Neurite outgrowth properties of *Calotropis procera*: In search for a neuroprotectant

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Abstract: In view of the well-documented medicinal properties of Calotropis *procera* (CP), the present study was designed to evaluate the neuroprotective effect of the extract. We have prepared a methanolic extract of Calotropis *procera* and screen varying concentration of CP (20, 30, 40, 50 and 70µg/ml) for the stimulatory potency on neurite outgrowth. The stimulatory effect of CP on neurite outgrowth was assessed in primary hippocampal neurons. Neurite lengths were measured using optika provison analysis software. Neuritogenesis was further analyzed by immunostaining by using specific neuronal marker β III-tubulin. The data show that neurite outgrowth from hippocampal neurons were significantly enhanced in the presence of CP (40µg/ml). The most stimulatory neurite outgrowth effects were appeared after 48hrs incubation of neurons with CP (40µg/ml). These data confirm that CP extract could promote *invitro* hippocampal neurite outgrowth in a dose-dependent manner. Our results indicate that CP can be used as a healthy dietary supplement for the cognitive functions of the brain.

Keywords: Neurite outgrowth, Calotropis *procera*, Neuroprotective.

INTRODUCTION

During development neuritogenesis is a significant event in neuronal path finding and the formation of synaptic connections (More *et al.,* 2012; Phan *et al.,* 2013). It is also important in neuronal plasticity and neuronal regeneration following injury (Hagg 2009; Wong *et a*l., 2012) and in the neurodegenerative conditions such as Alzheimer's, dementia and Parkinson's diseases (Kaneko and Sawamoto, 2009). Hence, treatments directing at stimulating neurite outgrowth and preserving the neurite network and synaptic connections are required.

The central dogma that nerve regeneration in mammalian central nervous system is irreversible, no longer exist. It is now accepted that under the presence of stimulatory substances the damaged neurons do regenerate (Pesavento *et al*., 2002) to some extent. Ever since the discovery of the neurotrophins they are being used in human clinical trials, however the use is limited due to the macromlecular character and difficulty in crossing the blood brain-barrier (Jonhagen, 2000; Tang *et al*., 2009).

Earlier studies have shown that by improving drug delivering system of neurotrophins belonging to the natural origin are extremely beneficial in preventing the neuronal loss (Pardridge, 2003). This leads to the major break through in the field of traditional medicine for the search of herbal compounds that have neurotrophic capability (Shibata *et al*., 2008).

Calotropis *procera*, is a prescribed folk medicinal herb belonging to the family Asclepiadaceae commonly known as Giant Swallow wort, milkweed and Arka. It is use in traditional system for the treatment of various diseases (Iqbal *et al*., 2005; Sharma, 2011; David *et al*., 2011). It has been reported that the title plant is used in the treatment of asthma, leprosy, eczema (Mainasara *et al.,* 2012), rheumatoid arthritis (Kumar and Roy, 2007) and also possess properties of anticancer, antipyretic, anti-inflammatory and anticoagulant (Dewan *et al.,* 2000; Magalhães *et al.,* 2010; Meena *et al*., 2011; Uddin *et al.,* 2012).

Therefore the need to conduct scientific investigations to ascertain the authenticity of the claims on the medicinal properties of this plant present study was conducted to evaluate neurite outgrowth stimulatory effects of Calotropis *procera* using primary hippocampal neurons.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle Medium (DMEM) (11965-084), B27 supplement (17504-044), and sodium pyruvate (11360-070) were purchased from Gibco (life technologies), USA. Poly-L-Lysine (PLL-P4832),

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Trypsin-EDTA (T3924) and FBS (F0804) were purchased from Sigma-Aldrich (USA). Pencillin-Streptomycin was purchased from Hyclone (SV30010). Secondary antibody (Alexa flour 568) was purchased from Gibco-Life technologies (USA).

Cell culture

Balb-C postnatal mice (P0) were obtained from the Animal center of ICCBS. All experimental procedures involving animals were conducted as per Institutional Animal Care guidelines. The hippocampi were quickly isolated from the brain in chilled HBSS solution under dissecting microscope and the tissue was treated with 0.25% trypsin-EDTA for 8 minutes at 37°C then triturated until the solution becomes homogenous. Cell suspension was centrifuged at 1000rpm for 10min at 4°C. The pallet was collected and the supernatant was discarded. Fresh medium containing 10% FBS in DMEM was then added in the pellet and mixed thoroughly. Cells were counted by hemocytometer and 10,000 neurons were seeded on each PLL-coated 10mm glass coverslips for 2hrs at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Dose dependent study

After 2hrs of cell plating, defined DMEM medium supplemented with 2% B27, 1% pen/strep, 1% sodium pyruvate were added to the neurons. CP extract was then added in varying concentrations (20, 30, 40, 50 and 70µg/ml) in the culture dishes containing the neurons.

Morphometric analysis

After 48hrs of incubation, neurons were observed under Nikon microscope (Nikon TE2000-U, Tokyo, Japan) and photographs were taken randomly from different fields of the well plate. Neurite outgrowth was measured using optika provison analysis software from all the groups. Neurite lengths were measured from the soma to the end of growth cone. All the bipolar, multipolar extensions from the neuron were measured and summed up. Mean average length of ten longest neurites and the sum of all neurite lengths were calculated. This helped us to analyze the overall morphological changes before and after the treatments. Neurites from at least 180 neurons were measured for each condition in three to four independent experiments and the results were pooled.

Immunostaining

The neurons were fixed with 4% PFA for 10min. Followed by washing with Kreb's Ringer Buffer (KRH) (3X) the cells were then permeabilized by incubating with 0.1% triton X-100 in KRH for 10min. After washing the cells were incubated with rabbit polyclonal antibody against βIII- tubulin (1:300) for 1hr at 37°C. After washing the cells were incubated for 45min at 37°C with goat anti-rabbit Alexa 568-conjugated IgG. Following rinsing with KRH 3X the cells were incubated with DAPI for 15min and mounted on glass slides by using an immumount.

STATISTICAL ANALYSIS

Statistical Software system (SPSS 19.0 Incorporation, Chicago, IL) was used for the statistics. All the experiments were done in triplicate and the results in this manuscript represent pooled data from three separate experiments. The data was analyzed using the nonparametric Mann–Whitney U-test and represented as means ±S.D. A P-value of <0.05 was regarded as significant.

RESULTS

In the present study dose response analysis was carried out for the methanolic extract of CP using neurite outgrowth assay to identify the optimum dose required for the growth and proliferation of hippocampal neurons. For this purpose, neurons from P0 mice pups were incubated with various doses (20, 30, 40, 50 and 70µg/ml) of CP in a defined culture medium for 48hrs at 37°C. The results were compared with the neurons cultured on PLL-coated well plates without the CP extract. Neurons were immunostained with the specific neuronal antibody against β III-tubulin (fig. 1).The data show morphological changes in hippocampal neurons in different concentrations (20-50µg/ml) after 48hrs Hippocampal neurons started to develop lamellipodia and extended the neurites after few hours of plating. The neurons after 2 days have acquired a particular morphology from unpolarzied to polarized stage. The neurites were long and multibranched rapidly grew in the presence of CP. However in control groups neurite extension was relatively slow when compared to the CP treated neurons. The data show a significant ($p<0.001$) dose dependent increase in the neurite outgrowth from 20-40µg/ml concentration when compared to the untreated controls (465.59±13.22). Although CP (50µg/ml) at higher doses also significantly increases the neurite length from the controls nevertheless the neurite length was not that pronounced (fig. 2A, B). The pooled data from 3 individual experiments showed that the mean average length of the 10 longest neurites at 50µg/ml (480±9.31) were reduced when compared to lower doses, 20µg/ml (487.84±12.9) and 40µg/ml (503.58±13.46) after 48hrs in vitro (fig. 2A). Moreover at even higher doses CP at 70µg/ml (469.42±17.37) remained non-significant towards the neurite length when compared to untreated controls (465.59±13.22).

Similar results were obtained when sum of the all the neurite lengths was measured at 20-40µg/ml concentrations. The sum of the neurite lengths at lower doses 20µg/ml (79907.67±1622.05), 30µg/ml (86407.49± 1895.996) and 40µg/ml (96555.94±758.65) were significantly increased ($p<0.001$) when compared to the untreated controls (70479.19±1819.94) after 48 hrs in vitro (fig. 2B).

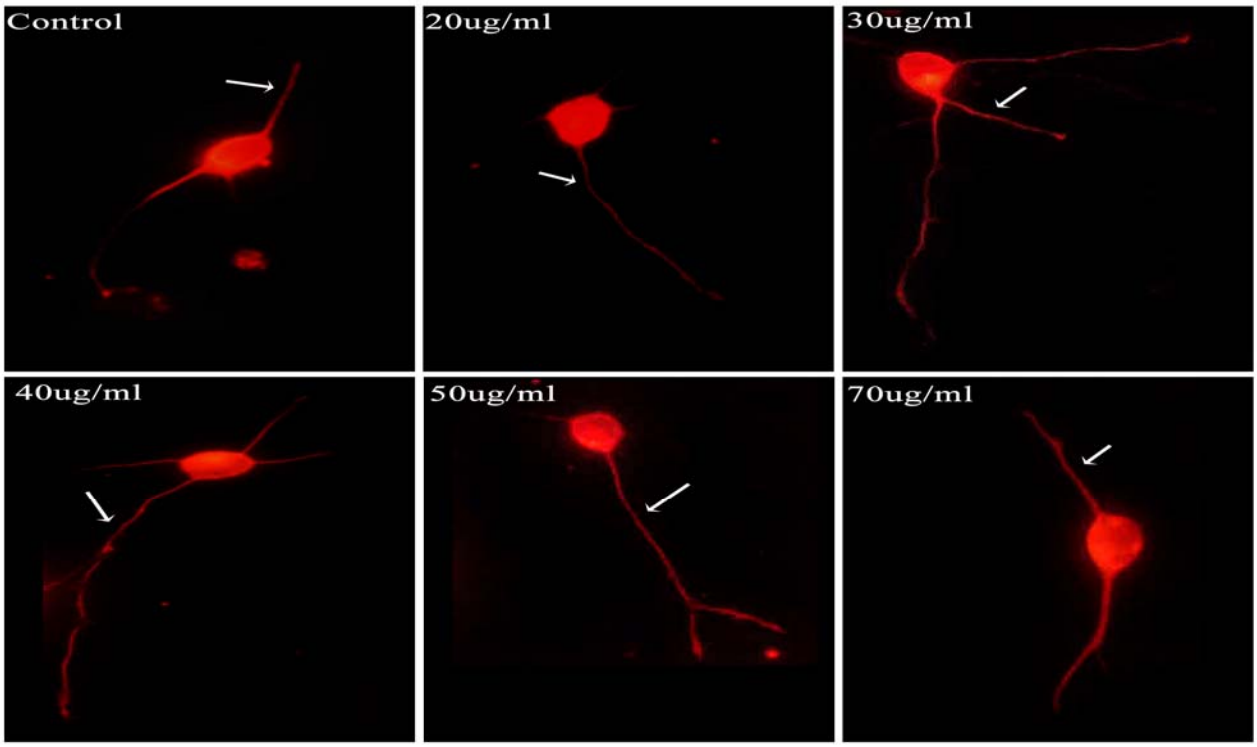

Fig. 1: Primary hippocampal neurons immunostained with ß-III tubulin. Neurons treated with different concentrations of CP (20-70µg/ml). Arrows indicate neurites. Scale bar= 20µm.

Furthermore CP at higher doses 50µg/ml (80288.56± 938.37) didn't show any significant effects on the sum of all the neurite lengths when compared to the untreated controls after 48hrs in vitro (fig. 2B). This suggested that CP has stimulatory effects only at lower doses (20-40 µg/ml) and inhibitory effects at higher doses (70µg/ml).

Overall, CP significantly increased the mean average lengths of the 10 longest ($p<0.001$) neurites at (20-50 µg/ml) and sum of all the neurites ($p<0.05$) at lower doses (20-40µg/ml).

DISCUSSION

The medicinal properties of Calotropis procera extract has been observed by a series of previously reported studies (Shivkar and Kumar, 2003). As axonal regeneration is very crucial and important for the fast recovery after nerve damage. Samy and colleagues (2012) have shown that an isolated protein from CP root inhibits the proliferation and activates the suppression of nuclear factor kappaB (NF-kB) in breast cancer cells. Numerous compounds have been isolated from herbal sources exhibit anticancer, anti-inflammatory, anti-tumorogenic properties without having adverse effects. These compounds are usually very helpful in designing and developing new drugs that could combat the serious illnesses.

However in this study, we further explored the effects of CP extract on the neurite outgrowth. As it is a well established fact, hippocampus belongs to the limbic system, have a primary role in the short-term memory and emotional regulation. Certain pathological abnormalities in hippocampus are associated with certain neurological ailments, such as Alzheimer's disease, dementia, Parkinson's disease and epilepsy. Therefore, neurons from hippocampus serve as a well-characterized model for investigating the effects of neuroactive compounds (Kimura *et al.,* 2004; Peng *et al.,* 2013).

Our experimental results revealed that treatment with CP extract for 48 hrs enhanced the neurite outgrowth from hippocampal neurons. Soumyanath and coworkers (2005) worked on another herbal extract Centella asiatica (CA) that has been used as a nerve tonic from centuries. They showed ethanolic extract of CA (100µg/ml) and nerve growth factor (NGF) induced an extensive neurite outgrowth in human SH-SY5Y cells through activating extra cellular-signal-regulated kinases (ERK) (Soumyanath *et al.,* 2005). It is also true in our case that shows the number of extensions and the length of neurites were found to be increased with increasing the concentration. However, at higher doses the neurite outgrowth tends to decrease or remained non-significant. This suggested that at higher doses CP has less neurite outgrowth effects probably due to the saturation of the

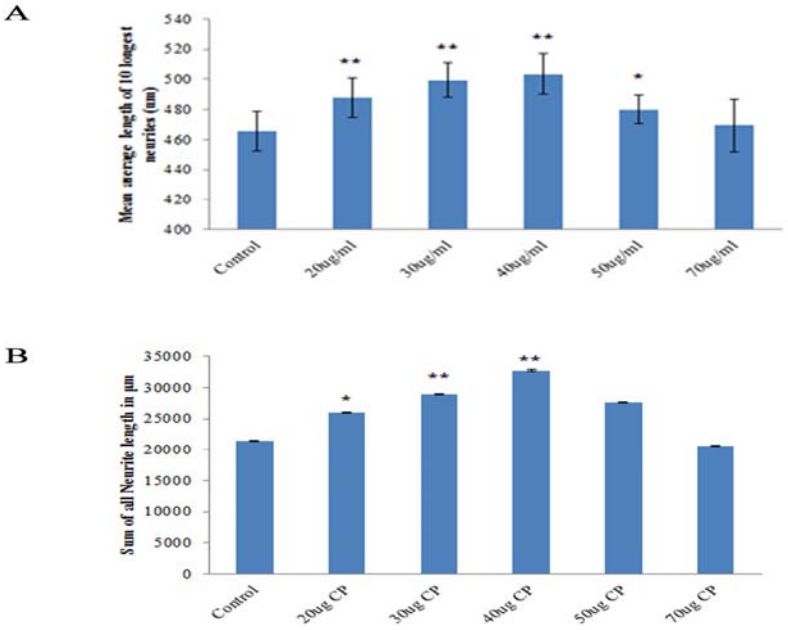

Fig. 2: Graphical representation showing effects of different concentration of CP on neurite lengths after 48hrs. (A) Mean average length of the 10 longest neurites (B) The sum of all the neurite lengths. The pooled results from three independent experiments are shown here. *p<0.05 and **p<0.001.

ligand-receptor availability he dose-dependent neurotrophic effects exerted by the CP extract on neurite outgrowth made it a very promising herbal extract to search the active compounds present in it. Therefore ingredients present in CP can accelerate the repair and regeneration of the neurites.

CONCLUSION

The extract of *Calotropis procera* showed potential in promoting neurite outgrowth in primary hippocampal neurons. The synergism of the various active entities in the methanolic extract may be responsible for the neurite outgrowth activity and further experiments are necessary to isolate and identify the compound (s). According to the previous studies the extract contains ketosteroids, polysaccharides, and polypeptides.

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