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Centella asiatica accelerates nerve regeneration upon oral administration and contains multiple active fractions increasing neurite elongation in-vitro

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Competing interests: Dr. Soumyanath is also an employee of Oregon's Wild Harvest, a company that might have a commercial interest in the results of this research. This potential conflict of interest has been reviewed and managed by the OHSU Conflict of Interest Research Committee.

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Abstract

Axonal regeneration is important for functional recovery following nerve damage. *Centella asiatica* Urban herb, also known as *Hydrocotyle asiatica* L., has been used in Ayurvedic medicine for centuries as a nerve tonic. Here, we show that *Centella asiatica* ethanolic extract (100 μg mL^{-1}) elicits a marked increase in neurite outgrowth in human SH-SY5Y cells in the presence of nerve growth factor (NGF). However, a water extract of *Centella* was ineffective at 100 μg mL^{-1}. Sub-fractions of *Centella* ethanolic extract, obtained through silica-gel chromatography, were tested (100 μg mL^{-1}) for neurite elongation in the presence of NGF. Greatest activity was found with a non-polar fraction (GKF4). Relatively polar fractions (GKF10 to GKF13) also showed activity, albeit less than GKF4. Thus, *Centella* contains more than one active component. Asiatic acid (AA), a triterpenoid compound found in *Centella* ethanolic extract and GKF4, showed marked activity at 1 μM (0.5 μg mL^{-1}). AA was not present in GKF10 to GKF13, further indicating that other active components must be present. Neurite elongation by AA was completely blocked by the extracellular-signal-regulated kinase (ERK) pathway inhibitor PD 098059 (10 μM). Male Sprague-Dawley rats given *Centella* ethanolic extract in their drinking water (300–330 mg kg^{-1} daily) demonstrated more rapid functional recovery and increased axonal regeneration (larger calibre axons and greater numbers of myelinated axons) compared with controls, indicating that the axons grew at a faster rate. Taken together, our findings indicate that components in *Centella* ethanolic extract may be useful for accelerating repair of damaged neurons.

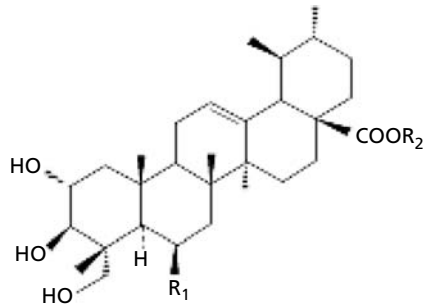
Introduction

Axonal degeneration can result from mechanical injury, exposure to a variety of occupational and environmental chemicals, neurodegenerative diseases, or from metabolic and inheritable factors. Recovery of function depends on regeneration of injured axons, which is usually a slow process. The speed of regeneration is critical since longer delays are associated with a worse prognosis for achieving full functional recovery. At present, there are no treatments available to enhance nerve regeneration and speed recovery of function in neurological injury and disease. Thus, the availability of therapies that could hasten this process would be clinically valuable. In this context, we have investigated the therapeutic potential of a botanical, *Centella asiatica* (L.) Urban herb (family Apiaceae). *Centella asiatica* (Indian pennywort) is highly regarded in Ayurvedic (traditional Indian) medicine as a rasayana, or rejuvenating herb (Brinkhaus et al 2000), reputed to increase intelligence and memory (Kapoor 1990). The herb has enjoyed growing popularity in the USA and other Western countries, where the aerial parts of the plant are sold as a herbal dietary supplement under the name Gotu kola (Newall et al 1996).

Potential neuropharmacological effects of *Centella asiatica* have been studied mainly in-vivo. *Centella asiatica* enhanced cognitive function in the rat (Veerendra Kumar & Gupta 2002) and an aqueous extract (200 mg kg^{-1} for 14 days) improved learning and memory, also reducing brain levels of malondialdehyde and increasing

levels of glutathione and catalase in the brain (Veerendra Kumar & Gupta 2002). Furthermore, aqueous extracts (100–300 mg kg^{-1} for 21 days) dose-dependently improved performance and normalized brain levels of malondialdehyde, catalase and glutathione in a rat model of Alzheimer's disease (Veerendra Kumar & Gupta 2003). The aqueous extract (100 and 300 mg kg^{-1}) decreased learning deficits in pentylenetetrazole (PTZ)-kindled rats; the higher dose also decreased seizure score in these rats (Gupta et al 2003). In addition, an antidepressant effect of a triterpene extract has been reported (Chen et al 2003) in mice using the forced swimming test. In man, a double-blind placebo controlled study in healthy subjects found that a single dose of the dried herb (12 g) reduced the acoustic startle response, suggesting that the herb may also have an anxiolytic effect (Bradwejn et al 2000). An early study in India (Appa Rao et al 1973) reported an improvement in general ability and behavioural patterns in mentally retarded children after 12 weeks of treatment with *Centella asiatica* dried herb (0.5 g). Finally, although the active components of *Centella asiatica* are unknown, asiatic acid (Figure 1), a triterpenoid ingredient, is neuroprotective in cell culture models of glutamate-induced (Lee et al 2000) and β-amyloid-induced neurotoxicity (Mook-Jung et al 1999; Jew et al 2000).

In this study, we examined the effects of *Centella asiatica* extracts on neurite elongation in-vitro and peripheral axonal regeneration in-vivo. First, we used human neuroblastoma SH-SY5Y cells to screen two types of *Centella asiatica* extract and the triterpene asiatic acid for their ability to stimulate neurite elongation in-vitro. Next, we examined the in-vivo efficacy of one of these extracts on peripheral nerve regeneration using the rat sciatic nerve crush model (Gold et al 1995; Wang et al 1997). In addition, we conducted in-vitro studies to explore the underlying mechanism involved. For these initial studies, we focused on the role of the mitogen-associated protein (MAP) kinase/extracellular-signal-regulated kinase (ERK) pathway in mediating the action of asiatic acid for three reasons. First, *Centella asiatica* contains several flavonoids and this class of compounds has recently been shown to activate ERK (Prouillet et al 2004). Second, asiatic acid is known to be present in *Centella asiatica* and this individual compound has been studied previously (see above). Third, our previous studies with FK506 (tacrolimus) have shown that its neurotrophic activity (for reviews, see Gold 1999, 2000a, b; Gold & Villafranca 2003) is dependent upon ERK activation (Gold & Zhong 2004). A preliminary report of some of this work has been presented (Soumyanath et al 2004).



	R_1	R_2
Asiatic acid	H	H
Asiaticoside	H	rhamnosyl-glucosyl-glucosyl
Madecassic acid	OH	H
Madecassoside	OH	rhamnosyl-glucosyl-glucosyl

Figure 1 Chief triterpenoid aglycones and saponin glycosides of *Centella asiatica*.

Materials and Methods

Preparation and analysis of botanical material

Extraction of Centella asiatica

Dried *Centella asiatica* was purchased in cut and sift form (Batch no. GOT-10072C-OGA; Oregon's Wild Harvest, Sandy, OR, USA). Its identity was verified by comparison of its thin-layer chromatography (TLC) profile with that previously reported (Wagner & Bladt 1996). Due to size limitations of the extraction equipment, five separate portions of the *Centella asiatica* herb were extracted in a similar fashion with ethanol and the extracts sequentially labelled GK1 to GK5 (GK extracts 1–5). For GK2, for example, *Centella asiatica* (242.7 g) was extracted by refluxing with ethanol (2 L) for 1 h. The initial ethanol extract was drained off, replaced with fresh ethanol (1 L) and refluxed for an additional 1 h. The two extracts were filtered, combined and evaporated to dryness on a rotary film evaporator to yield a dark green residue (9.93 g) designated GK2. All extracts (GK1 to GK5) showed essentially identical TLC profiles; GK1 was used in a preliminary study and only the results from GK2 to GK5 are reported here. A water extract was prepared by refluxing *Centella asiatica* (120 g) with water (1.5 L) for 2 h. The extract was filtered and freeze-dried to yield a residue (11.5 g), designated GKW1 (GK water extract 1).

Fractionation of Centella asiatica *GK2 extract*

GK2 was further separated into fractions using the technique of vacuum liquid chromatography (VLC) on silica gel (Houghton & Raman 1998) using silica gel 60 (Kieselgel 60; particle size 0.040–0.063 mm, 230–400 mesh). GK2 (4.05 g) mixed with a small amount of silica was layered over a silica bed (6.5 cm height × 9 cm diameter) prepared in a sintered glass funnel and overlaid with a thin (2 mm) layer of fresh silica. The column was eluted with a series of solvents of increasing polarity (Table 1). The fractions were collected separately and evaporated down using a rotary evaporator followed by a Savant Speedvac centrifugal evaporator to yield eleven fractions labelled as GK fraction 3 (GKF3) to GKF13 (Table 1).

Chromatographic analysis of Centella asiatica *extract*

TLC analysis of extracts and fractions was performed according to the literature (Wagner & Bladt 1996). High-performance liquid chromatography (HPLC) analysis was adapted from the literature (Inamdar et al 1996) and used an

Table 1 Preparation and neurite elongation stimulatory activity of fractions of *Centella asiatica* ethanolic extract

Hexane (mL)	Chloroform (mL)	Methanol (mL)	Acetone (mL)	Fraction no.	Weight (g)	Neurite length (μm)[#]
300	—	—	—	GKF3	0.28	134 ± 5*,†
150	150	—	—			
60	240	—	—	GKF4	0.07	202 ± 7*,†
—	300	—	—	GKF5	0.28	NV
—	270	30	—	GKF6	0.74	NV
—	240	60	—	GKF7	0.77	NV
—	210	90	—	GKF8	0.72	NV
—	180	120	—	GKF9	0.17	NV
—	150	150	—	GKF10	0.52	158 ± 6*
—	120	180	—	GKF11	0.36	152 ± 6*
—	90	210	—	GKF12	0.24	150 ± 7*
—	—	—	300	GKF13	0.12	168 ± 6*

Values for neurite length are means $\pm$ s.e.m. [#]Values for comparison: no treatment, 59 ± 1; NGF treated, 115 ± 4; GK2, 187 ± 7; FK506, 154 ± 5. *$P < 0.05$, compared with NGF; †$P < 0.05$, compared with FK506. NV, no viable cells found.

Econosil 5 μ C18 column (250 mm $\times$ 4.6 mm) with gradient elution (20% acetonitrile in water containing 0.1% acetic acid to 55% acetonitrile in water containing 0.1% acetic acid in 30 min followed by a linear increase to 90% acetonitrile in water containing 0.1% acetic acid in 15 min, followed by a return to start conditions in 10 min, and a 5-min equilibration period). The detection wavelength was 205 nm.

In-vitro studies on neurite elongation using SH-SY5Y human neuroblastoma cells

Cell culture

SH-SY5Y human neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM F12; GIBCO) supplemented with 20% fetal calf serum (FCS) (Hyclone), 50 IU mL^{-1} of penicillin and 50 mg mL^{-1} streptomycin (GIBCO) at 37°C with 5% CO_2. Cells were plated in six-well plates (Falcon) at 15 000 cells/well and treated with 0.4 μM aphidicolin (DNA polymerase inhibitor; Sigma). At 5 days, cells were washed and treated with nerve growth factor (NGF; 10 ng mL^{-1}) to induce neurite elongation, in the presence or absence of experimental test agents: *Centella asiatica* extracts or fractions (10–100 μg mL^{-1}), or asiatic acid (1 μM). To test the involvement of the MAP kinase/ERK pathway, the selective inhibitor PD 098059 (0.1–10 μM) (Gold & Zhong 2004; Liot et al 2004) was added 48 h after NGF, a protocol that we have previously reported does not inhibit NGF-induced neurite elongation (Gold & Zhong 2004). Media were changed at 48 h (replaced with fresh media with or without the compounds), and again at 96 h, and kept for an additional 72 h (total time, 168 h). Cells (15–20 per well) were examined daily and randomly photographed at 96 and 168 h.

Neurite elongation measurements

For analysis of neurite length, cells from duplicate wells (20 fields per well) were randomly photographed by an investigator blinded to the treatment groups (S. A. G.) at 96 and 168 h using a digital camera (Q-imaging Micropublisher 5.0) attached to a trinocular microscope (Nikon Diaphot). Neurite lengths were measured on computer-stored images (final magnification $\times$360) using a SummaSketch III (Summa graphics) digitizing tablet and BIOQUANT Classic 95 software (R&M Biometrics, Nashville, TN). Only those neurites greater than twice the cell body length were measured. Mean values at each time point were compared separately using a one-way analysis of variance and all subsequent pair-wise comparisons were performed using Tukey's test.

In-vivo evaluation of nerve regeneration using a sciatic bilateral nerve crush rat model

Animal surgery and drug administration

All studies were conducted according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Six-week old male Sprague-Dawley rats were used in these studies. Rats were anaesthetized with 2.5% halothane, the sciatic nerves exposed bilaterally and the nerve crushed twice (for a total of 30 s using a no. 7 Dumont jeweller's forceps) at the level of the hip. Rats were given either vehicle (water; n = 6) or *Centella asiatica* extract (GK3–GK5; n = 7) in two separate studies; extracts GK3–GK5 were prepared by an identical method to GK2 used in the in-vitro studies). The dried extract was dissolved in the rats' drinking water at a concentration of 2 mg mL^{-1}. Based upon the amount of water consumed, the average dose for each rat was calculated to be 300–330 mg kg^{-1} daily over the 18 days of study. Behavioural function and morphological measures were used to assess functional recovery and the rats were perfused with 5% glutaraldehyde at day 18 for histological examination. The experiment was conducted twice. As both generated similar results, only the data from one of the studies (GK5) are presented here.

Behavioural function assessment

A semi-quantitative scale was used for daily evaluation (for 18 days) by a blinded investigator (B. G. G.) of functional recovery: 0 = complete flaccid paralysis with the foot turned out upon walking and the toes curved; 1 = ability to right the foot and move the toes; 2 = ability to constantly walk on the foot; 3 = demonstrates toe-spread during walking; 4 = walks off heel and shows near normal toe-spread. Rats demonstrating intermediate ability were given partial scores (i.e., + = 0.25; ++ = 0.5; +++ = 0.75). The number of days following nerve crush until the rat demonstrated onset of an ability to right its foot and move its toes (termed onset; equivalent to 1, above), and the number of days until the rat demonstrated an ability to walk on its hind feet and toes (termed walking; equivalent to 2, above) were calculated for each rat.

For a more objective measure of functional recovery, toe-spread distances were measured. At day 18, toe-spread during walking was measured by marking the hind feet with tempera paint and allowing the rats to freely walk across a sheet of paper on an 8-cm wide and 60-cm long walled plank. Toe-spread was measured as the distance between the first and fifth digits, to the nearest 0.5 mm; three foot prints were analysed from each foot (Wang et al 1997).

Morphology

At 18 days after nerve crush, the rats were deeply anaesthetized with 4% halothane, heparinized and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 s followed by 1 L of 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and fixed at 4°C for 24 h. The following tissues were sampled: sciatic, peroneal, sural and tibial nerves at known (5 mm) distances from the crush site; tibial branches supplying the medial gastrocnemius and soleus muscles. Semi-thin sections (0.5 μm) were stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate, mounted on film-supported 75 mesh grids, and examined in a JOEL 100× electron microscope. Brains and spinal cords were sampled for pathological examination by light microscopy.

Morphometry

For quantitation of the numbers of myelinated axons and axonal calibres, cross-sections of the entire soleus nerve were photographed and printed at a final magnification of ×1350. The numbers of myelinated axons were counted and the area of the nerve determined by tracing the perineurium using a SummaSketch III (Summa graphics) digitizing tablet and BIOQUANT Classic 95 software (R&M Biometrics, Nashville, TN). From these data, the numbers of myelinated axons per 5000 μm^2 were calculated. Axonal areas of both myelinated and unmyelinated fibres were determined by tracing the axolemma using a SummaSketch III (Summa graphics) digitizing tablet and BIOQUANT Classic 95 software (R&M Biometrics, Nashville, TN). Histograms were constructed from these data and mean values were calculated.

Statistical analyses

For the neurite length data, the values from duplicate wells were not significantly different from each other and the values for each treatment group were combined. As the values were normally distributed, mean values were compared using a one-way analysis of variance followed by Newman–Keuls multiple comparison test for comparison of individual values (WINKS 4.62; TexaSoft, Cedar Hill, TX). For the behavioural function data, the values were compared using a one-way analysis of variance followed by an unequal variance *t*-test for comparison of individual values (WINKS 4.62; TexaSoft, Cedar Hill, TX). For the data on the number of days to onset and walking, toe-spread distances and axonal areas, mean values were compared using an unequal variance *t*-test (WINKS 4.62 professional edition). The behavioural functional recovery data and the axonal area histograms were also compared by calculating a Mann-Whitney *U*-statistic, which confirmed the results of the above tests that are reported here. Values are mean ± s.e.m.

Results

Neurite elongation

Centella asiatica ethanolic extracts GK1 (not shown) and GK2 (Figures 2 and 3) (100 $\mu g\,mL^{-1}$) elicited a marked increase in neurite elongation in the presence of NGF, which was significantly ($P < 0.05$) greater than FK506 (Figure 3). Like FK506, there was no activity in the absence of NGF (data not shown).

Each of the fractions derived from GK2 was also tested (100 $\mu g\,mL^{-1}$) for neurite elongation in the presence of NGF (Table 1). GKF3, GKF4 and GKF10–GKF13 all significantly increased neurite outgrowth compared with NGF alone, with the greatest activity occurring with GKF4. GKF5–GKF9, which all contained chlorophyll, were toxic to the SH-SY5Y neuroblastoma cells. The elution solvent for GKF4 (Table 1) and its TLC profile (data not shown) indicate that the compounds within it are non-polar. Consistent with the activity being attributable to non-polar compounds, a water-soluble extract (GKW1) was ineffective, being in fact inhibitory at 100 $\mu g\,mL^{-1}$ (Figure 3).

Asiatic acid, a triterpenoid compound found in *Centella asiatica* and a trace component of the GKF4 fraction, as determined by HPLC (Figure 4), also showed marked activity (Figures 2 and 3) at a concentration of 1 μM (0.5 $\mu g\,mL^{-1}$), albeit not as great as whole *Centella asiatica* extract at 100 $\mu g\,mL^{-1}$ (Figure 3). Furthermore, the MAP kinase kinase (MEK) inhibitor PD 098059, which inhibits MEK directly upstream of ERK in the MAP kinase pathway (Kaplan & Miller 2000), completely prevented the neurite-elongation activity of asiatic acid (Figures 2 and 3), without inhibiting NGF's activity (see Gold & Zhong 2004), revealing the ERK-dependency for this activity.

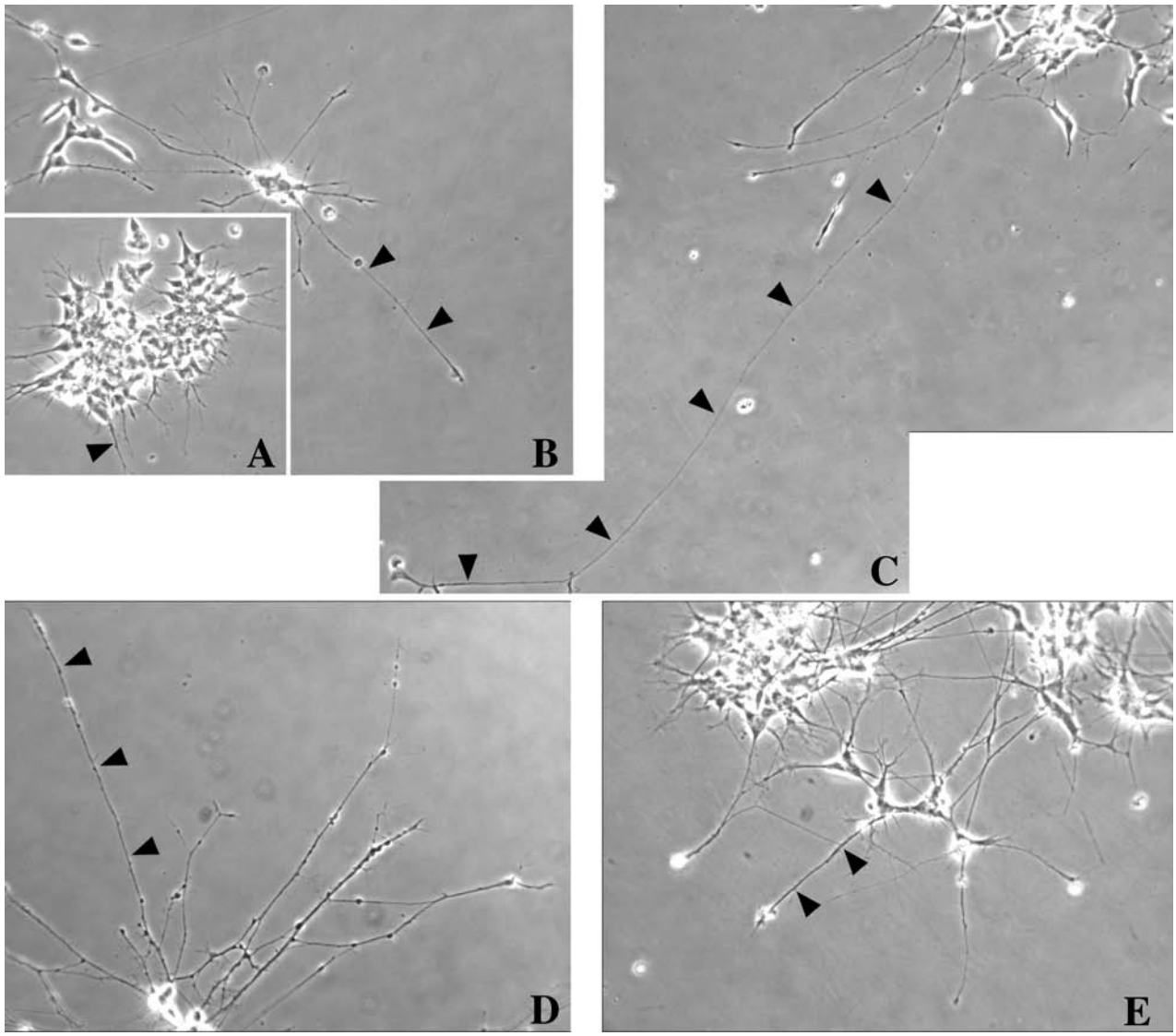


Figure 2 Representative micrographs of SH-SY5Y cells shown at 168 h after no treatment (A), treatment with NGF only (B), or treatment with NGF plus *Centella asiatica* ethanol extract GK2 (C), asiatic acid (D) or asiatic acid + PD 098059 (E). Undifferentiated cells exhibit only short processes (A), whereas those differentiated with NGF demonstrate process elongation (B). Elongation is markedly increased by *Centella asiatica* ethanol extract (C) and asiatic acid (D). Note that the activity of asiatic acid is inhibited by the MEK inhibitor PD 098059 (E). The number of arrowheads is indicative of process length. Magnification ×120.

Nerve regeneration

In-vivo functional recovery was observed earlier and progressed more rapidly in the GK5-treated rats ($n = 4$) compared with controls ($n = 3$) (Figure 5); based upon water consumption, the average dose of GK5 for each rat was calculated to be 330 mg kg^{-1} daily over the 18 days of study. The number of days until onset (level 1) and walking (level 2) were significantly ($P < 0.05$) reduced from 14.8 ± 0.17 and 17.3 ± 0.33, respectively, in control rats to 7.8 ± 0.32 and 10.8 ± 0.48, respectively, in GK5-treated rats. Footprints obtained at 18 days following nerve crush demonstrated a more normal appearance (greater toe-spread and less of a heel imprint) compared with vehicle-treated rats (not shown). The distance between the first and fifth digits was significantly ($P < 0.05$) larger in the GK5-treated rats compared with controls (14 ± 0.4 vs 10 ± 0.7 mm, respectively), being closer to toe-spread distances in uninjured, normal rats (18–19 mm).

Morphological examination was conducted at 18 days following axotomy. Regenerated axons in the soleus nerve from GK5-treated rats were larger in size and demonstrated more and thicker myelin sheaths compared with controls (Figure 6). Quantitation of the numbers of myelinated axons in the soleus nerve demonstrated a 675% increase in GK5-treated compared with water-treated rats. The distribution of axonal areas for all axons (myelinated and non-myelinated) showed a significant ($P < 0.05$; Mann–Whitney U-test) shift to the right for

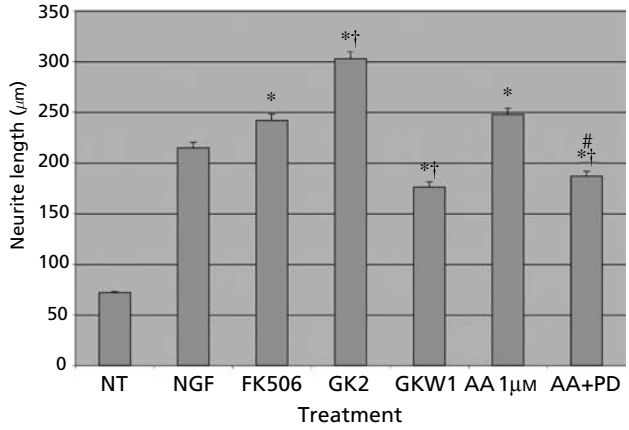


Figure 3 Mean values for neurite lengths obtained at 168 h from SH-SY5Y cells in the presence of NGF, except no treatment (NT). Note that asiatic acid (AA, 1 μM) demonstrates significant activity, which is prevented by the MEK inhibitor PD 098059, demonstrating the involvement of the ERK pathway. In contrast, a water-soluble extract (GKW1) exhibits no positive activity, being significantly inhibitory of NGF's activity. $^{*}P < 0.05$ compared with NGF; $^{\dagger}P < 0.05$ compared with FK506; $^{\#}P < 0.05$ compared with asiatic acid.

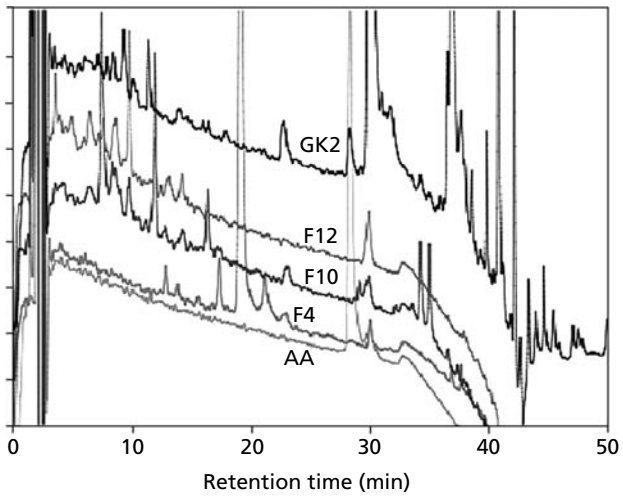


Figure 4 Reversed-phase HPLC analysis of *Centella asiatica* ethanolic extract (GK2), asiatic acid (AA) and VLC fractions GKF4, GKF10 and GKF12, all of which showed stimulation of neurite elongation in SH-SY5Y cells (see Table 1). Asiatic acid is present in GK2, and in trace amounts in GKF4 but not detectable in GKF10 or GKF12.

the GK5-treated compared with water-treated rats (data not shown), indicating the presence of larger-sized axons. The mean axonal area for all axons was 0.9 ± 0.30 (n = 3; number of axons = 350) from water-treated rats and 3.3 ± 1.50 (n = 4; number of axons = 626) from GK5-treated rats, representing a 251% increase over control values. Thus, regenerating axons in the GK5-treated rats were more advanced in their maturation, indicating that the axons arrived in the distal portion of the sciatic nerve at

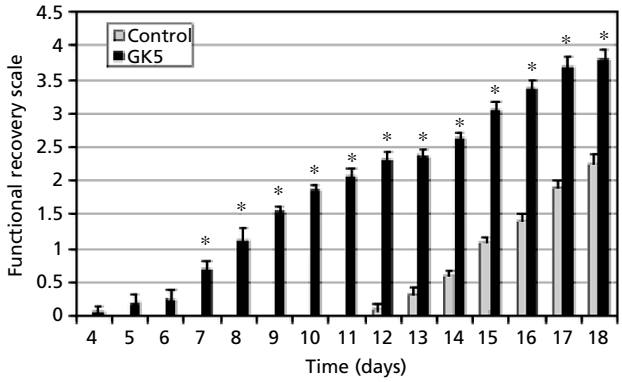


Figure 5 Oral administration of GK accelerates functional recovery in the rat sciatic nerve. Bar graphs showing mean functional recovery scores from rats given water only (n = 3) and GK5-treated (330 mg kg^{-1} daily; n = 4) rats. $^{*}P < 0.05$ GK5-treated rats compared with controls.

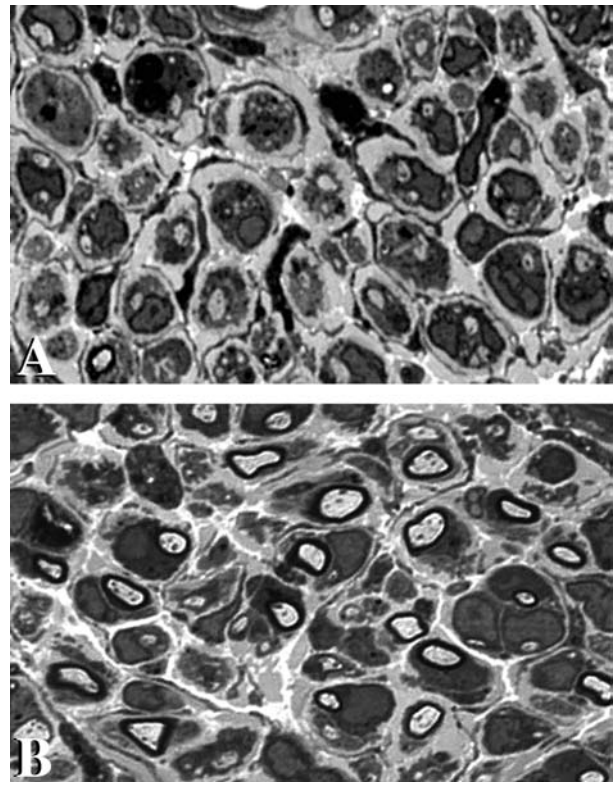


Figure 6 Light micrographs of soleus nerves from a water-treated rat (A) and a rat given oral administration of GK5 at a dose of 330 mg kg^{-1} (B). The GK5-treated rat exhibits larger-sized, more-myelinated regenerating axons in the soleus nerve at 18 days following nerve crush. Magnification ×600.

an earlier time (i.e., grew at a faster rate). Virtually identical results were obtained in a replicate study (n = 3/group) where the average dose of GK3 and GK4 for each rat was calculated to be 300 mg kg^{-1} daily over the 18 days of study.

Discussion

This is the first demonstration of the ability of *Centella asiatica* extracts to accelerate axonal regeneration in-vivo and promote neurite outgrowth in-vitro. An ethanolic extract, but not an aqueous extract, of *Centella asiatica* significantly increased neurite outgrowth in SH-SY5Y human neuroblastoma cells and was more active than the neuroimmunophilin ligand, FK506; as previously found with FK506 (Gold et al 1999), no activity was observed in the absence of NGF (data not shown), revealing that the material is not able to differentiate the cells into neuronal phenotype, but accelerates process elongation.

Multiple fractions of the ethanolic extract, which had varying polarity, were active in-vitro. The most active fraction, GKF4, contained asiatic acid, which also promoted neurite outgrowth in-vitro and this activity appeared to be dependent on MEK, suggesting ERK-dependency for this activity. While asiatic acid may be one of the compounds responsible for the in-vitro activity of the ethanolic extract, there are undoubtedly other compounds in *Centella asiatica* that promote neurite outgrowth. GKF4, for instance, contains significant amounts of compounds other than asiatic acid, which may contribute to activity, and the more polar fractions, GKF10–GKF13, which do not contain asiatic acid, also stimulate neurite outgrowth. Importantly, oral administration of the ethanolic extract of *Centella asiatica* stimulated axonal regeneration in the rat sciatic nerve crush model. Although toxicity was observed in-vitro with the chlorophyll-containing fractions GKF5–GKF9, no obvious toxic effects were observed in-vivo. These results indicate that compounds within an ethanolic extract of *Centella asiatica* promote axonal regeneration and warrant further study as novel therapies for treatment of peripheral neuropathies.

This is the first report assessing the effects of asiatic acid, a well-known triterpenoid component of *Centella asiatica*, on neurite elongation in-vitro. This compound is easily detectable by HPLC (Figure 4) in GK2, is a trace component of GKF4 and is absent in GKF10–GKF13. Asiatic acid showed marked activity (Figure 3) at a concentration of 1 μM (0.5 μg mL^{-1}) highlighting its role as one of the active components of *Centella asiatica*. Studies in man have shown that asiatic acid is orally bioavailable when given as part of a triterpene fraction of *Centella asiatica* (Grimaldi et al 1990), and when administered as its glycoside asiaticoside (Rush et al 1993), suggesting that these in-vitro results may well be clinically relevant.

The neurite elongation activity of asiatic acid was completely blocked by PD 098059, which is consistent with the very recent demonstration that asiatic-acid-mediated apoptosis in breast cancer cells is ERK dependent (Hsu et al 2004). ERK activation is known to be important for neurite elongation induced by neurotrophic factors, including NGF (Volonte et al 1993; York et al 1998; Yuen & Mobley 1999; Averill et al 2001; Boglári & Szeberényi 2001; Tsuji et al 2001; for review, see Kaplan & Miller 2000) and neurotrophin-3 (NT-3) (Aletsee et al 2001). Most relevant to this study, a number of natural compounds have been shown to increase neurite elongation via ERK activation, including FK506 (Price et al 2003; Gold & Zhong 2004) and genipin (Yamazaki et al 2004). Studies of functional involvement of the ERK pathway that rely on the MEK-inhibitory compound PD 098059 need to be interpreted with caution as many studies (e.g., see Bonni et al 1999; Liu et al 1999; Jin et al 2002) are based on the use at high (30–75 μM) concentrations that are not selective for MEK (Ed Hall, Pfizer Global Development, personal communication). Thus, our observation that relatively low (0.1–10 μM) concentrations of PD 098059, which are selective for MEK, inhibit asiatic acid's activity, definitively demonstrates the involvement of ERK in its action. This indicates that at least one of the active constituents of *Centella asiatica* (asiatic acid) involves this MAP kinase pathway. Taken together, our findings support the convergence of asiatic-acid-mediated events with known signalling pathways for neurotrophins (Lyons et al 1994; Kaplan & Miller 2000).

Interestingly, a water extract of *Centella asiatica* (GKW1) was inactive in the neurite elongation assay. This suggests that highly polar compounds are not involved in this activity and, further, that this activity is apparently not relevant to the in-vivo neuropharmacological effects reported by others using a water extract (see Introduction). However, the water extract may contain prodrug-type compounds that are metabolized to more active forms in-vivo, which would not be picked up during in-vitro testing. For example, the polar glycoside asiaticoside has been shown (Rush et al 1993) to be converted in-vivo to its less polar aglycone, asiatic acid, which we have shown to stimulate neurite elongation. While we have not tested asiaticoside in our in-vitro model, the closely related compound madecassoside (Figure 1) was inactive at 1 μM (data not shown).

Our results indicate that a number of compounds in *Centella asiatica* share asiatic acid's ability to stimulate neurite elongation in-vitro. Future studies are proposed to identify these other active compounds found in *Centella asiatica* and to define their mechanism of action. In addition to the triterpenes shown in Figure 1, *Centella asiatica* also contains flavonoids, volatile oils, phytosterols and alkaloids (Newall et al 1996; Brinkhaus et al 2000), an octadecanyl, hydroxy-pyrone (Srivastava & Shukla 1997), and additional triterpenes, such as asiaticoside B, centellasaponins A, B, C and D and the aglycone centellasapogenol A (Matsuda et al 2001a, b). It will be important to determine the plasma and brain concentrations of asiatic acid and any other active ingredients that are associated with therapeutic effects in-vivo to design human dosage schemes that achieve these therapeutic levels. Knowledge of the active constituents will provide a chemical handle both for new drug development, and for quality control and standardization of Gotu kola preparations on the market. It is known, for example, that the relative levels of the triterpene components can vary widely depending on the geographical source of the herb (Rouillard-Guellec et al 1997), and this will very likely have implications for therapeutic activity.

In summary, our findings clearly demonstrate the therapeutic efficacy of oral administration of the ethanolic extract of *Centella asiatica* for accelerating nerve regeneration in the peripheral nervous system in-vivo. Presently, there are no treatments available to enhance nerve regeneration and speed recovery of function in neurological injury and disease, with the possible exception of FK506. However, FK506 is neither practical nor ethical for such use outside of conditions necessitating immunosuppression (e.g., hand transplantation) due to its immunosuppressant activity. The availability of a safe and inexpensive agent would therefore not only be beneficial to patients, but also to society by greatly reducing hospital and workers' compensation costs. With further development, *Centella asiatica* and its active components represent an attractive, readily available means to speed nerve regeneration.

Conclusions

This study reports the discovery that an extract of the Ayurvedic herb *Centella asiatica* shows neurotrophic activity in-vitro and in-vivo. An ethanolic extract of the herb elicited a marked increase in neurite elongation in cultured human SH-SY5Y neuroblastoma cells. A non-polar fraction (GKF4) of the extract and a known triterpene component, asiatic acid, also showed significant activity. Relatively polar fractions (GKF10–GKF13), not containing detectable amounts of asiatic acid, were also active, albeit to a lesser degree, indicating that *Centella asiatica* contains more than one active component. Neurite elongation by asiatic acid was completely blocked by the ERK pathway inhibitor PD 098059 (10 μM). The in-vitro data were substantiated in an in-vivo model. Male Sprague-Dawley rats given *Centella asiatica* ethanolic extract in their drinking water (300–330 mg kg^{-1} daily) for 18 days following a sciatic nerve crush injury demonstrated more rapid functional recovery and increased axonal regeneration (larger calibre axons and greater numbers of myelinated axons) compared with controls, indicating that the axons grew at a faster rate. Taken together, these findings demonstrate that multiple components in *Centella asiatica* ethanolic extract contribute to its neurotrophic activity and may be useful for accelerating nerve regeneration in human neurological disorders.

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