochemical and pharmacological research and drug development. Paraguay and Thailand possess abundant natural resources of medicinal plants. However, ethnobotanical, phytochemical as well as pharmacological studies on medicinal plants of Paraguay and Thailand were reported very rare, and these medicinal plants have not been sufficiently developed and utilized as yet. In the course of our investigations of pharmacologically active substances from natural resources,[5,6] we have devoted our attention to the medicinal plants of Paraguay and Thailand possessing a NGF-potentiating activity since these are expected to be potentially useful for the medical treatment of dementia.[7] Furthermore, utilizing bioactivity-guided separation, the

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detailed studies of active constituents for these medicinal plants are of great value for generating a new type of drugs aimed at medical treatment for the serious neurological disorders, such as human immunodeficiency virus associated dementia (HAD), Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS).[8—10] This review mainly focuses on our recent findings about searching for constituents with neurotrophic factor-potentiating activity from medicinal plants.

DISCOVER NEW NATURAL RESOURCES WITH NEUROTROPHIC FACTOR-POTENTIATING SUBSTANCES

The methanol extracts of 23 medicinal plants and their fractions were examined for the effects on the NGF-mediated neurite outgrowth from PC12D cells to evaluate their NGF-potentiating activities (Table 1). In the methanol extracts, *Verbena littoralis*, *Scoparia dulcis*, *Artemisia absinthium* and *Garcinia xanthochymus* showed obviously potentiating activities of NGF-induced neurite outgrowth from PC12D cells. In the ethyl acetate fractions, *V. littoralis*, *S. dulcis*, *Sida cordifolia*, *A. absinthium* and *G. xanthochymus* markedly enhanced the neurite outgrowth from PC12D cells, and *V. littoralis* was the most powerful in the tested samples. In the water fractions, *V. littoralis*, *S. dulcis*, *S. cordifolia*, *A. absinthium* and *Bystropogon mollis* showed the weak activities. *Gochnatia polymorpha* and *Piper fulvescens* presented cytotoxicity in PC12D cells.

IRIDOID AND IRIDOID GLYCOSIDES

The crude extract of *V. littoralis* has been shown to potentiate NGF-induced neurite outgrowth from

Table 1. Effects of Extracts and Fractions on the NGF-mediated Neurite Outgrowth from PC12D Cells

Plants	Neurite-bearing cells (% of control)		
	MeOH extract	EtOAc fraction	H_2O fraction
Paraguay plants			
1. *Achyrocline alata* DC.	24.77±2.34	15.08±3.15	8.52±1.74
2. *Anthemis mixta* Linn.	16.34±1.87	14.57±1.52	10.71±2.12
3. *Artemisia absinthium* Linn.	50.53±7.44**	41.53±4.63**	51.43±3.17**
4. *Eugenia uniflora* Linn.	6.25±0.99	14.19±1.53	6.61±2.33
5. *Eucalyptus* sp.	12.87±1.52	24.27±6.32	2.13±1.40
6. *Lippia alba* (Miller) N. E. Brown	14.84±2.03	36.89±2.77**	27.64±7.10
7. *Verbena littoralis* H. B. K.	59.93±1.83**	79.98±4.65**	42.24±1.46*
8. *Aristolochia triangularis* Cham et Schleht	9.28±1.13	7.96±1.06	31.42±7.23
9. *Sida cordifolia* Linn.	32.14±1.03**	43.99±4.01**	37.57±1.54**
10. *Cassia angustifolia* Vohl.	18.01±0.76*	16.29±0.84	25.38±5.41
11. *Scoparia dulcis* Linn.	87.76±4.07**	78.89±4.07**	22.53±3.68*
12. *Cecropia adenopus* Mart.	13.93±1.24	19.54±2.53	1.92±1.13
13. *Bystropogon mollis* H. B. K.	22.68±2.95*	25.46±3.29*	34.34±5.01**
14. *Cymbopogon citratus* Stapf.	27.48±3.68**	33.79±4.84**	14.95±2.09
15. *Gochnatia polymorpha* (Less) Cab.	14.48±4.78	—	—
16. *Acanthospermum australe* O. K.	15.0±3.89	16.67±2.62	13.78±5.21
17. *Baccharis gaudichaudiana* DC.	19.47±3.54	16.43±4.02	12.34±3.78
18. *Operculina macrocarpa* (L.) Urban.	15.12±3.21	33.76±4.10	12.98±4.84
19. *Rosa blanksiae* Ait.	16.79±2.54	16.89±3.21	12.98±4.08
20. *Piper fulvescens* DC.	—	—	—
Thailand plants			
21. *Garcinia xanthochymus* Hook. f.	65.98±2.65**	73.25±4.65**	13.23±3.25
22. *Cratoxylum cochinchinense* (Lour.) Bl.	16.25±4.24	15.20±2.56	12.22±2.57
23. *Garcinia thorelii* Pierre.	14.93±3.23	15.12±1.24	14.28±2.78

All extracts and fractions of medicinal plants at the concentration of 30 μg/ml were administered. Proportion of neurite-bearing cells was expressed as a percentage against the control with NGF (30 ng/ml, 100%) in the absence of extracts and fractions. Significant differences were compared with NGF (2 ng/ml, 13.83±1.14%) in the absence of extracts and fractions. * $p<0.05$, ** $p<0.01$, $n=3$. "—" expressed that the cells were killed by the cytotoxicity of extracts and fractions.

PC12D cells. This extract was chromatographed by monitoring the potentiation of NGF's action, to give four new iridoid or iridoid glycoside compounds, littoralisone (**1**),[11] gelsemiol 6′–*trans*–caffeoyl-1-glucoside (**2**),[12] 7a-hydroxygelsemiol (**3**) and 6-methoxysemperoside (**4**),[13] together with five known compounds, gelsemiol (**5**), 7a-hydroxysemperoside aglucone (7a-OHSA, **6**),[14] gelsemiol 3-*O*-*β*-D-glucoside (**7**), 7a-hydroxysemperoside (**8**),[15] and brasoside (**9**) (Fig. 1).[11] In particular, littoralisone, which possess the NGF-potentiating activity, is a unique heptacyclic iridolactone bearing four-, five-, six-, and nine-membered rings. It was first description that the biosynthesis of the four-membered ring may be formed by the intramolecular [2+2] cycloaddition of trans-cinnamate moiety on the convex side of iridolactone. Moreover, gelsemiol and 7a-OHSA showed significant activity enhancing the NGF's action in PC12D cells. Neurite outgrowth was not caused by both the compounds in the absence of NGF. However, gelsemiol markedly enhanced an increase in the proportion of neurite-bearing cells and an extension of the neurite length in the presence of NGF. It is interesting that in the presence of NGF, 7a-OHSA markedly enhanced the elongation of the neurite length, whereas the increase in the population bearing neurites was not affected by it. The data may be interpreted in that gelsemiol enhanced NGF-signaling pathways resulting in an increase in the population bearing neurites and neurite elongation, but that 7a-OHSA preferentially potentiated the NGF-signal transduction of neurite elongation (Figs. 2 and 3). Gelsemiol and 7a-OHSA may provide useful pharmacological tools for studying the mechanism of neurotrophic action of NGF.

DIHYDROCHALCONE DIMERS, FLAVONOIDS AND FLAVONOID GLYCOSIDES

Two new dihydrochalcone dimers, verbenachalcone (**10**)[16] and littorachalcone (**11**)[17] were isolated from *V. littoralis*, together with two known flavonoid compounds, 4-hydroxywogonin (**12**) and 8,3′-dimethoxy-5,7,4′-trihydroxyflavone (**13**) (Fig. 1).[17] Verbenachalcone and littorachalcone are the first examples of the dimeric dihydrochalcone with a biphenyl ether linkage. These dihydrochalcone dimers showed strong NGF-potentiating activity (Fig. 4). Verbenachalcone has been regarded as lead compound and successfully been synthesized because of its unique chemical structure and NGF-potentiating

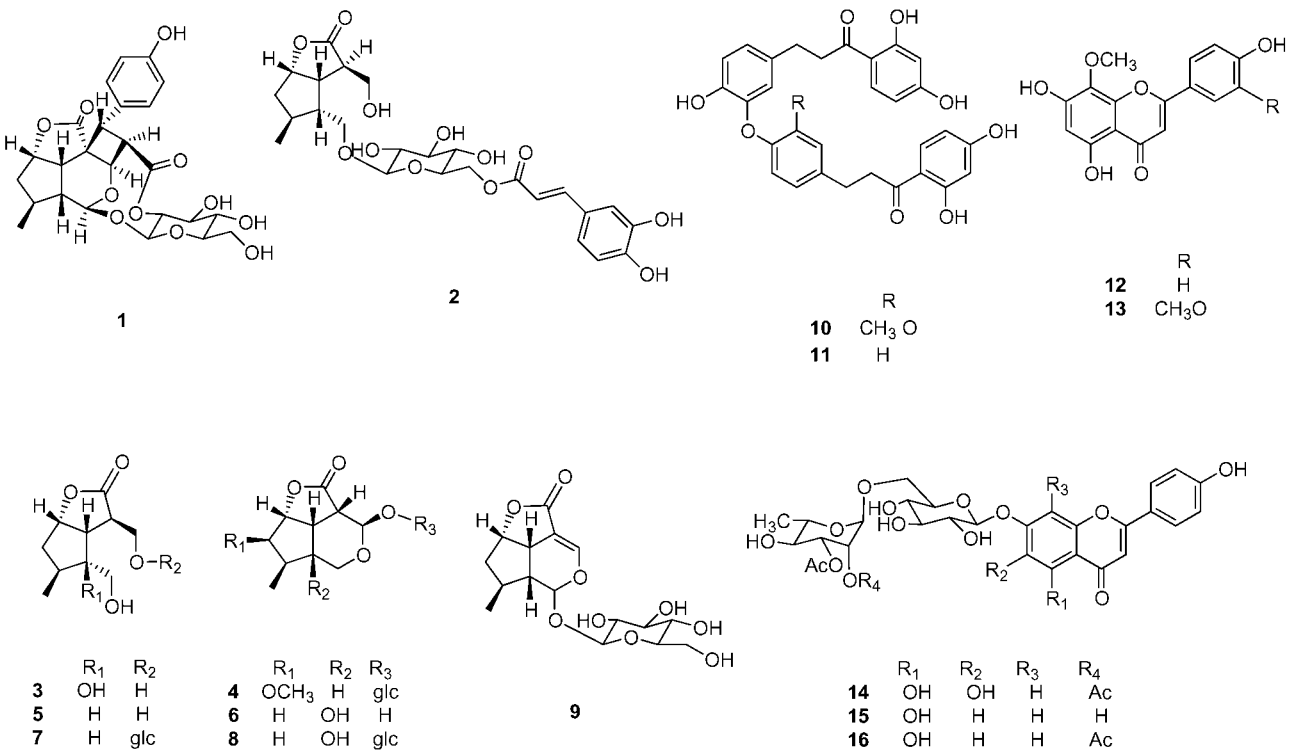

Fig. 1. The Structures of Compounds **1**—**16**

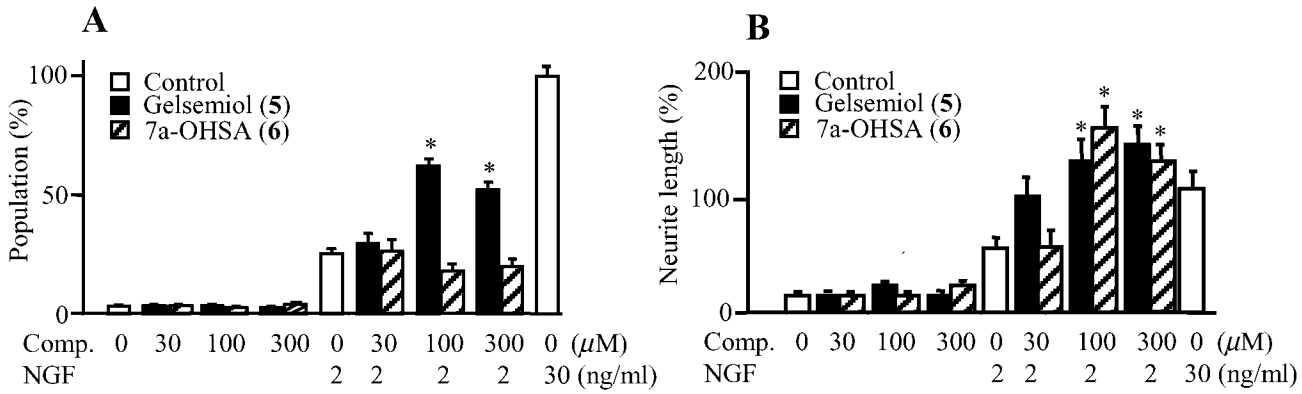

Fig. 2. Effects of Gelsemiol (**5**) and 7a-Hydroxysemperoside Aglucone (**6**, **7a**-OHSA) on the Proportion (A) and the Neurite Length (B) of Neurite-Bearing PC12D Cells in the Presence or Absence of NGF

The proportion and the neurite length of neurite-bearing cells were expressed as a percentage against the maximum response to NGF (30 ng/ml, 100%) in the absence of gelsemiol (**5**) and 7a-OHSA (**6**). Statistically significant difference from the control (2 ng/ml NGF) in the absence of compounds **5** and **6** is indicated in the figure: $^{*}p<0.01$.

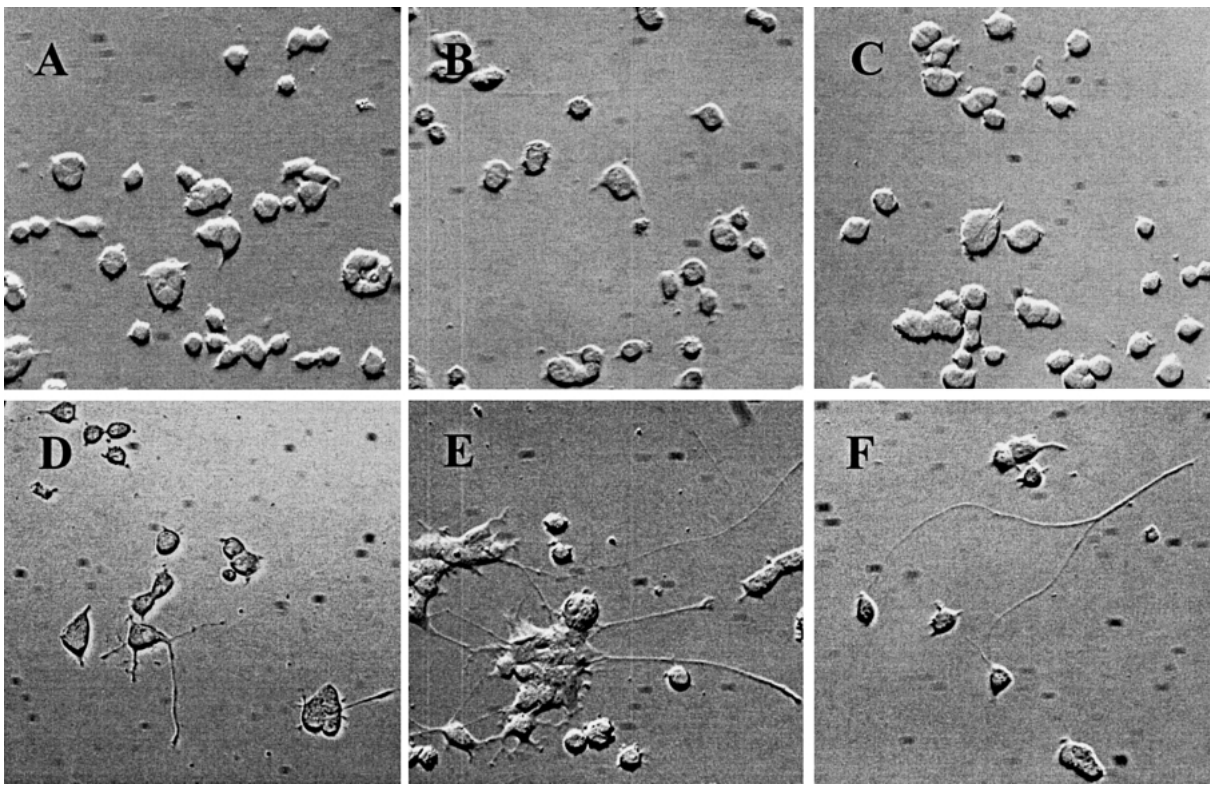

Fig. 3. Effects of Gelsemiol (**5**), 7a-OHSA (**6**) and NGF on the Morphology of PC12D Cells

The cells were treated 48 h without (A, D) or with gelsemiol (**5**, 100 μM) (B, E) or 7a-OHSA (**6**, 100 μM) (C, F) in the absence (A, B and C) or the presence (D, E and F) of NGF (2 ng/ml).

activity.[18] We have testified that PD98059 (10 and 20 μM), a potent mitogen-activated protein (MAP) kinase kinase inhibitor, blocked the verbenachalcone (40 μM)-induced enhancement of the neurites outgrowth from PC12D cells by NGF (2 ng/ml), suggesting MAPK-dependent action of verbenachalcone (Fig. 5). Western blot analysis was done in order to elucidate the site of action of verbenachalcone. Verbenachalcone increased markedly the phosphorylation of p-42/44 MAP kinase in PC12D cells in the presence or absence of NGF (Fig. 6). The detailed mechanism study that verbenachalcone enhances NGF's action is in progress.

Furthermore, three new acetylated flavonoid glyco-

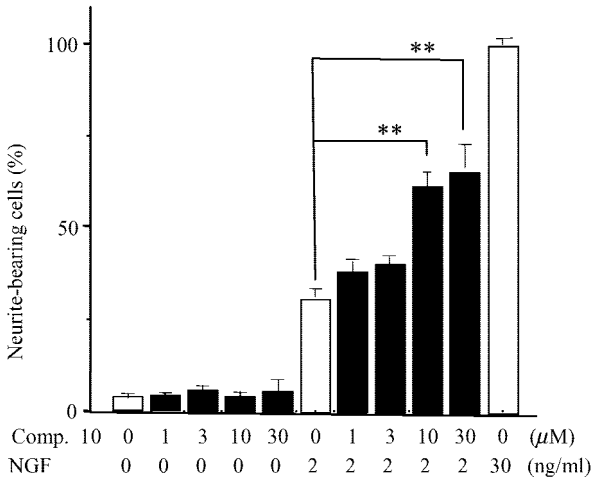

Fig. 4. Effect of Verbenachalcone (**10**) on the Proportion of Neurite-Bearing PC12D Cells in the Presence or Absence of NGF

The proportion of neurite-bearing cells is expressed as a percentage against the maximum response to NGF (30 ng/ml, 100%) in the absence of verbenachalcone. Values are mean±S.E. from four experiments. A statistically significant differences (*$p<0.05$, **$p<0.01$) from the control (NGF 2 ng/ml) in the absence of verbenachalcone was apparent.

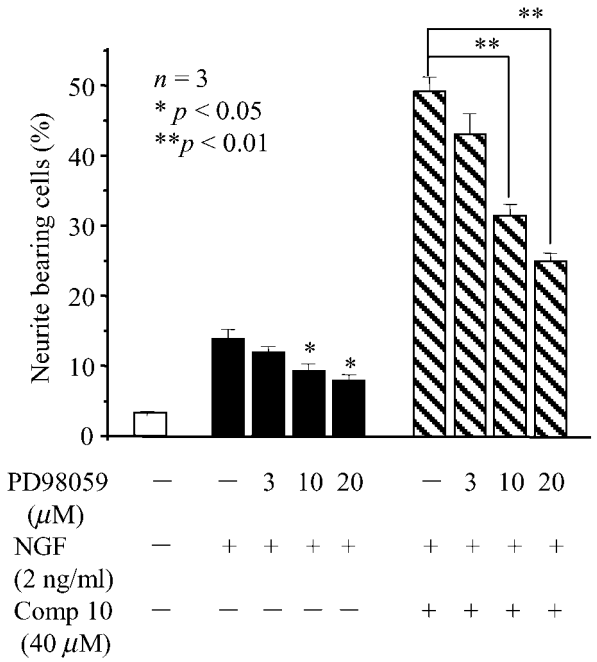

Fig. 5. Effect of PD98059 on the Verbenachalcone-Induced Enhancement of Neurite-Bearing Cells by NGF

Neurite-bearing cells are expressed as a percentage against the maximum response to NGF (30 ng/ml, 100%) in the absence of verbenachalcone. Each point represents the mean±S.E. from three experiments. Statistically significant difference from the control in the absence of PD98059 is indicated in the figure: *$p<0.05$, *$p<0.01$.

sides, 5,6,4′-trihydroxyflavone 7-*O*-α-L-2,3-di-*O*-acetyl-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**14**), apigenin 7-*O*-α-L-3-*O*-acetyl-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**15**) and apigenin 7-*O*-α-L-2,3-di-*O*-acetyl-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**16**) (Fig. 1), were isolated from *S. dulcis*. Compounds **15** and **16** showed an enhancing activity of NGF-mediated neurite outgrowth in PC12D cells.[19]

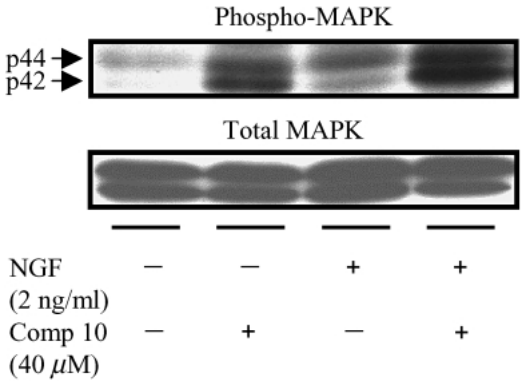

Fig. 6. Dual Phosphorylation of p-42/44 MAPK in Response to NGF and/or Verbenachalcone

p-42/44 MAPK was detected by Western blot analysis with anti-dually phosphorylated p-42/44 MAPK antibody (top) or anti-total p42/44 MAPK antibody (bottom). The cell lysate samples (16 μg of protein/lane) were separated on 12.5% SDS-PAGE gel.

STEROLS AND TRITERPENOIDS

Two new sterol compounds, stigmast-5-ene 3β,4β,7α,22α-tetraol (**17**) and stigmast-5-ene 3β,7α,22α-triol (**18**),[20] and 10 known triterpenoid compounds (**19**—**28**) (Fig. 7) were separated from *V. littoralis*. The structural difference between compounds **17** and **18** was found at C-4 position only at where **17** has a hydroxyl group but **18**, though compound **18** showed intense NGF-potentiating activities, on the contrary, **17** was inactivity. The pharmacological activity potentiating NGF's action of triterpenoid compounds **19**—**28** were evaluated (Table 2). The percentage order of neurite-bearing cells on enhancing NGF's action in PC12D cells was **25** (10 μM, 143.54±8.06%) >**19** (1 μM, 112.12±2.37%) >**24** (30 μM, 100.43±5.41%) >**20** (10 μM, 89.77±10.12%) >**25** (3 μM, 85.41±4.71%) >**26** (30 μM, 80.10±5.44%) >**19** (3 μM, 74.48±1.78%) >**24** (10 μM, 43.63±5.41%) > other derivatives. Among these triterpenoid compounds ursolic acid (**19**) and oleanolic acid (**25**) showed the most powerful activities. Study of the structure-activity relationships showed that during the ursane type triterpene compounds (**19**—**24**), ursolic acid (**19**) and 3β-hydroxyurs-11-en-28,13β-olide (**24**) exhibited significant activity and 2α,3α-di-

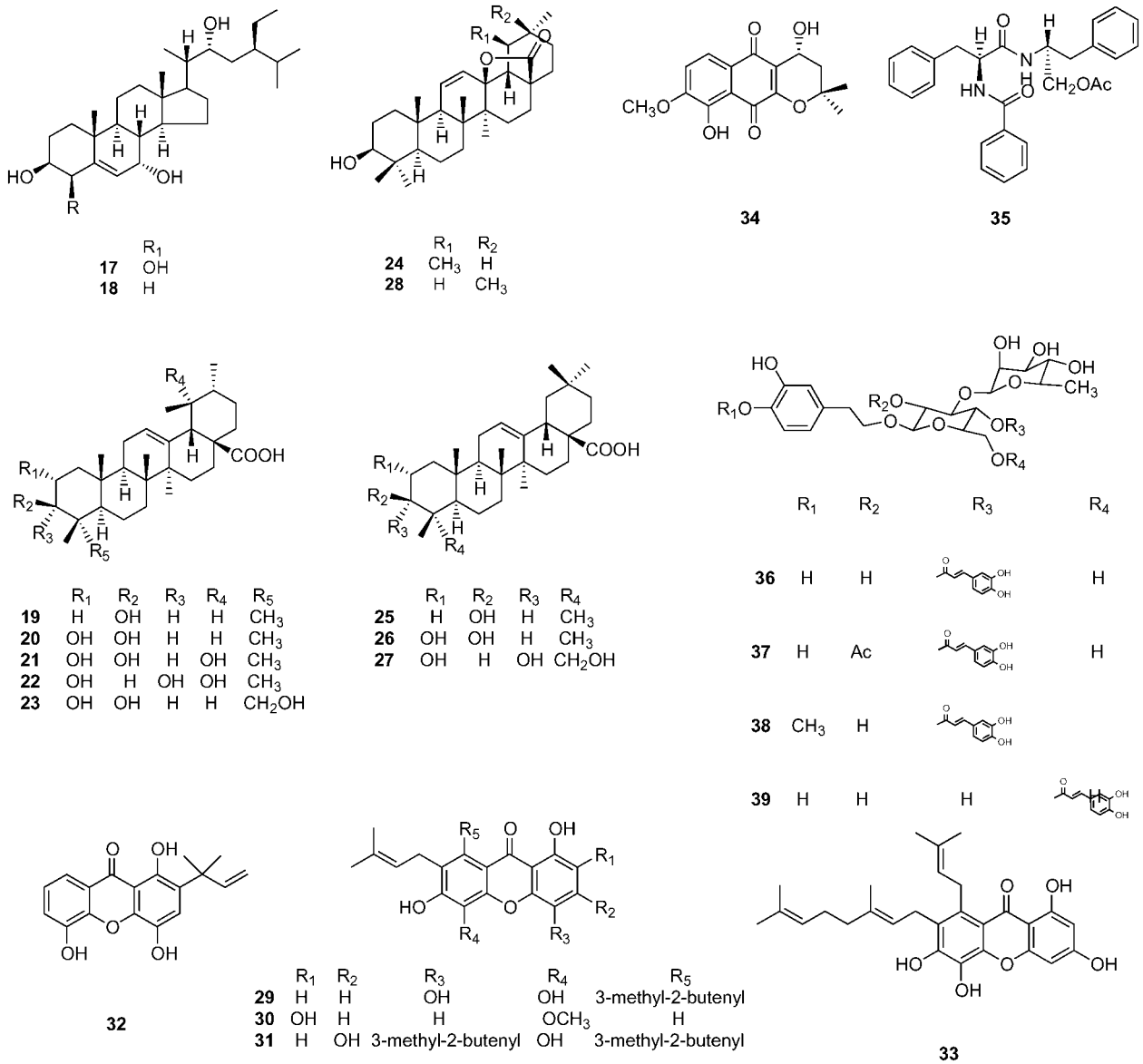

Fig. 7. The Structures of Compounds **17**—**39**

hydroxyurs-12-en-28-oic acid (**20**) was secondary, but the other compounds, which possess one hydroxyl group at C-19, C-23, or C-24 position more than ones, were not found activity for this examination. For oleanane type triterpene compounds (**26**—**28**), oleanolic acid (**25**) and 2α,3β-dihydroxyolean-12-en-28-oic acid (**26**) showed powerful activities. However, the other compounds were inactivity. These results suggest that with the increase of hydroxyl group number the activity will be weakened or lost in this bioassay system, accordingly.

PRENYLATED XANTHONES

Three new prenylated xanthones, 1,4,5,6-tetrahydroxy-7,8-di(3-methylbut-2-enyl)xanthone (**29**), 1,2,6-trihydroxy-5-methoxy-7-(3-methylbut-2-enyl) xanthone (**30**) and 1,3,5,6-tetrahydroxy-4,7,8-tri(3-methyl-2-butenyl)xanthone (**31**), were isolated from the wood of *G. xanthochymus* along with two known xanthones, 12b-hydroxy-des-D-garcigerrin A (**32**) and garciniaxanthone E (**33**) (Fig. 7).[21,22] Compound **29**—**33** showed a markedly enhancing activity of NGF-mediated neurite outgrowth in PC12D cells.

OTHER CONSTITUENTS

We also isolated (4*R*)-4,9-dihydroxy-8-methoxy-α-lapachone (**34**),[13] asperglaucide (**35**),[15] acteoside (**36**), 2′-acetylacteoside (**37**), jionoside (**38**), isover-

Table 2. Enhancing NGF's Effects on Stimulating Neurite Outgrowth in PC12D Cells with Compounds **19**—**28**

Compound	Concentration	Neurite-bearing cells (%)
NGF	2 ng/ml	14.08±0.55
	30 ng/ml	100.00±1.28
Ursolic acid (**19**)	1 μM	112.12±2.37**
	3 μM	74.48±1.78**
2α,3β-dihydroxyurs-12-en-28-oic acid (**20**)	10 μM	89.77±10.12**
	30 μM	—
2α,3β,19α-trihydroxyurs-12-en-28-oic acid (**21**)	10 μM	8.79±0.09
	30 μM	18.07±1.26
2α,3α,19α-trihydroxyurs-12-en-28-oic acid (**22**)	10 μM	19.19±1.34
	30 μM	24.69±0.33*
2α,3β,23-trihydroxyurs-12-en-28-oic acid (**23**)	10 μM	14.56±0.65
	30 μM	15.59±0.15
3β-hydroxyurs-11-en-28,13β-olide (**24**)	10 μM	43.63±4.93*
	30 μM	100.43±5.41**
Oleanolic acid (**25**)	3 μM	85.41±4.71**
	10 μM	143.54±8.06**
2α,3β-drihydroxyolean-12-en-28-oic acid (**26**)	10 μM	20.67±3.54
	30 μM	80.10±5.44**
2α,3α,23-trihydroxyolean-12-en-28-oic acid (**27**)	10 μM	7.93±0.40
	30 μM	12.56±0.26
3β-hydroxyolean-11-en-28,13β-olide (**28**)	10 μM	15.42±1.02
	30 μM	17.85±1.44

Cells were incubated in the presence of NGF (2 or 30 ng/ml) alone and in the presence of compounds **19**—**28** plus NGF (2 ng/ml) for 48 h before being fixed with 2% glutaraldehyde (37°C, 1 h). The results were reported by the relative ratio of neurite-bearing cells to NGF 30 ng/ml (100%) and expressed as a mean±S.E. ($n=12$). A statistically significant difference (* $p<0.05$ or ** $p<0.01$) from the control (NGF 2 ng/ml) in the absence of compounds **19**—**28** were apparent. The death of cells was expressed by symbol "—".

bascoside (**39**) (Fig. 7),[12] (+)-lyoniresinol 2a-*O*-β-D-glucopyranoside (**40**)[15] from *V. littoralis* and eugenyl β-D-glucopyranoside (**41**)[19] from *S. dulcis*, respectively. (4*R*)-4,9-dihydroxy-8-methoxy-α-lapachone markedly enhanced an increase in the proportion of neurite-bearing cells in the presence of NGF.

CONCLUSION

23 medicinal plants were examined on NGF-potentiating activities in PC12D cells and found that four plants, *V. littoralis*, *S. dulcis*, *A. absinthium* and *G. xanthochymus*, markedly enhanced the neurite outgrowth induced by NGF from PC12D cells. Furthermore, 15 new natural products (**1**—**4**, **10**—**11**, **14**—**18**, **29**—**33** and **34**) were isolated from *V. littoralis, S. dulcis* and *G. xanthochymus*, respectively. The results of pharmacological trails demonstrated that 16 compounds (**1**, **5**—**6**, **10**—**11**, **18**—**19**, **24**—**26**, **29**—**34**) possess the markedly pharmacological activity enhanced NGF's action. These substances are of great value for generating a new type of drugs aimed at medical treatment for the serious neurological disorders.

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