Neuroprotective capabilities of *Vitex negundo* in primary hippocampal neurons

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Abstract: Vitex *negundu* (Vn) is a well-known aromatic shrub commonly used as a traditional folk medicine famous for its potential pharmacological and biological activities. Several chemical compounds are extracted and identified from the different parts of the Vn such as leaves, root, seeds and flowers. Number of researches reported the herb as antimicrobial, anti-androgenic, anti-osteoporotic, and anti-tumour, anti-cancer, anti-inflammatory, anti-oxidant, anti-hyperglycemic and hepatoprotective. The effects of Vn on neurite outgrowth have not been identified till now. Therefore present study was designed to investigate the neurite outgrowth effects of Vn extract in hippocampal neurons. Neurons from P0 mice were isolated and cultured in defined medium containing the different concentrations of Vn (20, 30, 40, 50, 100, 150 and 200 µg/ml) for 48 hrs. The presence of the neurites was confirmed by using ßIII-tubulin antibody which specifically labels only the neurites. Morphometric analysis was done by using Optika Pro-Vision software. The data show that Vn at 30 and 40 µg/ml significantly increased the mean average length of the longest neurite whereas at 150 and 200 µg/ml it significantly decreased the mean average length of the 10 longest neurite in hippocampal neurons. Nevertheless Vn did not show any significant effects on the sum of all the neurite lengths at any concentrations tested. Taken together the result shows that methanolic extract of Vn has potential to produce long neurites at 30 and 40 µg/ml and therefore can be act as a neuroprotective agent in the future drug development.

Keyword: Neurite outgrowth, Neuronal regeneration, *Vitex nigundo*, Hippocampal neurons, Immunostaining, morphometery, neuroinflammation.

INTRODUCTION

Vitex *negundu* (Vn) is a large aromatic shrub which is also known as five leaved chaste tree widely distributed in various regions of the world such as south Asia, Indonesia, china, Japan, Pakistan, India, east Africa and South America (Gautam *et al*., 2010 and Zheng *et al*., 2015). All parts of the plant, leaves, roots and seeds have been largely used as a folk medicine in south and south East Asia (Padua *et al*., 1999). Number of volatile compounds have been identified from the crude extract of Vn that possess ant-inflammatory, anti-oxidant, anti-fungal, anti-bacterial, analgesic, anti-rheumatism (Zheng *et al*., 2009b; Zheng *et al*., 2014a), antitumor (Zhou *et al*., 2009), insecticidal (Kamaraj *et al*., 2009; Kamaraj *et al*., 2010), antimicrobial (Kamruzzaman *et al*., 2013), anti-androgenic, anti-osteoporotic, anti-hyperglycemic and heptoprotective properties. Ethanolic and Water extract of fresh mature leaves of Vn has been reported for significant analgesic activity in rat and mice (Dharmasiri *et al*., 2003). Methanolic extract of Vn (leaves) shows potential anti-inflammatory and anti-oxidant effects that prevent tissue damage by scavenging free radical approach (Kulkarni *et al*., 2008). Another study reported the significant anti-inflammatory effects of fresh mature leaf extract of Vn in formaldehyde and carrageenan induced rat paw oedema (Chawla *et al*.,1992). Several other chemical compounds have been isolated from the plant mainly flavanoids, terpenoids and lignans that are reported to have convincing medicinal properties (Zehng *et al*., 2015). A type of lignan 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxyme-thyl-7-methoxy-3,4-dihydro-2-naphthaldehyde from Vn documented as a potent analgesic, anti-inflammatory, anti-oxidant, anti-tumor and immuno-stimulatory compound (Zehng *et al*., 2009). Usually compounds that have anti-inflammatory effects are good therapeutic agents that can help to cure neurodegenerative diseases. Therefore present study was designed to investigate Vn effects on the neurite outgrowth from hippocampal neurons. The identification of the therapeutically effective dose concentration of Vn would lead to the development of a promising anti-inflammatory agent to treat neurodegenerative disorders and helps us to recover the altered morphology of neurons.

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MATERIALS AND METHODS

Animal ethics

All experiments in this study were performed according to established ethical guidelines on embryos or postnatal pups derived from timed pregnant Balb-C mice (Animal House Facility, ICCBS). The animals were housed in cages in a standard barrier facility and maintained on a 12 h light: 12 h dark cycle. All experiments in this study were performed according to the established guidelines. All experiments were performed in PCMD, ICCBS, University of Karachi.

Cell culture

Hippocampai from brains were dissected out from the neonatal P0-1 mice. Meninges were removed and small hippocampus tissues were digested with 0.25% trypsin EDTA solution (Sigma Aldrich) for 10-12 min at 37°C. Mechanical digestion was performed by gently tapping and re-pipetting the suspension. Trypsinization was stopped by adding culture medium. Cells were centrifuged at 1000 rpm for 10 min at 4°C. Supernatant was discarded and cell pellet was resuspended into the fresh medium. Cells were seeded into four well plates which were previously coated with poly-L-lysine (PLL 0.01%, Sigma Aldrich) for 2 hrs at room temperature. Neurons were cultured in a medium composed of 10%FBS, 1% glutamax, 1% penicillin/streptomycin and 1% sodium pyruvatein Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1). Cells were incubated for 48 hours at 37°C in 5% CO_2 humidified atmosphere.

Design of the study

Neonatal mice pups of postnatal day P0-1were selected. The lengths of neurites were measured after 48 hours of neuronal growth with and without herbal extract Vn. Only the free growing neurons with short and long axons were measured from the soma of the cell to the tip of neurite. Cells having neurites less than 20 µm were excluded from the study. Neurons that were too close and meeting their neurites to soma of other neurons were also excluded from the study. This study was completed in 2.5 years.

Dose response curve

Stock solution of 1mg/ml is prepared by dissolving methanolic extract of Vn in de-ionised water. The solution was filtered and aliquots were stored at -20°C. Hippocampal neurons were seeded in a density of 10,000 cells per well. Different selected doses of 20µg/ml, 30µg/ml, 40 µg/ml, 50 µg/ml, 100µg/ml, 150µg/ml and 200 µg/ml were added into each well. Controls received only culture medium. All dilutions were prepared in the culture medium. The effect of different doses of drug on hippocampal neurons was observed after 48hrs of incubation at 37°Cin 5% CO_2 humidified atmosphere.

Morphometery

After 48 hours of incubation images were captured through a camera attached to the microscope. Length of neurite was measured from the point of emergence at the cell body to the tip of axon terminal, bipolar and multipolar extension were measured and sum up for consideration of a single neurite length. 10-12 different fields from each well is focused and captured. Quantification of the morphological parameters was carried out using Optika provision analysis software. At least 150 cells per treatment were analysed in each experiment, and results were pooled from at least 3 different experiments.

Immunostaining

Hippocampal neurons were stained with rabbit anti β-III tubulin (Merk Millipore) antibody diluted in Kreb's Ringer buffer/0.01 % BSA. Triton X-100 (0.1%) is added to permeablize the membrane. Cells were fixed with 4% PFA for 20min and were incubated for 1 hour with primary antibody. Counter staining with secondary antibody was done by using Alexa flour 588 for 45 min. Each step followed washing with Kreb's Ringer buffer. Cover slips were mounted by using an immumount on clean glass slide store it in a cool dry place at 4°C.The stained pictures were captured by using fluorescence microscope.

STATISTICAL ANALYSIS

Experiments were repeated at least 3 times and the data is represented as Means ± SD. Mann Whitney U-test was applied to evaluate the significance level. A p-value of <0.05 is considered as statistically significant.

RESULTS

We have studied the role of methanolic extract of Vn on the hippocampal neurite outgrowth isolated from the P1 mice and cultured in a defined medium for 48 hours. Neurons were cultured in the presence of different doses of Vn extract (20, 30, 40, 50, 100, 150, 200 µg/ml). Neurite outgrowth was measured after 48 hours. The neurites were identified by using specific antibody ß-III tubulin which recognizes and labels only neurites from the neurons. The present study show that Vn at lower doses i.e. 30 and 40µg/ml significantly ($P<0.05$, $P<0.0001$ respectively) increased the mean average length of the 10 longest neurites (fig. 1). However there was a slight non-significant increase in the longest neurite lengths at 50 µg/ml dose (fig. 2). Whereas at 100µg/ml there was a slight non-significant decrease in the longest neurite lengths but at higher concentrations i.e. 150 and 200µg/ml Vn significantly ($P<0.005$, $P<0.0001$ respectively) inhibited the mean average lengths of the 10 longest neurites (fig. 2). On the other hand sum of all the neurites show a different effects compared to the 10 longest

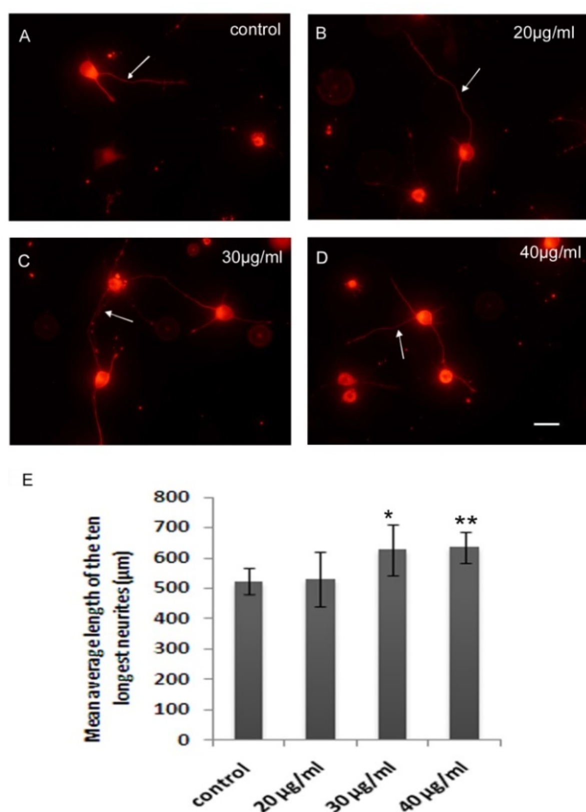

Fig. 1: The effect of lower doses of Vitex *negundu* on neurite outgrowth. *A-D)* Neurons were cultured with or without the presence of different doses of Vn. Neurons are stained with β-III tubulin antibody. *F)* The graph shows the mean average length of the ten longest neurites. Significant increase in neurite lengths was observed at dose 30 and 40 µg/ml *p value < 0.05% and **p<0.0001. Scale Bar = 20µm.

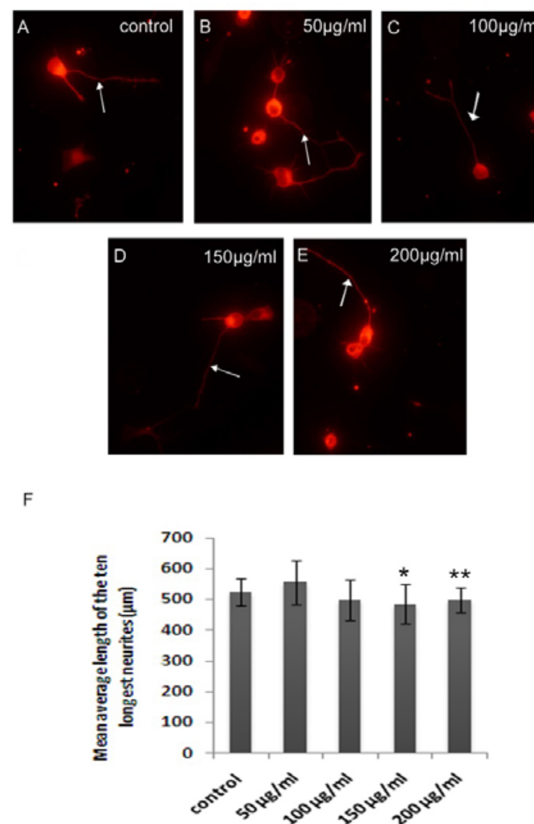

Fig. 2: The effect of higher doses of Vitex *negundu* extract on neurite outgrowth. *A-E)* Cells are stained with β-III tubulin antibody. *F)* Graph representing the mean average length of the ten longest neurites. Significant decrease was observed at 150 and 200 µg/ml concentrations. *P<0.005, **P<0.0001. Scale Bar = 20µm.

neurite lengths. Current data did not show any significant effects of Vn on total neurite outgrowth. Even though total neurite lengths were higher at 40µg/ml still the effects was non-significant (fig. 3). This has suggested that Vn have ability to produce long neurites rather to induce sprouting.

DISCUSSION

More than 120 chemical compounds are purified from the Vn in which main contribution of flavonoids, terpenoids, steroids and lignans is reported. These chemical compounds are demonstrated to have potential biological and pharmacological activities. A compound tris (2, 4-ditert-butylphenyl) phosphate is isolated from the leaves of Vn significantly inhibit the carageenin induced paw edema in rat *(Zheng et al.,* 2015). Further investigations indicated that agunoside isolated from Vn inhibit the expression of pro-inflammatory cytokines in a dose dependent manner *(Vinuchakkaravarthy et al., 2011*). Moreover, Phenylnaphthalene type lignans also reported to have potent nitric oxide scavenging activities in LPS-stimulated RAW 264.7 cells *(Zheng et al., 2015).*In the present study the neurites were cultured on the poly-D-lysine (PDL) coated coverslips which represents the minimal substrate. The methanolic fraction of Vn was added after 30 mins of culturing the neurons. Neurite outgrowth was measured after 48 hrs. Neurons cultivated only on PDL coated cover slip was considered as the controls. Two parameters of neurite outgrowth was used (1) The mean average length of the 10 longest neurites, which shows the effectiveness of the extract to stimulate the neurite outgrowth within 48 hrs (2) The sum of all the neurites, which shows the overall effectiveness of the extract to generate maximum numbers of neurites within 48hrs. In the present study the effects of Vn on generating long neurites made it as a potential therapeutic target in neurodegenerating diseases in which neuritis looses their

functions either by degradation of myelin sheaths or by neuronal death or loss of synaptic connections or by the death of oligodendrocytes. Hence any compound that has ability to induce neurite outgrowth can provide neuroprotection against many CNS diseases.

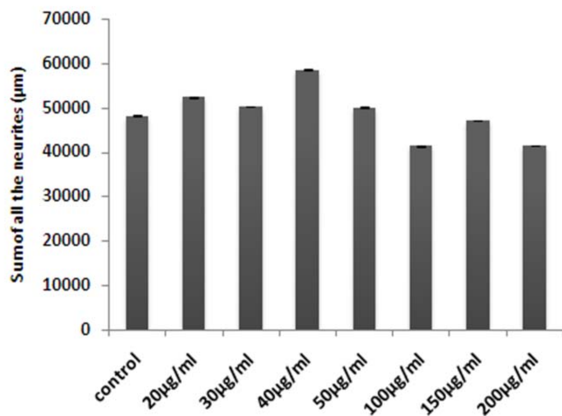

Fig. 3: The effect of higher doses of Vitex negundu extract on the sum of all the neurites. Graph represents the total sum of all the neurites lengths on different doses of Vn. There were no significant effects of Vn on the sum of all the neurite lengths.

CONCLUSION

Data at hand suggested that methanolic leaf extract of Vn stimulates the neurite growth in hippocampal neurons at 30 and 40µg/ml concentrations, whereas it inhibits the neurite lengths at 150 and 200µg/ml. Therefore it is suggested that Vn have ability to generate long neurites.

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