

## The Effect of *Centella asiatica* (L.) Urban on the Organotypic Model of Spinal Cord Injury (Kesan *Centella asiatica* (L.) Urban pada Model Organotip Kecelakaan Saraf Tunjang)

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### ABSTRACT

*Centella asiatica* (L.) Urban (CA) is a well-known plant used to improve brain and memory functions in traditional medicine. Scientifically it was proven to show neurogenic effect on neural cell lines and in rat's hippocampus. Its effect on spinal cord (SC) neurons, however, have not been studied. Aim of this study was to investigate the effects of raw extract of CA (RECA) on neurite outgrowths in an organotypic model of SC injury (OMSCI). OMSCI was prepared using SC slices obtained from postnatal-day 8 rat pups. Spinal cord tissues were embedded in gelatine gel and sliced to produce 300 µm thick slices. These slices were 100% viable for 8 days in culture. RECA, in concentrations of 0-800 µg/mL was added to the OMSCI media for 7 days, followed by immunostaining for TUJ-1 and GFAP. The investigated parameters were mean neurite count, mean neurite length, mean longest neurite and growth ratio. The tested RECA concentrations showed no cytotoxicity. ANOVA and Kruskal-Wallis tests showed no significant difference between groups in all the tested parameters. This may be due to low content of neurotrophic bioactive compounds content in the extract, which probably due to differences in geographical location, extraction method and absence of neurotrophic factors in the media. In conclusion, the tested RECA concentration were found to be safe; but without notable neurotrophic effects on the spinal cord organotypic model as demonstrated in this study.

**Keywords:** *Centella asiatica*; interface culture; neurite measurements; neurotrophin

### ABSTRAK

*Centella asiatica* (L.) Urban (CA) adalah tumbuhan terkenal yang digunakan untuk meningkatkan fungsi otak dan ingatan dalam perubatan tradisi. Secara saintifik, ia terbukti menunjukkan kesan neurogenik pada sel-sel sel saraf dan hipokampus tikus. Kesannya pada neuron saraf tunjang (ST), bagaimanapun, belum dikaji. Tujuan penyelidikan ini adalah untuk mengkaji kesan ekstrak mentah CA (RECA) ke atas neurit dalam model organotip kecederaan ST (MOKST). MOKST telah disediakan menggunakan kepingan ST yang diperolehi daripada tikus berumur 8 hari posnatal. Tisu saraf tunjang dibenamkan dalam gel gelatin dan dihiris untuk menghasilkan kepingan berketebalan 300 µm. Kepingan ini menunjukkan 100% kebolehhidupan apabila dikultur selama 8 hari. RECA, dengan kepekatan 0-800 µg/mL telah ditambahkan kepada media MOKST selama 7 hari, diikuti dengan pewarnaan imunologi untuk TUJ-1 dan GFAP. Parameter yang dikaji adalah purata bilangan neurit, panjang neurit, neurit terpanjang dan nisbah pertumbuhan. Kepekatan RECA yang diuji tidak menunjukkan kesitotoksikan. Ujian ANOVA dan Kruskal-Wallis tidak menunjukkan perbezaan yang signifikan antara kumpulan bagi semua parameter yang diuji. Ini mungkin disebabkan oleh kandungan bioaktif neurotrof yang rendah dalam ekstrak, yang mungkin disebabkan oleh perbezaan lokasi geografi, kaedah pengekstrakan dan ketiadaan faktor neurotrof dalam media. Kepekatan RECA yang diuji didapati selamat; namun tidak menunjukkan kesan neurotrof pada model organotip saraf tunjang seperti yang diperhatikan dalam kajian ini.

**Kata kunci:** *Centella asiatica*; kultur antara-muka; neurotrofin; pengukuran neurit

### INTRODUCTION

Spinal cord (SC) injury (SCI) occurs due to trauma sustained by the victims of motor vehicle accidents (66%), falls (28%), violence (4%), sports (2%) and other accidents in homes or work places (Ibrahim et al. 2013). The trauma itself results in primary injury to the SC when pieces of vertebral bones puncture the tissue causing disruption of descending and ascending nerve tracts connecting the brain to the peripheral motor and visceral organs (Sadowsky et al. 2002). The event was then followed by secondary injury in

which inflammation and oxidative damage further destroys the tissue. Finally chronic injury occurs when a permanent structure called glial scar, formed from activated astrocytes, further exert a physical barrier for any regenerative process to occur to reconnect the nerve tracts (Norenberg et al. 2004; Silva et al. 2014). Steroids are the first medication given to patient to halt excessive inflammatory reactions to prevent further damage. Surgical treatment such as decompression laminectomy is done to alleviate pressure from the SC. Rehabilitative exercise regimen must then

be undertaken by the patients to strengthen un-paralyzed muscles and maximize their function to compensate for the loss of function of paralyzed limbs. However, no conclusive cure has been found (Ramer et al. 2014).

Therefore, research on novel pharmacological agents are actively being pursued to find a solution for SCI. One branch of that is looking into herbal extracts and in this study, a raw extract of *Centella asiatica* (L.) Urban (RECA) was investigated for potential neurotrophic effect. *Centella asiatica* (L.) Urban (CA) is known in Malay traditional medicine as *pegaga* and is widely taken as a health supplement and in Indian and Chinese traditional medicine, as a brain tonic, to improve memory (Bhavna & Jyoti 2011; Nurlaily et al. 2012; Shinomol et al. 2011). Its key bioactive markers are the triterpenoids; madecassoside, asiaticoside, madecassic acid and asiatic acid (Alqahtani et al. 2015; Lokanathan et al. 2016; Othman 2003; Puttarak & Panichayupakaranant 2012). Other extracts of CA have been tested on human neuroblastoma cell lines such as SH5Y-SY and IMR-32 with both showed increased neurite outgrowths (Soumyanath et al. 2005; Wanakhachornkrai et al. 2013). When given via oral feeding to rats, upon dissection of the rat's hippocampus, it was found that there were increased dendritic arborisation in the centre for memory (Rao et al. 2008).

In this study, the model of choice for investigation of neurotrophic potential of RECA is a 3D *in vitro* model of the spinal cord called the organotypic spinal cord injury model (OMSCI). This model has advantage over the conventional cell culture model as it is produced with minimal processing of the sample, no digestion involved, uses primary tissue, maintains cell-to-cell connections and its extracellular matrix, and is three dimensional. These features make it an ideal *in vitro* model that mimics *in vivo* environment (Gähwiler et al. 1997; Morrison et al. 1998; Shamir & Ewald 2014; Simoni & Yu 2006; Stoppini et al. 1991; Sypecka et al. 2015; Weightman et al. 2014; Yang & Xiong 2012).

## MATERIALS AND METHODS

### PREPARATION OF RAW EXTRACT OF *CENTELLA ASIATICA* (L.) (RECA)

The leaves of *Centella asiatica* (L.) plant grown in Pulau Pinang, Malaysia was used to prepare raw extract of *Centella asiatica* (L.), RECA (UiTM; voucher specimen no. CA-K017). The plant was washed, cleaned and dried in the oven at 40°C. 50 kg powdered *Centella asiatica* (L.) leaves was extracted in five batches. In each batch, 10 kg of *Centella asiatica* (L.) powder was extracted with 95% denatured ethanol (60 L ethanol + 40 L deionized water) for 8 h at 60°C temperature. 14.8 L of concentrated liquid extract was obtained and was freeze-dried to obtain 7.96 kg of dried extract (15.92% yield). Dried extract was stored at room temperature until further use. Bioactive compounds of RECA was determined using High Performance Liquid Chromatography (HPLC).

### PRODUCTION OF ORGANOTYPIC SPINAL CORD INJURY MODEL

Wistar rat pups aged 8 postnatal-days were decapitated, sprayed with 70% alcohol and their vertebra was exposed. Then, the laminae on both sides of the spinous processes of the vertebra was cut and the spinous process was removed to expose the SC using microsurgical scissors. Then all the spinal nerves on both sides of the SC were cut before SC was removed from the spinal canal of the vertebra and immediately placed in cold Hank's Balanced Salt Solution (HBSS) (4°C). The SC is then placed in a square block gel mould and 10% gelatine (Sigma-Aldrich, USA) solution was poured into the mould. The mould with SC and gelatine was then placed in ice to slowly cool it down for 15 min. Once the gel has solidified, it was removed from the mould and glued to the vibratome stage with tissue glue cyanoacrylate (Loctite 404; Henkel Corp., Rocky Hill, Conn., USA). The embedded SC was sliced using Leica Vibratome VT1000. The vibratome blade frequency and amplitude were set at a constant of notch 1 and 10, respectively. The slice thickness of 300 µm and transverse sectioning were found to be optimal in terms of slice intactness and those slices were used throughout this study. Only intact slices were collected in cold HBSS and further cultured organotypically.

### CULTURE WITH RECA

Organotypic culture of the SC slices performed on culture inserts placed in a 6-well plate. Two types of culture medium were prepared for organotypic culture of the SC, with horse serum (HS) and without serum (serum free - SF). 100 mL medium with HS was prepared with the ratio of 50% DMEM-HG, 1% HEPES, 25% HBSS, 25% HS, 1% glutamax and 1% antibiotic-antimycotic. SF-medium was prepared in the ratio 75% DMEM/F12, 25% HBSS, 1% glutamax, 5 mL/mL glucose, 1% antibiotic-antimycotic, 1% N2 and 1% B27. All culture media and supplements were purchased from GIBCO, USA, unless stated otherwise. Briefly, 1 mL of organotypic culture media was pipetted into each well and the culture inserts were placed into the wells. Then, three SC slices were then placed in a culture insert. The plate containing the culture inserts were incubated at 37°C and 5% CO<sub>2</sub>. The remaining solid gelatine melted in the incubator and was pipetted out during media change. After 24 h of culture, RECA was supplemented into the culture media in different concentrations (0, 100, 200, 400 and 800 µg/mL) and media change was done every day (Soumyanath et al. 2005; Wanakhachornkrai et al. 2013). Medium with HS was used in the first 5 days of culture, while SF-medium was used in the remaining 3 days.

### WHOLE MOUNT IMMUNOHISTOCHEMISTRY

After 8 days in culture, the slices were immune-stained. Briefly, for each step, 500 µL of each reagent was pipetted onto the culture inserts and 500 µL below the inserts. The slices were washed with DPBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 30 min at

4°C. Then, they were washed with washing solution (0.1% Tween 20 in 1% goat serum in DPBS) for 3 times, 10 min each, permeabilized with 0.5% Triton X for 40 min and then blocked with 10% goat serum (10 mL goat serum in 90 mL DPBS) for 1 h at 37°C. The slices were then incubated with primary antibody solution (1:500 for GFAP and 1:1000 for TUJ-1) overnight at 4°C. The following day, the antibody solution was discarded and then the slices were incubated with secondary antibody solution was added (Goat anti-rabbit IgG FITC and Goat anti-Mouse IgG Alexa Fluor 594 with 1% goat serum solution at ratios of 1:300) and plates placed at 37°C for 1 h. Nuclei were then counterstained with DAPI solution (1:15000) at room temperature for 40 min. After that, the slices were washed three times with DPBS and observed under fluorescence microscope.

#### NEURITES MEASUREMENT

Analysis was done using images taken with Nikon Eclipse Ti Fluorescence microscope (Nikon, Japan) and the NIS-element software. 5 regions of interest (ROI) were marked on each SC slice at the edge of the SC slices where the neurite outgrowths sprouted out. Each ROI measures 200 × 200 μm. 4 parameters were measured: mean neurite length, mean neurite count, mean longest neurite and mean growth ratio for each group. For mean neurite length, the length of each neurite in each ROI was measured and the mean value for the ROI is calculated. This mean value is added up with the mean value of the other ROI and divided by 5 to find the mean value for the slice. Then, the mean value for the slices are added up and divided by the number of slices to find the mean neurite length value for the group. The same method is used to calculate mean neurite count for the group. Instead of lengths, the number of neurites in each ROI is measured. For the mean longest neurite, the longest neurite measured in each ROI are added up and divided by the number of ROI to find the mean longest neurite for the slice. Then, the mean longest neurite of all the slices are

added up and divided by the number of slices to find the mean longest neurite for the group. Lastly, growth ratio for each slice is calculated by measuring the surface area of the slice and its neurite outgrowth divided by the surface area of the slice alone. The growth ratios of the slices are added up and divided by the number of slices to find the mean growth ratio for the group.

#### STATISTICAL ANALYSIS

Anova and Kruskal-Wallis test was done with SPSS software to identify the statistically significant differences between the groups ( $n=5$ ,  $p<0.05$ ).

#### RESULTS

##### CHEMICAL ANALYSIS OF RECA

HPLC shows four bioactive compounds detected in RECA (Figure 1) which comprised of madecassoside (0.0060%), asiaticoside (0.0035%), madecassic acid (0.0020%) and asiatic acid (0.0017%).

##### EFFECT OF RECA ON NEURITES

As the SC organotypic slices were thick, z-stacking and z-projection of fluorescence images of the SC slices after organotypic culture with RECA and whole mount immunohistochemistry were performed (Figure 2). The microscopic analysis showed neurite outgrowths from the SC slices in all the groups indicating RECA causes no toxicity to neurite outgrowths. However, no clear differences in the parameters of the neurites can be seen with the eye and therefore these images were then analysed with *NIS-Elements* microscope imaging software (Nikon, Japan) for measurements of the four parameters (Figure 3). Results from ANOVA and Kruskal-Wallis test on the measured data showed no significant differences detected

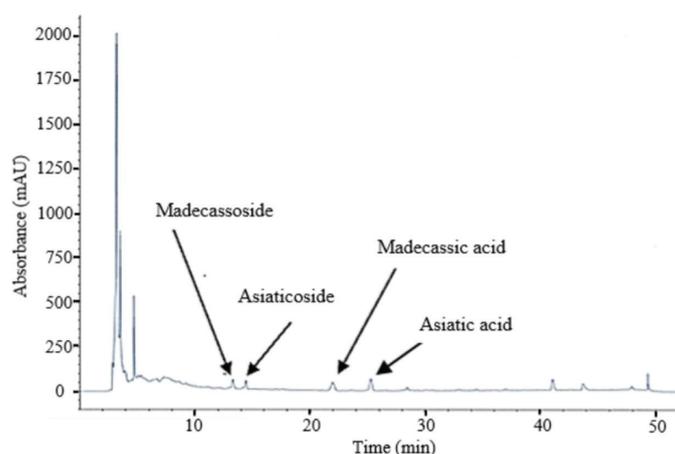
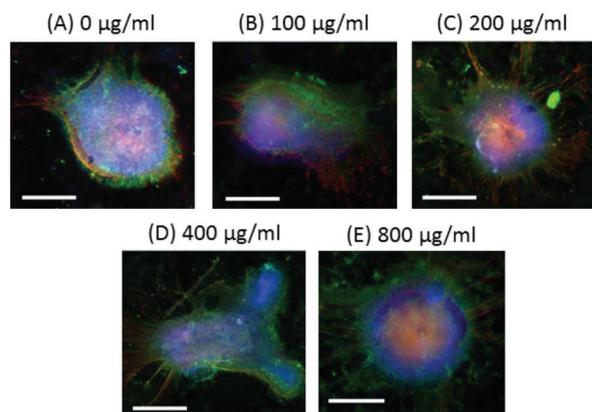


FIGURE 1. HPLC analysis of bioactive compounds of RECA (Reproduced with permission from *Regenerative Research* 5(2) 2017 10-18. Copyright Tissue Engineering and Regenerative Medicine Society of Malaysia)



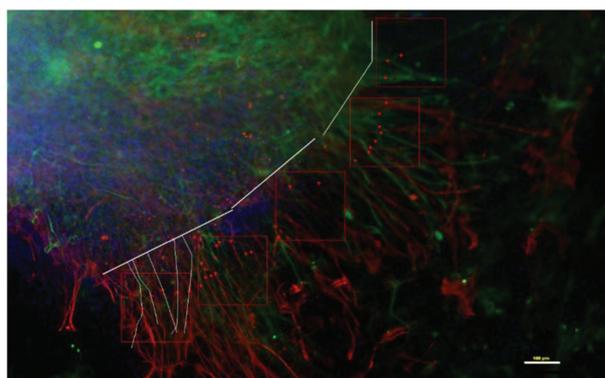
Bar represent 1000 µm

FIGURE 2. Projection of the z-stack fluorescent images of spinal cord slices and its neurite outgrowths. The slices were cultured in media containing RECA at A) 0 µg/mL, B) 100 µg/mL, C) 200 µg/mL, D) 400 µg/mL and E) 800 µg/mL. Blue: cell nucleus, green: neurite outgrowths, red: astrocytes

between groups for all Mean Neurite Length, Mean Longest Neurite, Mean Neurite Count and Mean Growth Ratio (Figure 4).

#### DISCUSSION

These results indicated that concentrations of RECA tested on organotypic spinal cord slice cultures showed no neurotrophic effect on neurite outgrowths. Several



Bar represent 100 µm

FIGURE 3. Enlarged (10×) fluorescent image of a representative spinal cord slice and its neurite outgrowths for the group cultured with 100 µg/mL of RECA. The red boxes are the ROIs and the red dots mark neurite outgrowths for neurite count. White lines are the measurement of length of the neurite outgrowths. Blue: cell nucleus, green: neurite outgrowths, red: astrocytes

factors could have contributed to this result. Firstly, it could be due to the content of bioactive substances in the extract, which varies between extracts depending on their geographical origin, extraction method, solvent polarity and ratio. For example, studies done by Putarak and Panichayupakaranant (2017) and Zhang et al. (2009) showed that CA taken from different parts of Thailand and China was shown to have different amounts of the bioactive contents. Azwanida (2015) and Chew et

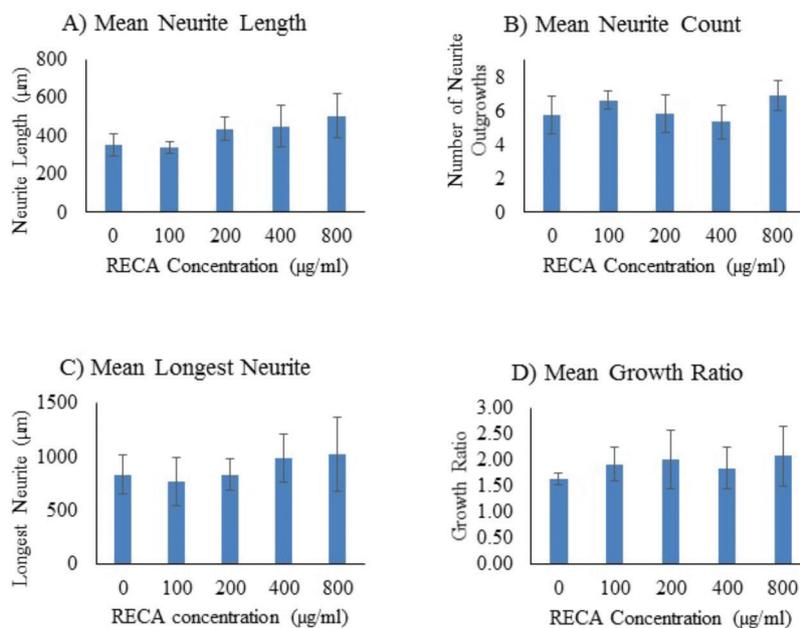


FIGURE 4. (A) Mean neurite lengths vs. RECA concentration. The mean neurite length increases as the concentrations of RECA increase ( $n=6$ ), (B) mean neurite count vs. RECA concentration. No trend can be seen in the mean neurite count as the RECA concentration increases ( $n=6$ ), (C) Mean longest neurite vs. RECA concentration. A slight increasing trend can be seen in the mean longest neurite as the RECA concentration increases ( $n=5$ ) and (D) Mean growth ratio vs. RECA concentration. No trend occurs in the mean growth ratio as RECA concentration increase ( $n=6$ ). No significant difference was detected between groups and  $p>0.05$  in all the above parameters

al. (2011) explained that different extraction methods, types of plant samples (fresh or dried), nature of samples (grounded or powdered), and the extraction protocols (air dried, oven, microwave and freeze drying, maceration, infusion, percolation and decoction, Soxhlet extraction or hot extraction) can result in differential bioactive content of the final extract (Azwanida 2015; Chew et al. 2011; Yung et al. 2010). Finally, Soumyanath et al. (2005) tested the effect of different solvent polarity and ratios of solvents on the final extract's ability to impart neurotrophic activity on neuronal cells. They found that solvent polarity and ratios affect significantly on the neurotrophic ability of the extract. Therefore, it could be possible that RECA does not contain enough amount of neurotrophic substance in it due to its different geographical origin, extraction method or solvent used from the CA extracts reported to have neurotrophic effects. Wanakhachornkrai et al. (2013) reported enhanced neurite outgrowth in IMR-32 cells with a CA extract called ECa233 which is produced in Thailand and is standardized to contain 80% triterpenoids with 52% madecassoside and 32% asiaticoside (Wanakhachornkrai et al. 2013). The CA extract used in this study, RECA, is produced from CA plants in Pulau Pinang, Malaysia and once analyzed with high performance liquid chromatography (HPLC) was shown to contain 0.006% madecassoside and 0.0035% asiaticoside (Omar et al. 2017). These substances could be the ones imparting neurotrophic effect on neuronal cells and their low concentration in RECA could be the reason for RECA's lack of neurotrophic activity.

Another factor that could have affected the outcome of this study is the presence or absence of known neurotrophic substance such as nerve growth factor (NGF). Neurotrophic factors are proteins with functions during development, to regulate neuron cell survival and for mature nervous system, synaptic function and plasticity, cell fate differentiation, controlling proliferation, differentiation and axonal growths (Huang & Reichardt 2001). In the study done by Soumyanath et al. (2015), SH5Y-SY cells were cultured with different concentrations of an ethanolic CA extract of various polarity in the presence of NGF. They found that neurotrophic effect of the extract only presented itself when combined with NGF and its degree was more in the group with NGF alone. The CA extract without NGF group showed no neurotrophic activity (Soumyanath et al. 2005). This indicates that CA extract could be acting in synergy with NGF rather than possessing neurotrophic activity on its own. Therefore, the absence of NGF in this study could be the reason that RECA did not show neurotrophic activity.

Although this study was not able to demonstrate the neurotrophic effect of raw extract of CA (RECA) on neurite growths from the SC, the evidence of neurotrophic effects of other extracts of CA on other nerve cells and on rat models are proven in various publications (Lokanathan et al. 2016). Therefore, further research should be carried out, either by using established standardized extracts

such as ECa 233 or other extracts from other geographic origin, processing methods or solvents and the bioactive compounds content that have been determined to be higher. Furthermore, the effect of other neurotrophic factors such as Brain-derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT-3) should also be studied. As implicated by Gurcan et al. (2016) and Jiang et al. (2016), the bioactive components of CA namely asiaticoside, madecassoside, asiatic acid and madecassic acid can also be tested individually using this model. This will facilitate accurate identification of the neurotrophic bioactive substance to be developed into therapeutics.

## CONCLUSION

RECA, which was not toxic to neuronal cells up to 800 µg/mL, was found to show no neurotrophic activity in this setting. Although the neurite length showed an increasing trend with increasing concentration of RECA, the difference between groups were not significant. The neurite count and growth ratio also showed a non-consistent pattern. This could be due to low concentration of bioactive neurotrophic substance in RECA, specifically madecassoside and asiaticoside. Therefore, further studies investigating the effect of the individual bioactive compounds and the standardized CA extract on the organotypic model of spinal cord injury is necessary.

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