Axon- or dendrite-predominant outgrowth induced by constituents from Ashwagandha

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We previously reported that the methanol extract of Ashwagandha (roots of *Withania somnifera* Dunal) induced dendrite extension in a human neuroblastoma cell line. In this study, we found that six of the 18 compounds isolated from the methanol extract enhanced neurite outgrowth in human neuroblastoma SH-SY5Y cells. Double immunostaining was performed in rat cortical neurons using antibodies to phosphorylated NF-H as an axonal marker, and to MAP2 as a dendritic marker. In withanolide A-treated cells, the length of NF-H-positive processes was significantly increased compared with vehicle-treated cells, whereas, the length of MAP2-positive processes was increased by withanosides IV and VI. These results suggest that axons are predominantly extended by withanolide A, and dendrites by withanosides IV and VI. *NeuroReport* 13:1715–1720

Key words: Ashwagandha; Axon; Dendrite; *Withania somnifera*; Withanolide A; Withanoside IV; Withanoside VI

INTRODUCTION

Even with the tremendous advancement that medicine has achieved, dementia is still an untreatable disease. While acetylcholine esterase inhibitors are primarily used in the treatment of dementia, they only delay the progression of dementia rather than actually restore brain function. Both types of dementia, Alzheimer's disease (AD) and cerebrovascular, are caused by neuronal degeneration and atrophy. Recently, methods for inhibiting the cause of (AD) namely the accumulation of amyloid-β peptide, have been investigated. These have included γ-secretase inhibitors [1,2] and amyloid-β peptide vaccination [3,4]. Removing the cause of dementia may prevent the progression of the disease, but cannot correct any severe disfunction of the brain that has resulted from the dementia. Therefore, facilitating synaptogenesis and reconstructing neuronal networks may be a successful strategy in the ultimate therapeutics of dementia. Although it is difficult to repair neurons or restore cell numbers after neurodegeneration has occurred in the CNS, the formation of new synapses is possible through the activation of the remaining immature neurons. Synaptic formation occurs in two steps: neurite outgrowth and the maturation of axons and dendrites. Drugs activating these two steps could initiate a recovery of brain function.

Ashwagandha (roots of *Withania somnifera* Dunal), a representative drug of Ayurvedic medicine, has been used for imparting long life, youthful vigour and intellectual power [5]. Recently, Ashwagandha was reported to protect stress-induced neuronal loss in the hippocampus of rats [6] and to improve cognitive deficits induced by scopolamine in mice [7]. We previously showed that the methanol extract of Ashwagandha extended multipolar neurites in human neuroblastoma SK-N-SH cells, and that these neurites were mainly dendrites [8]. As the constituents of Ashwagandha, sitoindosides IX and X were reported to facilitate memory and learning in normal rats [9], and the mixture of sitoindosides VII-X and withaferin A is known to enhance cholinergic function and learning in ibotenic acid-treated rats [10]. However, no compound with neurite outgrowth activity has been identified. This study was conducted to isolate neurite outgrowth-promoting compounds from the methanol extract of Ashwagandha and to investigate their effect on neurite extension and neurite maturity, i.e., the differentiation to axons and dendrites.

MATERIALS AND METHODS

Isolation of compounds: Ashwagandha was purchased from Jaipur, India in 1999. A voucher specimen of Ashwagandha was deposited with TMPW number 19975 in the Museum of Materia Medica, Research Center for Ethnomedicines, Institute of Natural Medicine. The crushed roots (1.8 kg) were extracted three times with 1.5 l methanol (MeOH) under reflux for 3 h each to give 206.9 g of dry extract. A portion (186.0 g) of this extract was suspended in 1.5 l water and partitioned five times with 1.5 l chloroform ($CHCl_3$) and 1.5 l *n*-butanol (*n*-BuOH) successively to give $CHCl_3$-soluble (20.7 g) and *n*-BuOH-soluble (34.6 g) frac-

tions. A portion (15.0 g) of the $CHCl_3$-soluble fraction was suspended in ethanol (EtOH) and centrifuged. The supernatant solution was subjected to column chromatography on Sephadex LH-20. Elution with MeOH-H_2O (1:1 and 2:1), MeOH and EtOH gave five fractions: fraction I (1.21 g), II (6.52 g), III (4.30 g), IV (1.19 g) and V (0.55 g). Fraction III was subjected to column chromatography on silica gel to give β-sitosterol and daucosterol. Fraction II was subjected to column chromatography on silica gel, Sephadex LH-20, and ODS successively to give withanolide A (170.0 mg), lycium substance B (2.0 mg), (20*S*, 22*R*)-5α,27-dihydroxy-6α,7α-epoxy-1-oxowitha-2, 24-dienolide (1.5 mg), withacoagin (4.0 mg), withaferin A (9.0 mg) and withanolide D (90.0 mg). Finally, (20*S*, 22*R*)-3α, 6α-epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide (4.3 mg) was purified by HPLC eluted with MeOH/0.1% trifluoroacetic acid-H_2O (7:3). The *n*-BuOH-soluble fraction was subjected to column chromatography on a Diaion HP-20 column eluted with H_2O, MeOH-H_2O (3:7 and 3:2) and MeOH to furnish fractions VI–IX (27.20 g, 2.82 g, 4.45 g and 0.14 g).

Fractions VII and VIII were further subjected to repeated column chromatography on silica gel, Sephadex LH-20, and ODS. Withanosides VIII (5.3 mg) and IX (6.5 mg) were obtained from fraction VII after purification by HPLC. Similarly, HPLC of fraction VIII yielded (20*S*, 22*R*)-4β, 5β, 6α, 27-tetrahydroxy-1-oxowitha-2, 24-dienolide (5.1 mg), withanoside III (3.1 mg), physagulin D (7.6 mg), coagulin Q (2.0 mg) and withanosides IV (33.1 mg), V (9.8 mg) and VI (41.6 mg). The chemical structures of all compounds isolated were determined by ^{1}H-, ^{13}C-NMR and MS analyses [11].

Cell culture: A human neuroblastoma cell line, SH-SY5Y (Riken, Tsukuba, Japan), was grown in minimum essential medium (Gibco BRL, Rockville, USA) with 5% fetal bovine serum at 37°C in a humidified atmosphere of 90% air/10% CO_2.

Quantification of neurite outgrowth: SH-SY5Y cells were plated at a density of 1.4×10^4 cells/cm^2 in a 24-well culture dish (Falcon, Franklin Lakes, USA). The extract or compounds were added to the culture medium once at the start of the culture. The vehicle solution was 0.1% dimethyl sulfoxide (DMSO). Cells (100–300) were counted in four areas 650×430 μm each, and the percentage of cells with neurites > 50 μm was calculated.

Primary culture: Embryos were removed from pregnant Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) at 18 days of gestation (E18). All animals were handled according to The NIH Guide for the Care and Use of Laboratory Animals (NIH publications). The cortices were dissected and the dura mater was removed. The cells were chopped, dissociated and plated into 4-well chamber slides (Falcon) at a density of 1.3×10^5 cells/1.7 cm^2, coated with poly-D-lysine (5 μg/ml), and grown at 37°C in a humidified atmosphere with 10% CO_2. The next day, test compounds or mouse β-NGF (Austral Biologicals, San Ramon, USA) were added.

Immunocytochemistry [8]: Primary cultured cortical neurons were fixed and double-immunostained with monoclonal antibody to MAP2 (MAP2a and 2b; dilution 1:200, Chemicon, Temecula, USA) as a dendrite marker and antiserum to phosphorylated neurofilament-H (dilution 1:1000, anti-NF-H rabbit antiserum, Affiniti, Exeter, UK) as an axon marker. Alexa Fluor 488-conjugated goat anti-mouse IgG (dilution 1:100, Molecular Probes, Eugene, USA) and Cy3-labeled donkey anti-rabbit IgG (dilution 1:100, Chemicon, Temecula, USA) were used as second antibodies. The slides were mounted with Aqua Poly Mount (Polysciences, Warrington, USA), and viewed with a confocal laser scanning microscope (LSM-GB200-IMT-2, Olympus, Tokyo, Japan).

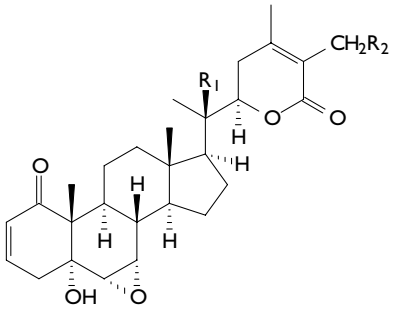
Image analysis of immunostaining: The fluorescence images were captured by a confocal laser scanning microscope. The size of one image was 600×800 μm and four images were captured per treatment. The length of parts of neurites positive for MAP2, phosphorylated NF-H, or both was measured by an image analyzer (Scion Image, Scion, Frederick, USA) for each cell, and the measurements were performed in 30 cells per treatment.

Data analysis: Statistical comparisons were made by Student's *t*-test. $p < 0.05$ was considered significant. The means of the data are presented together with s.e.m.

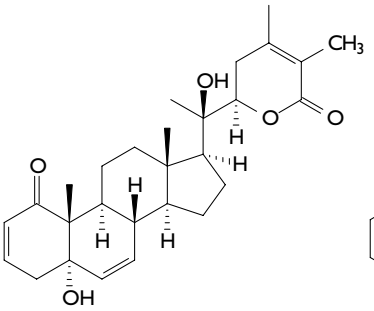
RESULTS

Compounds isolated from the methanol extract of Ashwagandha: From the $CHCl_3$-soluble fraction, we isolated seven withanolides: withanolide A, lycium substance B, (20*S*, 22*R*)-5α, 27-dihydroxy-6α, 7α-epoxy-1-oxowitha-2, 24-dienolide, withacoagin, withaferin A, withanolide D and (20*S*, 22*R*)-3α, 6α-epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide, and two kinds of sterols, β-sitosterol and daucosterol. From the *n*-BuOH-soluble fraction, we isolated nine withanolides: (20*S*, 22*R*)-4β, 5β, 6α, 27-tetrahydroxy-1-oxowitha-2, 24-dienolide, withanoside III, physagulin D, coagulin Q and withanosides IV, V, VI, VIII and IX. The chemical structures of these compounds are shown in Fig. 1. Among these 18 compounds, (20*S*, 22*R*)-3α, 6α-epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide, and withanosides VIII and IX were new compounds [11].

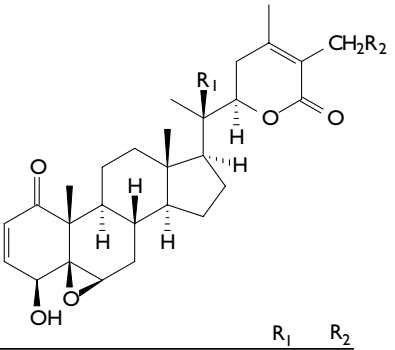
Effect of isolated compounds on neurite extension in SH-SY5Y cells: Each of these 18 compounds or the methanol extract of Ashwagandha were added to the culture medium at a concentration of either 1 μM or 5 μg/ml, respectively. Six days after the treatment, the percentages of cells with neurites were counted (Fig. 2a). Withanolide A, (20*S*, 22*R*)-3α, 6α-epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide, (20*S*, 22*R*)-4β, 5β, 6α, 27-tetrahydroxy-1-oxowitha-2, 24-dienolide, coagulin Q, withanoside IV and withanoside VI caused neurite extension. We tested the reproducibility of the effects of six compounds (Fig. 2b). Treatment with each of these compounds and the methanol extract extended neurites significantly. The other compounds which were inactive in Fig. 2a also had no effect on neurite outgrowth in other neuroblastomas, SK-N-SH and IMR-32 cells (data not shown). Withaferin A and withanolide D were found to be neurotoxic at a concentration of 1 μM.



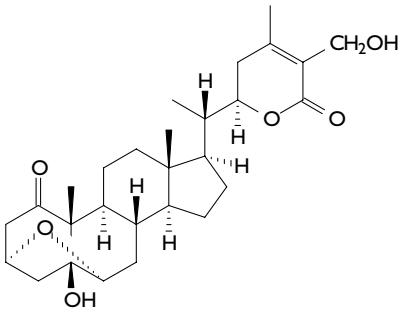
		R_1	R_2
Withanolide A	(WL-A)	OH	H
Luycium substance B	(LS-B)	H	H
(20*S*, 22*R*)-5α, 27-dihydroxy-6α,7α-epoxy-1-oxowitha-2, 24-dienolide	(27HWL)	H	OH



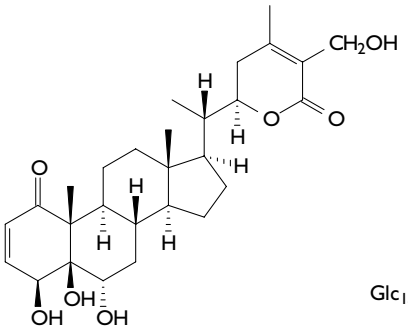
Withacoagin (WC)



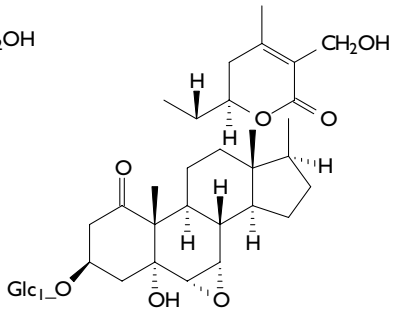
		R_1	R_2
Withaferin A	(WF-A)	H	OH
Withanolide D	(WL-D)	OH	H



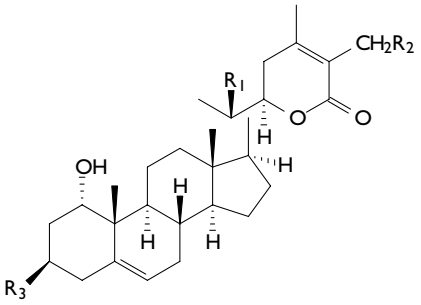
*(20*S*, 22*R*)-4β, 5β, 27-trihydroxy-3α, 6α -epoxy-1-oxowitha-24-enolide (EWL)



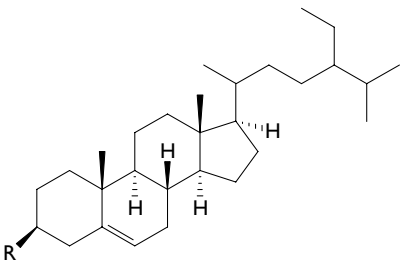
(20*S*, 22*R*)-4β, 5β, 6α, 27-tetrahydroxy-1-oxowitha-2, 24-dienolide (THWL)



Withanoside III (WS-III)



		R_1	R_2	R_3
Physagulin D	(PS-D)	H	OH	O–^{1}glc
Coagulin Q	(CO-Q)	OH	H	O–^{1}glc
Withanoside IV	(WS-IV)	H	OH	O–^{1}glc^6–^{1}glc
Withanoside V	(WS-V)	H	H	O–^{1}glc^6–^{1}glc
Withanoside VI	(WS-VI)	OH	H	O–^{1}glc^6–^{1}glc
* Withanoside VII	(WS-VII)	H	O–^{1}glc	O–^{1}glc^6–^{1}glc
* Withanoside IX	(WS-IX)	H	O–^{1}glc^6–^{1}glc	O–^{1}glc^6–^{1}glc



		R
β-Sitosterol	(SS)	OH
Daucosterol	(DS)	O–^{1}glc

Fig. 1. Structures of compounds isolated from the methanol extract of Ashwagandha. (20*S*, 22*R*)-3α, 6α-epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide, and withanosides VIII and IX were new compounds (*).

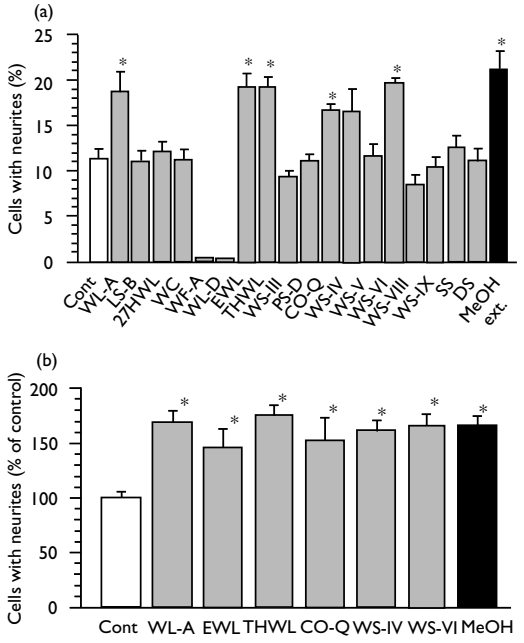


Fig. 2. Effects of compounds isolated from Ashwagandha on neurite outgrowth in SH-SY5Y cells. (**a**) Cells were treated with each of 18 compounds at a concentration of 1 μM (hatched columns) or the methanol extract of Ashwagandha at a concentration of 5 μg/ml (closed column) or vehicle (Cont, 0.1% DMSO, open column) at the start of the culture. Six days after treatment, we determined the percentages of cells with neurites > 50 μm in length. Values represent the means ± s.e.m. of four areas. $*p < 0.05$ *vs* control. (**b**) Neurite outgrowth effects of WL-A, EWL, THWL, CO-Q, WS-IV, WS-VI and the methanol extract were repeatedly tested. Concentrations were same as in (a). Values represent the means ± s.e.m. of 3–5 experiments. $*p < 0.05$ *vs* controls. Abbreviated names of compounds are shown in Fig. 1.

Of the six compounds which we obtained, (20*S*, 22*R*)-3α, 6α epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide, (20*S*, 22*R*)-4β, 5β, 6α, 27-tetrahydroxy-1-oxowitha-2, 24-dienolide and coagulin Q were present in insufficient amounts for use in consecutive experiments. Therefore, we focused on withanolide A and withanosides IV and VI.

Maturity of neurites induced by three active compounds To investigate the expression of axonal and dendritic markers in extended neurites, double-immunostaining for phosphorylated neurofilament-H (NF-H) and MAP2 was performed in E18 rat cerebral cortex neurons. The cortical neurons were treated with withanolide A, withanoside IV or withanoside VI at a concentration of 1 μM or NGF at a concentration of 100 ng/ml, and were fixed and stained 6 days after treatment (Fig. 3a), because maximum effects of each compound were shown at 1 μM and from 5 days after treatment in SH-SY5Y cells (data not shown). Immunostaining with phosphorylated NF-H antibody (axonal marker) is shown in red, whereas the green stain indicates MAP2 (dendrite marker) expression. A merged yellow color indicates the co-expression of both proteins. Withanolide A-, withanoside IV-, withanoside VI- and NGF-treated cells extended longer neurites than did vehicle-treated cells. Compared with vehicle-treated cells, withanolide A-treated cells significantly extended long neurites that stained positive for phosphorylated NF-H, whereas withanosides IV- or withanoside VI-treated cells significantly extended MAP2-positive neurites (Fig. 3b). NGF-treated cells significantly extended MAP2-positive neurites, while phosphorylated NF-H-positive neurites were not extended so much. Co-expression of NF-H and MAP2 was detected in cell bodies and proximal parts of neurites, whereas the specific expression of NF-H or MAP2 was obvious in distal ends of neurites. We considered that the differentiation into axons or dendrites was not sufficient in these co-expressed parts. Therefore, lengths of neurites expressing specifically NF-H or MAP2 were measured in Fig. 3b.

DISCUSSION

The present study demonstrated that withanolide A, (20*S*, 22*R*)-3α, 6α-epoxy-4β, 5β, 27-trihydroxy-1-oxowitha-24-enolide, (20*S*, 22*R*)-4β, 5β, 6α, 27-tetrahydroxy-1-oxowitha-2, 24-dienolide, coagulin Q, and withanosides IV and VI, which were isolated from the methanol extract of Ashwagandha, had neurite outgrowth stimulating activity in SH-SY5Y cells. The effect of the comparatively high levels of the compounds, withanolide A and withanosides IV and VI on neurite extension was investigated.

Neurite outgrowth by withanolide A, withanosides IV and VI observed in undifferentiated SH-SY5Y cells was also induced in rat cortical neurites. This suggests that those three compounds enhance neurite formation in immature neurons. Treatment with withanosides IV and VI at a concentration of 1 μM induced MAP2-positive neurites in rat cortical neurons, suggesting that withanoside IV- and VI-treated cells predominantly extend dendrites. Accordingly, these results suggested that the dendrite extension effect of the methanol extract of Ashwagandha on SK-N-SH cells [8] is due to its major compounds, withanosides IV and VI. However, withanolide A-treated cells unexpectedly extended axons. Namely, the treatment with withanolide A at 1 μM induced phosphorylated NF-H-positive neurites in rat cortical neurons. Our preliminary experiment showed that oral administration of withanolide A increased the axon extension in the mouse cerebral cortex (data not shown), suggesting that withanolide A may induce axonal formation *in vivo* as well as *in vitro*.

In the present study, we used NGF as a positive control for the neurite outgrowth effect on cortical neurons, since it has been reported that NGF induced axons and dendrites in CNS neurons [12,13], and TrkA, a high affinity NGF receptor, is expressed in the cerebral cortex [14,15]. In the present experiment, however, NGF significantly induced dendrites in cortical neurons, but not axons. Withanolide A is the unique compound that predominantly induced axons. Several NGF agonists, including neotrofin [16,17] and xaliproden [18] have been studied as anti-Alzheimer's disease drugs. In addition, several compounds have been isolated from natural products and shown to be neurite outgrowth inducers [19,20]. However, those compounds showed effects by simultaneous addition with NGF, but not without NGF. The damaged brain contains a variety of neurons that are sensitive or insensitive to NGF. Considering that with-

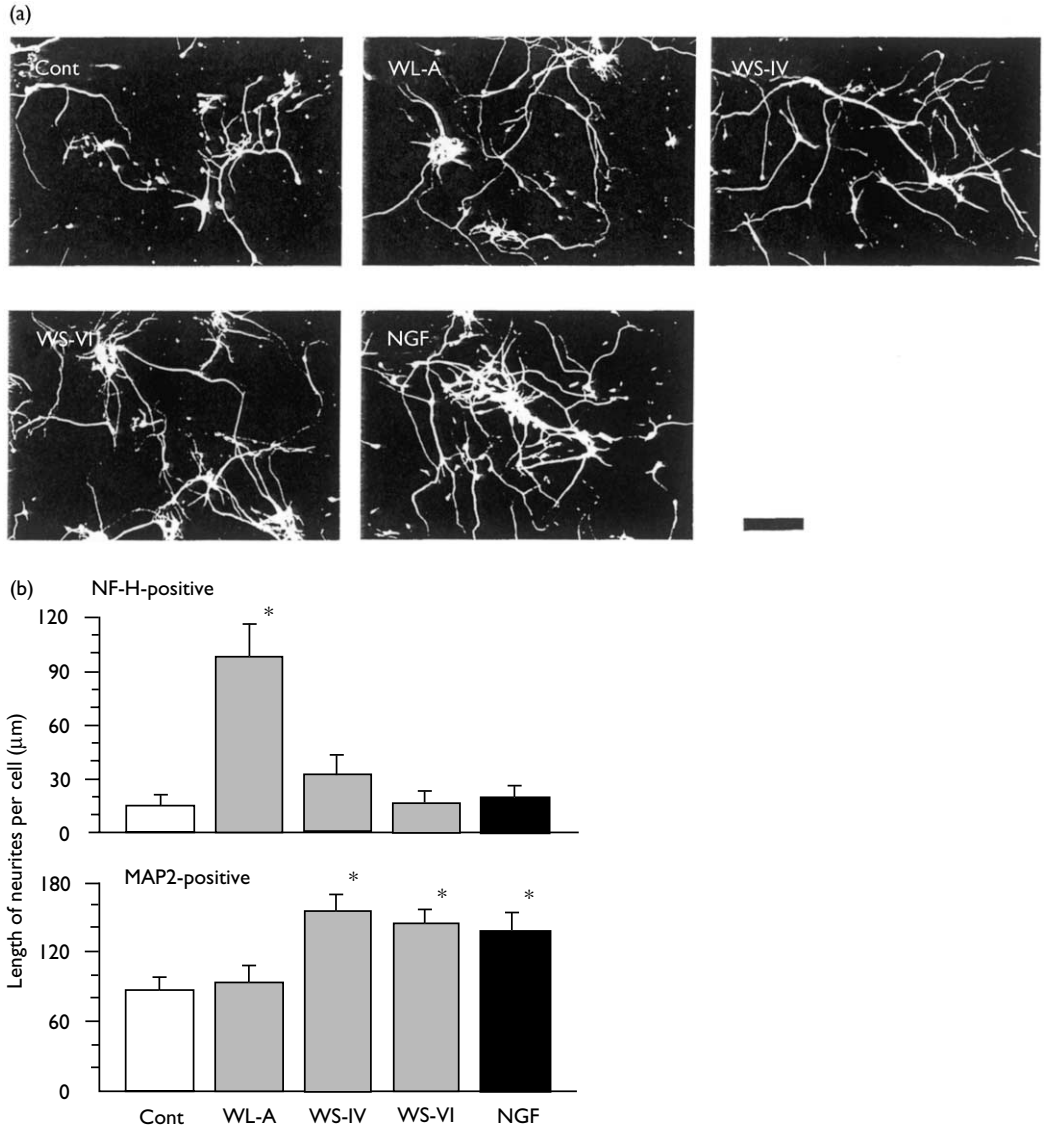


Fig. 3. Effects of three active compounds on the expression of phosphorylated NF-H and MAP2 in rat cerebral cortex neurons. (**a**) Cells were cultured for 24 h and then treated with withanolide A (WL-A), withanoside IV (WS-IV) or withanoside VI (WS-VI) at a concentration of 1 μM, NGF at a concentration of 100 ng/ml or vehicle (Cont). Six days after the treatment, the cells were fixed and double-immunostained for phosphorylated NF-H (red color) and MAP2 (green color). Co-localization of both stains appears yellow. Bar = 100 μm. (**b**) Length of phosphorylated NF-H-positive neurites (red color) and MAP2-positive neurites (green color) were measured in vehicle (Cont)-, WL-A-, WS-IV-, WS-VI- or NGF-treated neurons. Values represent the means ± s.e.m. of 30 cells. *$p < 0.05$ *vs* control.

anolide A, and withanosides IV and VI induced outgrowth of axons and dendrites, respectively without NGF addition, the formation of both axons and dendrites in a damaged brain may be efficiently controlled by a mixed cocktail of these compounds in various ratios.

As shown in Fig. 3, co-expression of NF-H and MAP2 was mostly seen in proximal parts of neurites. This may indicate that differentiation into axons or dendrites is not completed up to 7 days *in vitro*. In the early stage of differentiation, MAP2 expression was detected in not only dendrites but also proximal parts of axons [21], and the co-expression of MAP2 and phosphorylated NF-H was detected in both dendrites and axons [22]. Therefore, we should investigate the effects of three compounds on the neurite maturity in long-term cultured (> 7 days) neurons.

No clear structure–activity relationship could be found among the compounds isolated from the methanol extract of Ashwagandha. β-Sitosterol and daucosterol, which do not have a lactone ring at C20, showed no neurite outgrowth activity, while the sterol-type compounds with a lactone ring at C20 were active. It is possible that the lactone ring is one of the essential elements for this activity. Although the configuration of the A/B ring junction (*cis* or *trans*) and a substitution at C5 and C6 (α- or β-hydroxy group at C5, or a double bond between C5 and C6) could be considered factors which determine conformational differences, the

neurite outgrowth activity was not affected by these differences.

Traditionally, Ashwagandha has been considered to have no side effects. However, the methanol extract of Ashwagandha showed neurotoxicity at high doses ($> 50\,\mu g/ml$) in our experiments (data not shown). This neurotoxicity of the extract may have been caused by the neurotoxic constituents withaferin A and withanolide D at least in part. Therefore, isolated active compounds but not the extract form should be used for the purpose of facilitating neurite maturity.

We clarified that six compounds isolated from the methanol extract of Ashwagandha showed neurite outgrowth activity, and among them, withanolide A predominantly induced axonal formation, and withanosides IV and VI induced dendritic formation. In order to elucidate whether the neurites induced by these compounds contribute significantly to synaptic formation and the restoration of the neuronal network, we must now continue this investigation at both the tissue and behavioral levels.

CONCLUSION

We previously reported that the methanol extract of Ashwagandha (roots of *Withania somnifera* Dunal) induced dendrite extension in a human neuroblastoma cell line. In this study, we isolated 18 compounds from that extract and found that six compounds had neurite outgrowth activity in human neuroblastoma SH-SY5Y cells. Among them, withanolide A and withanosides IV and VI enhanced neurite formation in rat cortical neurons. Double-immunostaining indicated that withanolide A and withanosides IV and VI predominantly induced axons and dendrites, respectively.

REFERENCES

1. Moore CL, Leatherwood DD, Diehl TS *et al. J Med Chem* **43**, 3434–3442 (2000).
2. Dovey HF, John V, Anderson JP *et al. J Neurochem* **76**, 173–181 (2001).
3. Janus C, Pearson J, McLaurin J *et al. Nature* **408**, 979–982 (2000).
4. Morgan D, Diamond DM, Gottschall PE *et al. Nature* **408**, 982–985 (2000).
5. Sivarajan VV and Balachandran I. *Ayurvedic Drugs and their Plant Sources*. New York: International science publisher; 1994, pp. 65–66.
6. Jain S, Shukla SD, Sharma K *et al. Phytother Res* **15**, 544–548 (2001).
7. Dhuley JN. *Phytother Res* **15**, 524–528 (2001).
8. Tohda C, Kuboyama T and Katsuko K. *Neuroreport* **11**, 1981–1985 (2000).
9. Ghosal S, Lal J, Srivastava R *et al. Phytother Res* **3**, 201–206 (1989).
10. Bhattacharya SK, Kumar A and Ghosal S. *Phytother Res* **9**, 110–113 (1995).
11. Zhao J, Nakamura N, Hattori M *et al. Chem Pharm Bull* **50**, 760–765 (2002).
12. Studer L, Spenger C, Luthman J *et al. J Comp Neurol* **340**, 281–296 (1994).
13. McAllister AK, Lo DC and Katz LC. *Neuron* **15**, 791–803 (1995).
14. Lee T-H, Kato H, Chen S-T *et al. Stroke* **29**, 1687–1697 (1998).
15. Miller MW and Pitts AF. *Brain Res* **852**, 355–366 (2000).
16. Taylor EM, Yan R, Hauptmann N *et al. J Pharmacol Exp Ther* **293**, 813–821 (2000).
17. Di Iorio P, Virgilio A, Giuliani P *et al. Exp Neurol* **169**, 392–399 (2001).
18. Hurko O and Walsh FS. *J Neurol Sci* **180**, 21–28 (2000).
19. Nishiyama N, Cho SI, Kitagawa I *et al. Biol Pharm Bull* **17**, 509–513 (1994).
20. Li P, Matsunaga K and Ohizumi Y. *Biol Pharm Bull* **23**, 890–892 (2000).
21. Sharp DJ, Yu W and Baas PW. *J Cell Biol* **130**, 93–103 (1995).
22. Pennypacker K, Fischer I and Levitt P. *Exp Neurol* **111**, 25–35 (1991).

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