Vitex negundo induces an anticonvulsant effect by inhibiting voltage gated sodium channels in murine Neuro 2A cell line

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Abstract: *Vitex negundo* (Vn) extract is famous for the treatment of neurological diseases such as migraine and epilepsy. These neurological diseases have been associated with abnormally increased influx of sodium ions into the neurons. Drugs that inhibit voltage gated sodium channels can be used as potent anti-epileptics. Till now, the effects of Vn on sodium channels have not been investigated. Therefore, we have investigated the effects of methalonic fraction of Vn extract in Murine Neuro 2A cell line. Cells were cultured in a defined medium with or without the Vn extract (100 μg/ml). Sodium currents were recorded using whole-cell patch clamp method. The data show that methanolic extract of Vn inhibited sodium currents in a dose dependent manner (IC50 =161μg/ml). Vn (100 μg/ml) shifted the steady-state inactivation curve to the left or towards the hyper polarization state. However, Vn did not show any effects on outward rectifying potassium currents. Moreover, Vn (100 μg/ml) significantly reduced the sustained repetitive (48±4.8%, P<0.01) firing from neonatal hippocampal neurons at 12 DIV. Hence, our data suggested that inhibition of sodium channels by Vn may exert pharmacological effects in reducing pain and convulsions.

Keywords: Neurons, sustained repetitive firing, sodium channels, *Vitex negundo.*

INTRODUCTION

Vitex negundo (Vn), is a famous herbal plant which has been traditionally used extensively in Indian Ayurveda, Arabic Unani medicine and traditional Chinese medicine. A number of scientific evidences revealed that Vn extract possesses a number of beneficial effects. Numerous pharmacological effects of Vn for the treatment of neurological problems including pain (Telang *et al*., 1999), convulsions (Gupta *et al*., 2005) and anxiety (Adnaik *et al*., 2009) have been previously reported. These neural deficits are thought to be associated with increased influx of sodium ions than normal physiological conditions. Increased sodium ions through voltage-gated sodium channels generates an increased sodium currents that results in the facilitation of repetitive firing and prolong depolarization of neurons (Yan and Li, 2011). Therefore any compound that blocks sodium channels or currents may act as a potent analgesics (Dib-Hajj *et al*., 2009) or anticonvulsant drugs (Large *et al*., 2009). For example, phenytoin, carbamazepine (Kuo, 1998) and lidocaine have similar mechanism of action on sodium channels. These drugs decreased the cellular excitability by reducing the influx of sodium in to neurons. Normally, inward movement of sodium ions is responsible to depolarize cellular membrane to initiate as well as the propagate action potentials. Action potentials, in turn, form the electrophysiological basis of the functioning of the brain. Therefore, voltage gated sodium channels plays a key role in the excitation of neuron and underlying brain functions. Moreover, modulation of these channels is involved in behavioral and pathological conditions. The aim of the study is to delineate the changes exerted by the Vitex *negundo* extract on voltage-gated sodium channels in Neuro 2A cell line.

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MATERIALS AND METHODS

Plant material and preparation

Vitex negundo (Vn) plant was dried and grinded into powder. For the preparation of the methanolic extract, powdered Vn was soaked in methanol (MeOH) at room temperature for 30 days. After drying out solvent using rotary dryer, sample was filtered through a 400-mesh cloth to collect the extract and kept at 4°C till the analysis.

Cell culturing

Murine Neuro 2A neuroblastoma cells were obtained from American Type Culture Collection, ATCC (Manassas, VA, USA). Neuro 2A were thawed and centrifuged for 10 min at 1000 RPM at 4°C. Supernatant was discarded and pellet was collected. Fresh Dulbecco's modified eagle medium (DMEM) was then added in to the pellet, mixed properly and washed with phosphate buffer saline (PBS, pH 7.3) Cells were counted by heamocytometer and seeded 2000 cells on each 22 mm

cover slips. Primary hippocampal neurons were isolated from 1-2 days old mice pups. Hippocampal tissues were cut into small pieces and incubated with 0.05% trypsin/EDTA at 37°C for 15 mins. Digested tissues were then triturated until the solution becomes homogenous. Cell suspension was centrifuged for 10 min at 1000 RPM at 4 ^{0}C and pellet was collected. Cells were seeded at a density of 15000 on each cover slip. Cells were incubated with 1% fetal calf serum (FCS), 1 % Pen/Step, 0.5 mM glutamax and 2% B27 supplement (Gibco chemicals) in DMEM medium. Cells were incubated in 5% CO_2 incubator at 37°C.

Patch clamp recording

Whole-cell patch clamp technique was used to record currents from cultured Neuro 2A cells. The cover-slip containing cultured cells was transferred into a bathing chamber on the stage of inverted microscope. Cells were perfused with artificial cerebrospinal fluid (ACSF) containing 140mM NaCl, 5mM KCl, 2mM $MgCl_2$, 10mM glucose, and 10mM HEPES and pH was adjusted to 7.3. Borosilicate glass pipettes were pulled using a Brown-Flaming P-97 electrode puller (Sutter Instruments, USA) with a 2-3 µm diameter. Pipettes gave a resistance of 4 to 5 MΩ when filled with a solution containing 140mM KCl, 10mM NaCl, 10mM EGTA, 2mM $MgCl_2$, and 10mM HEPES, pH 7.2. Patch clamp recordings were carried out using HEKA EPC 10 amplifiers (HEKA Instruments, Inc., New York). Cells were discarded when variation seen more than 10% of cell capacitance and series resistance. Only those cells were used, that has resting membrane potential (RMP) more negative than -50 mV.

To record sodium and potassium currents, cells were held at -110 mV for 300 msec from holding potential -60 mV and then voltages changed from -60 to +60 mV pulses. For recording of inactivation currents, cells were held at 0 mV followed by pre-pulse command potential started from -100 to -10 mV. Recorded currents were then normalized with maximum values and plotted against pre-pulse potentials and fitted to Boltzmann equation.

$$I / I_{max} = 1 / (1+\exp (V_{1/2} - V_{pre})/k))$$

Where I_{max} is the maximum current, V_{pre} is the prepulse potentials and $V_{1/2}$ is the potential at which inactivation is half-maximal.

For action potential recording, neurons were stimulated at 50-100 pA current for 400 msec in current clamp mode.

STATISTICAL ANALYSIS

Data represented as Means ± SD. Statistical significance was calculated with student t-test and values of $p < 0.05$ were considered as significant.

RESULTS

Effects of Vn on sodium currents

Sodium currents from voltage-gated sodium channels were recorded in whole-cell mode from cultured Neuro 2A cells. Cultured Neuro 2A cells were incubated with or without Vn (100 µg/ml). Present study show that methanolic fraction of Vn significantly (P<0.05) inhibit the peak amplitude of sodium currents in concentration dependent manner. Dose-dependent inhibition curve was illustrated in the fig. 1B and current-voltage relationship was summarized in fig. 1C. We have calculated IC50 value from this graph was to be 161µg/ml.

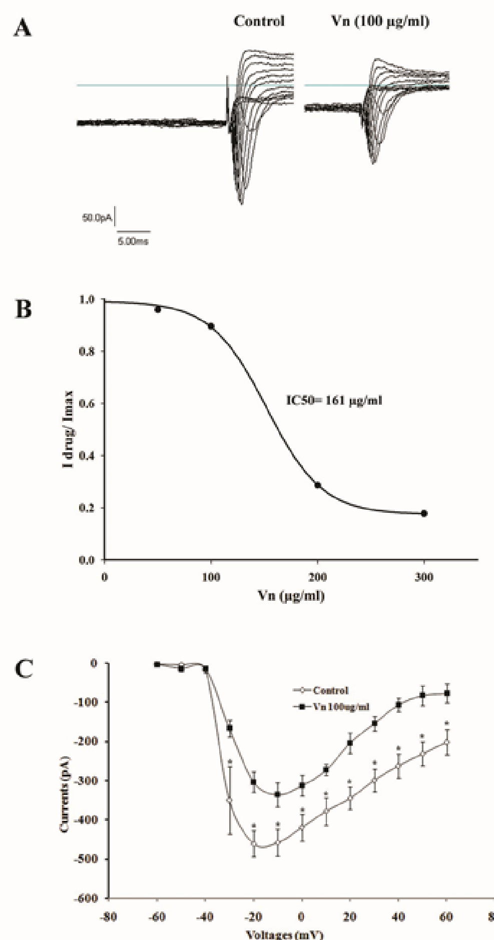

Fig. 1: Voltage-gated sodium currents in Neuro 2A cells were reduced by Vn. (A) Representative traces of sodium currents activated by depolarization pulse (−60 to +60mV in 10mV increments at a holding potential of −60mV) with and without the presence of Vn (100 µg/ml). (B) Dose response curve of sodium currents inhibition by Vn. (C) I-V relationship of sodium currents. Results represent means ± SD from 3–7 cells in each group. Similar results were obtained 3 or more experiments.

Since many sodium channel blockers inhibit sodium channels in the inactivation state, we investigated the

effect of Vn extract on voltage dependence of inactivation. The normalized peak currents were plotted against command voltages and all recordings were fitted according to the Boltzmann function. Vn extract significantly shifted the $V_{1/2}$ of inactivation by approximately 7mV ($V_{1/2}$ for control -75.2 mV and for Vn -82.4 mV) in the direction towards the hyper polarization (n=5, P<0.05). The results show that Vn decreases the availability of the channel by binding to its inactivated state (fig. 2).

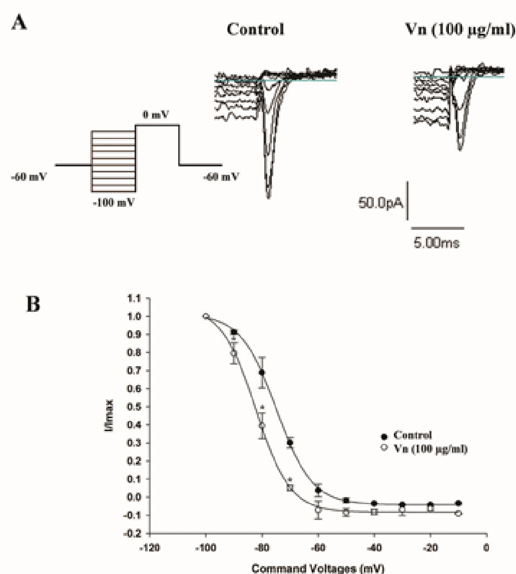

Fig. 2: Effects of Vn on steady-state inactivation of sodium channels in Neuro 2A cell line. (A) The steady-state inactivation currents traces with and without Vn (100 µg/ml). left panel shows the pulse protocol for recording of inactivation currents. (B) Graph represents inactivation curve of sodium channels. The currents showing above were normalized and fitted by the Boltzmann equation. Results are means ± SD from 5 cells in each group.

Effects of Vn on Potassium Channels

The main members of potassium channel expressed in Neuro 2A cells are Kv1.1 and Kv2.1 (Leung *et al*., 2011). Therefore, we recorded potassium currents from Neuro 2A cells treated with Vn. The data show no significant difference in the potassium curents from Neuro 2A cells after the application of Vn (fig. 3) was observed when compared to the untreated controls.

Vn reduced excitability of Neurons

Results from Neuro 2A showed that Vn reduced currents from sodium channels. This means that it must have an affect on the firing of a neuron. A further set of experiments (*n*=5) was performed to investigate how excitability of neuron is affected by the treatment of Vn extract. We recorded the action potentials from cultured primary hippocampal neurons. As Neuro 2A is a neuroblastoma cell line that does not show action potential that is why we have used primary hippocampal neurons to see the effect of Vn on the excitability. Current study show that Vn significantly reduced the neuronal firing rate (Control 20.8 ±2.88 Hz Vs Vn 10 ±2.5 Hz; P<0.01) when compared from the un-treated controls. Table 1 shows the effect of Vn on different parameters of action potentials in cultured neurons.

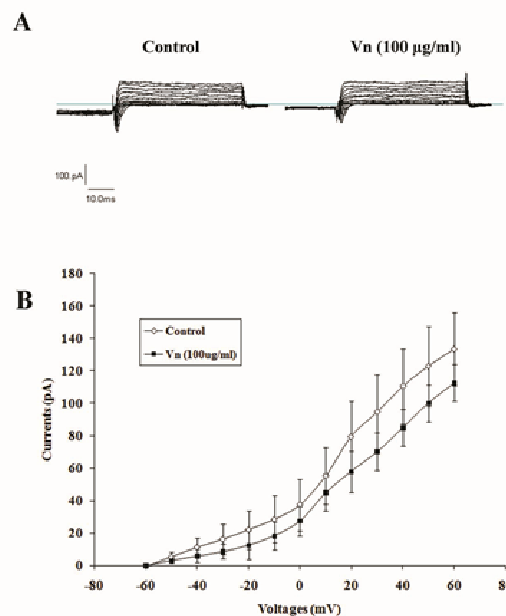

Fig. 3: Vn did not affect voltage-gated potassium currents in Neuro 2A cell line. (A) Outward rectifying K^+ currents were stimulated by -60mV to +60mV pulses and Vn (100µg/ml) was added to the bath solution. The traces shows the currents obtained before and after the application of Vn on Neuro 2A cells (B) indicates the current-voltage (I-V curve) relationship of voltage gated K^+ currents.

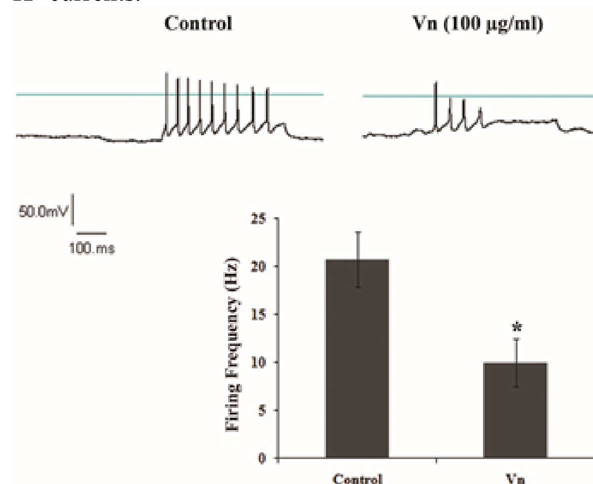

Fig. 4: Effects of Vn extract on action potential of cultured neurons. (A) Representative traces of sustained or repetitive firing of neurons. 50-100 pA current was injected to induced action potential train in the neuron.

(B) Bar graph showing the effect of Vn on firing rate of neuron. Data were represented as means ± SD. *p< 0.01 from their respective controls.

Table 1: Table showing the parameters of action potential from neonatal hippocampal neurons. Vn extract significantly reduces the firing frequency of the neurons. RPM stands for resting membrane potential; amplitude was measured at highest peak of action potential and frequency is the firing rate of action potential per second. Data represented as means ± SD. *p<0.01 from their respective controls.

	Control	Vn
RMP	-72.66±3.05 (mV)	-64.6±5.50 (mV)
Amplitude	25.33±5.50 (mV)	19.3±1.15 (mV)
Frequency	20.83±2.88 (Hz)	10±2.5* (Hz)

DISCUSSION

Our data, for the first time, reported that Vn suppressed voltage-gated sodium channels in neuronal cells in a concentration-dependent manner. Lidocaine is a known drug used as local anesthetics blocks sodium channels (Hille, 2001; Leung *et al*., 2010a). In addition number of drugs also reported for blockage of sodium channels in Neuro 2A cells for example, osthol, a herbal compound which has antihypertensive and neuroprotective effects (Leung *et al*., 2010a) and diphenidol, an anti-emetic agent and used as local anesthetic, inhibit specifically sodium channels in Neuro 2A cells (Leung *et al*., 2010b). Because blocking of sodium channel is associated with analgesic (Dick *et al*., 2007) and anti-convulsant effects therefore we suggest that Vn may partly contribute to reduce the intensity of pain and convulsions (Tandon and Gupta, 2005). Voltage-gated sodium channels are highly expressed in Neuro 2A cells while voltage-gated calcium channel's expression is very low. As Neuro 2A cells do not show action potentials because they are still in neuroblastoma stage and did not differentiated properly i.e. why primary hippocampal neurons were utilized to analyze the effect of Vn on sustained repetitive firing.

Sodium channels are in resting and closed state at potentials lower than the resting membrane potentials but they open briefly and then inactivate when the membrane potential is depolarized. Therefore, hyperpolarized membrane potentials are required to re-open sodium channels from inactivated states. It has been shown that many local anesthetics like lidocaine and phenytoin binds to the inactivation state of voltage-dependent sodium channels. Our results show that Vn cause a shift towards the hyperpolarization state in the voltage-dependence of inactivation of sodium channels. This shift to the left in the inactivation curve suggested Vn has higher binding affinity to the inactive state of the sodium channel. It is a known fact that blockers of voltage-dependent sodium channel, tetrodotoxin act as a neuroprotective. In this regard, tetrodotoxin shows significant neuroprotection in numerous models (Tasker *et al*., 1992 and Weber and Taylor, 1994) suggested that neuroprotection can be achieved if only sodium channels are blocked or inhibited. It is possible that the reduction of voltage-gated sodium channels by Vn that we observed in our experiments may also contribute to the neuroprotective effects.

Our data supported by previously well established mechanism that anti-analgesic and anti-epileptic drugs may have ability to inhibit sodium channels. Sodium channel-blocking effect of Vn may provide this herbal medicine as an analgesic and anticonvulsant agent for future remedy.

CONCLUSIONS

Vn has now been shown for the first time to suppress sodium currents in a concentration-dependent manner in cultured Neuro 2A cell line. Our finding provides that Vn can exert anticonvulsant, antidepression and anxiolytic effects in patients and in experimental animals.

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