

Spinogenesis and Synaptogenesis Effects of the Red Seaweed *Kappaphycus alvarezii* and Its Isolated Cholesterol on Hippocampal Neuron Cultures

Gabriel Tirtawijaya¹, Md. Nazmul Haque², Jae Sue Choi³, Il Soo Moon², Maria Dyah Nur Meinita⁴, Jae-Suk Choi⁵, and Yong-Ki Hong¹

¹Department of Biotechnology and ³Department of Food and Life Science, Pukyong National University, Busan 48513, Korea

²Department of Anatomy, College of Medicine, Dongguk University, Gyeongju, Gyeongbuk 38066, Korea

⁴Faculty of Fisheries and Marine Science, Center for Maritime Biosciences Studies, Jenderal Soedirman University, Purwokerto, Jawa Tengah 53123, Indonesia

⁵Division of Bioindustry, Silla University, Busan 46958, Korea

ABSTRACT: Neurotrophic factors promote the formation of spines and synapses in neuron development and maintenance. Synaptic connections enhance memory in the brain. In this study, the effects of *Kappaphycus alvarezii* ethanolic extract (EKA) and its isolated cholesterol (iCHOL) on spinogenesis and synaptogenesis of hippocampal neurons were evaluated. Compared with the vehicle, both EKA and iCHOL significantly promoted generation of dendritic filopodia (2.4- and 2.2-fold, respectively) and spine (1.7- and 1.4-fold) formations in spinogenesis; they also increased presynaptic (3.6- and 2.6-fold), postsynaptic (2.5- and 2.9-fold), and cocolonized (3.8- and 3.0-fold) puncta, which enhances synaptic function ($P < 0.05$). Further, EKA- and iCHOL-treated neurons showed significantly improved functional presynaptic plasticity (1.6- and 1.4-fold, respectively, at 17 days *in vitro*; $P < 0.05$). These results indicate that *K. alvarezii* facilitates neuronal development, and support its use as a functional food to reduce neurological disorders and prevent brain aging via helping to reconstruct partially damaged neural networks.

Keywords: cholesterol, hippocampal neuron, *Kappaphycus alvarezii*, spinogenesis, synaptogenesis

INTRODUCTION

Aging-related neurodegenerative diseases can be prevented and treated by administration of neurotrophic factors (Weissmiller and Wu, 2012). These diseases are marked by the progressive loss of neurons in the central nervous system along with cognitive decline. Neurotrophic factors are important for facilitating the survival, maintenance, and development of neuron cells (Sampaio et al., 2017).

The red seaweed *Kappaphycus alvarezii* (commercially known as cottonii) is well-known for its ability to produce carrageenan. The tropical seaweed is native to the Philippines and has distributed to other countries in Asia, Africa, America, and Oceania (McHugh, 2003). *K. alvarezii* grows well in 30~35 ppt salinity at 25~30°C and with moderate water movement under 0.5~1.0 m. In 2017, approximately 8.5 million tons of cottonii were produced

by aquaculture in Indonesia (FAO, 2019). A great advantage of this seaweed is its high abundance and fast growth. In addition to its benefits as a source of carrageenan, *K. alvarezii* is reported to have preventive effects on diet-induced metabolic syndrome (Wanyonyi et al., 2017). *K. alvarezii* also possesses cardiovascular protective (Matanjun et al., 2010), anti-inflammatory (Ranganayaki et al., 2014), antimicrobial (Prabha et al., 2013), antioxidant (Nagarani and Kumaraguru, 2012), and wound healing (Fard et al., 2011) activities. According to Pangestuti and Kim (2011), the neuroprotective effects of *K. alvarezii* are derived from its antioxidant, anti-neuroinflammatory, and anti-cholinesterase activities.

In our previous studies of 34 seaweeds collected from Indonesia, an ethanolic extract of *K. alvarezii* (EKA) possessed the highest neurotrophic activities by accelerating neurite growth (Tirtawijaya et al., 2016). The EKA con-

Received 10 June 2019; Accepted 30 September 2019; Published online 31 December 2019

Correspondence to Yong-Ki Hong, Tel: +82-51-629-5862, E-mail: ykhong@pknu.ac.kr

Author information: Gabriel Tirtawijaya (Graduate Student), Md. Nazmul Haque (Researcher), Jae Sue Choi (Professor), Il Soo Moon (Professor), Maria Dyah Nur Meinita (Professor), Jae-Suk Choi (Professor), Yong-Ki Hong (Professor)

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tained several kinds of neurotrophic compounds including sterol, ispronidine, oleic acid, stigmast-4-ene-3,6-dione, oxysterol, and campesterol (Tirtawijaya et al., 2018). Development of dendritic morphology in neuronal cytoarchitecture has a role in synapse formation and, subsequently, synapses promote further dendritic arbor (Cline, 2001). Synaptic vulnerability is a crucial factor in neurological diseases. In neurodegenerative diseases, disruption of synaptic form and function occurs comparatively early, preceding the onset of degenerative changes in the neuronal cell body (Gillingwater and Wishart, 2013). Synapse-specific neuroprotective strategies will be required to provide effective therapeutics to delay or halt the progression of neurodegenerative conditions. Thus, we investigated the effects of EKA and its isolated cholesterol (iCHOL) on dendritic morphology in spinogenesis and synaptogenesis of hippocampal neuron cells, and we identified the major sterol compound from *K. alvarezii* as cholesterol.

MATERIALS AND METHODS

Preparation and extraction of the seaweed *K. alvarezii*

Fresh thalli of the carrageenophyte *K. alvarezii* were collected from an aquaculture farm in West Nusa Tenggara (8°42'18.3"S, 116°46'45.9"E), Indonesia, in 2017 and 2018. Thalli were thoroughly rinsed with seawater followed by freshwater to eliminate all debris, and dried in the shade for 3 days. The dried tissues were ground to a powder using a grinder and stored in the dark at 18°C until use. A voucher specimen was deposited in the author's laboratory (Y.K. Hong). The methods for extraction and fractionation of EKA followed the procedure described by Tirtawijaya et al. (2018).

Identification of active compounds in *K. alvarezii*

In our previous study, we found that one of the major neurotrophic compounds in EKA was a sterol-related compound (Tirtawijaya et al., 2018). To identify the sterols present in this study, we fractionated EKA to collect active neutral, moderately polar, and basic fractions in accordance with their polarity (Harborne, 1998). We combined the 3 fractions, and then separated them by reverse-phase (RP) high-performance liquid chromatography (HPLC). We then carried out an isocratic program using a mixture of acetonitrile, methanol, and ethyl acetate (1:1:3) as the mobile phase. The mixture was eluted through a reverse-phase C18 column (1 cm i.d. × 25 cm) (Ultrasphere; Beckman Coulter, Fullerton, CA, USA) with a flow rate of 2 mL/min and detection was by a refractive index detector (Shodex RI-101 detector; Showa Denko K.K., Tokyo, Japan) and each peak was dried completely under a stream of nitrogen gas. Samples were dis-

solved in chloroform-*d* (CDCl₃) prior to analysis of ¹H (600 MHz) and ¹³C (151 MHz) nuclear magnetic resonance (NMR) spectra using a JNM-ECP 600 NMR spectrometer (JEOL, Tokyo, Japan). The structures of the isolated compounds were identified and confirmed as being identical to the spectral data reported by Khatun et al. (2017) and Nasir et al. (2011).

Primary culture and treatment of hippocampal neurons

All animal care and procedures were carried out in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committee of the College of Medicine, Dongguk University, Korea. The ethics committee approved this study under protocol IACUC-2015-002. Fetuses were collected at embryonic day 19 of pregnant rat. The brains were dissected to collect the hippocampal tissues, and hippocampal neuron cells were prepared as described previously (Goslin et al., 1998). Briefly, the prepared cells were plated in 24-well plates, which had a poly-DL-lysine-coated coverslip in each well, at a density of approximately 1 × 10⁴ cells/cm² for morphometric studies of early development, or a density of 2 × 10⁴ cells/cm² for spinogenesis and synaptogenesis analyses. For spinogenesis and synaptogenesis analyses, neurons were seeded in neurobasal medium containing 10% fetal bovine serum. After incubation for 4 h, the medium was changed for serum-free neurobasal medium. Half the volume of the medium was replaced with fresh medium (containing extract, isolated compound, or dimethyl sulfoxide) every 4 days to maintain the cells.

Neuronal staining

At indicated days *in vitro* (DIV), neurons on coverslips were stained with green fluorescent Vybrant DiO (Benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate; Molecular Probes, Eugene, OR, USA) to observe the development of filopodial (DIV 12) and spines (DIV 17). For synapse formation, cell cultures at DIV 17 were fixed using a paraformaldehyde-methanol fixation procedure (Moon et al., 2007). The fixed cells were stained using primary antibodies against synaptic vesicle protein 2 (SV2; mouse monoclonal, 1:1,000; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and postsynaptic density protein-95 (PSD-95; rabbit polyclonal, 1:1,000; Upstate Biotechnology, Inc., Lake Placid, NY, USA). After incubation overnight at 4°C, cells were stained for 1 h with secondary antibodies at room temperature. To visualize synaptic vesicles and recycling activities at presynaptic terminals, neurons were incubated in depolarizing extracellular solution containing 10 μM FM1-43 dye [N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide; Molecular Probes] for 3 min and washed 3 times (5 min each) with FM1-43-free solution.

Image acquisition and analysis

Images (1,388×1,039 pixels) were acquired by phase-contrast and epifluorescence microscopy using a Leica Research Microscope DM IRE2 (Leica Microsystems AG, Wetzlar, Germany) equipped with a high-resolution charge-coupled device camera (CoolSNAP™, Photometrics Inc., Tucson, AZ, USA) and Leica FW4000 software (Leica Microsystems AG). DiO stained or immunostained cells were observed under a 100× oil immersion objective lens. FM1-43 stained presynaptic vesicles were observed under a 40× objective lens. The digital images were processed using Adobe Photoshop CS6 (Adobe Inc., San Jose, CA, USA) and analyzed using ImageJ software (version 1.45, National Institute of Health, Bethesda, MD, USA) for morphometric assessment, arbitrary intensity values assessment, and quantification. Filopodia were morphologically identified as dendritic protrusions with a long slender structure, without a head, and of a length of more than 2 μm, whereas spines were classified as dendritic protrusions with a bulbous head attached to dendrites by a narrow stalk with a length of less than 2 μm (Harris and Spacek, 2001). The number of primary neurites (NPN), total length of primary neurites (TLPN), and length of the longest neurite (LLN) were measured for morphometric studies of early development.

Data analysis

Data were expressed as means±standard error (SE) of at least three independent experiments. Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons *post hoc* test using an SPSS 17.0 program (SPSS Inc., Chicago, IL, USA). Data were considered statistically significant at $P < 0.05$.

RESULTS

Identification of the main sterol in *K. alvarezii*

K. alvarezii powder was extracted with ethanol and successively fractionated into 5 main classes of constituents in accordance with their polarity. All the active fractions of neutral, moderately polar, and basic fractions were combined and then re-fractionated by RP-HPLC. One major active peak at a retention time of 11.5 min was collected and dried, generating a white crystalline powder. This peak was tentatively assumed to be the same sterol compound identified in previous studies using gas chromatograph (GC)-mass spectrometry (MS). The presence of cholesterol as a single compound in the peak was confirmed by injecting several sterols including the standard cholesterol (sCHOL) reagent (C8667, Sigma-Aldrich Co., St. Louis, MO, USA) into the RP-HPLC; the symmetric peak spike was observed at the same retention time of

11.5 min. The structure of the isolated sterol from *K. alvarezii* was re-confirmed based on ¹H-NMR and ¹³C-NMR data analysis. The spectroscopic data of ¹H-NMR (600 MHz, CDCl₃) were δ: 5.37~5.34 (1H, m), 3.52 (2H, t), 2.37~2.27 (3H, m), 2.26~2.19 (2H, m), 1.98 (4H, m), 1.86~1.90 (6H, m), 1.65~1.40 (22H, m), 1.38~1.30 (8H, m), 1.25 (10H, s), 1.18~1.05 (16H, m), 1.02~0.96 (10H, m), 0.92 (8H, d), 0.86 (13H, m), and 0.68 (6H, s). The ¹³C-NMR (151 MHz, CDCl₃) spectrum was δ: 140.84 (s), 121.81 (s), 71.96 (s), 56.84 (s), 56.22 (s), 50.27 (s), 42.45 (s), 42.38 (s), 39.85 (s), 39.59 (s), 37.33 (s), 36.58 (s), 36.26 (s), 35.87 (s), 31.99 (s), 31.97 (s), 31.74 (s), 28.31 (s), 28.17 (s), 24.37 (s), 23.97 (s), 22.97 (s), 22.64 (s), 21.16 (s), 19.48 (s), 18.79 (s), and 11.94 (s). These spectral data completely matched data for cholesterol. Therefore, we concluded that the most abundant sterol from *K. alvarezii* is cholesterol.

To quantify the amount of cholesterol (as a quantification indicator) in *K. alvarezii* tissue, standard calibration solutions ranging from 0.625 to 5 mg/mL were prepared to construct a linear regression curve; calibration curve y (μRIU) = 6.68x (mg/mL) + 1.49 showed a correlation coefficient R^2 of 0.999 ($R^2 > 0.99$ indicates linearity). Using this method for determination, the amount of cholesterol in the EKA was found to be 4% (w/w), which represents 20 mg/100 g dry weight of *K. alvarezii* powder.

Cholesterol supported neurotrophic activity

We investigated at which concentration of iCHOL extracted from *K. alvarezii* possesses optimal neurotrophic activity. iCHOL was added to the culture media at concentrations ranging from 1 to 30 μg/mL. At concentrations of 1 to 10 μg/mL, NPN (Fig. 1A) and TLPN (Fig. 1B) significantly increased compared with the vehicle ($P < 0.05$), whereas LLN (Fig. 1C) did not show any significant difference. iCHOL showed an inhibitory effect at the concentrations of 20 and 30 μg/mL, demonstrating a significant decrease in all the neurotrophic parameters ($P < 0.05$). Thus, the optimal concentration of iCHOL was 10 μg/mL; this concentration supported growth of TLPN (1.3-fold) and increased NPN (1.2-fold) compared with the vehicle.

In addition, we compared the neurotrophic activities of iCHOL, sCHOL, and EKA. We added 10 μg/mL iCHOL and sCHOL of 10 μg/mL and the optimal concentration 1 μg/mL of EKA (Fig. 1D). We did not find any significant differences between iCHOL and sCHOL on neurotrophic activities, but EKA treatment increased TLPN 1.2- and 1.2-fold above those in the iCHOL and sCHOL groups, respectively ($P < 0.05$). Similar results were observed for iCHOL and sCHOL on neurotrophic activity. In addition, more potent activities were shown for EKA with several neurotrophic compounds than a single cholesterol compound.

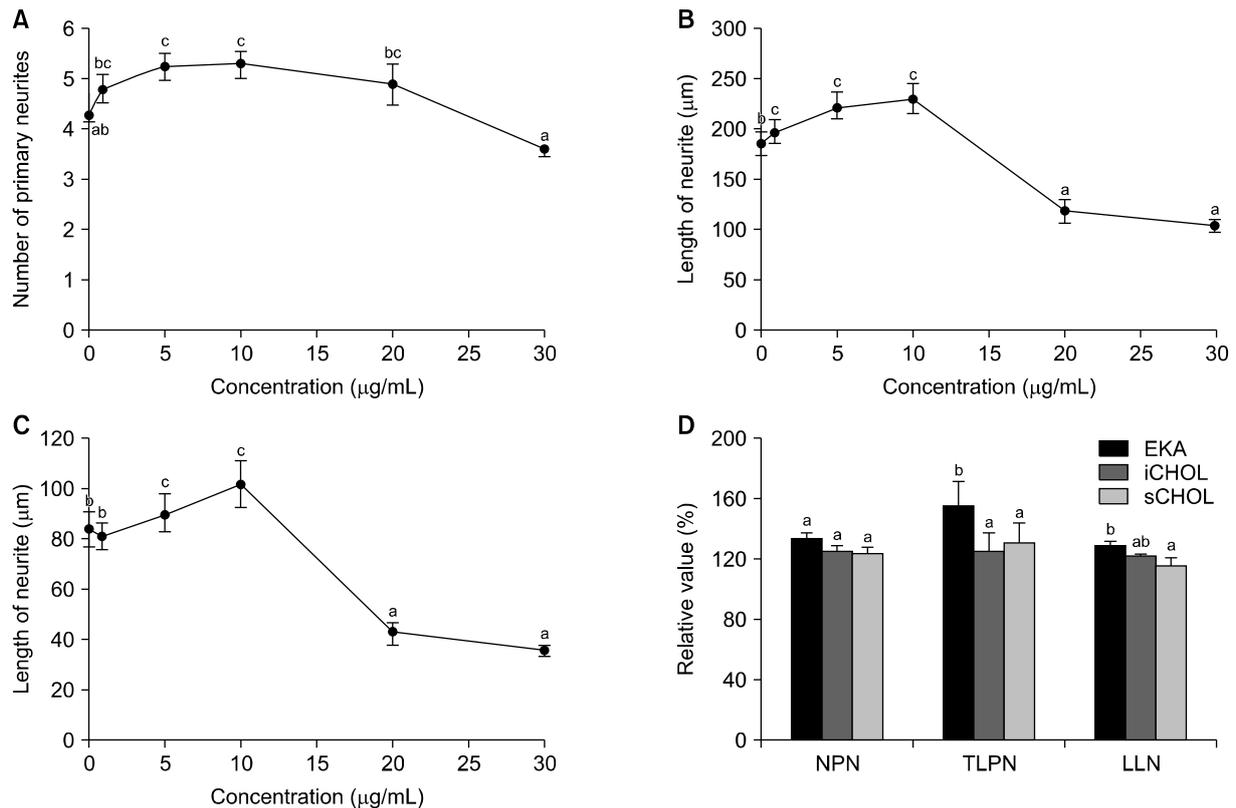


Fig. 1. Effects of *Kappaphycus alvarezii* ethanolic extract (EKA), isolated cholesterol (iCHOL), and standard cholesterol (sCHOL) on hippocampal neurons at 3 days *in vitro*. Optimization of iCHOL for (A) number of primary neurites (NPN), (B) total length of primary neurites (TLPN), and (C) length of the longest neurite (LLN). "0" indicates vehicle. Comparison of EKA, iCHOL, and sCHOL on relative values of NPN, TLPN, and LLN (D). Data represent means \pm SE ($n=50$ neurons). Different letters (a-c) indicate statistically significant differences between the means ($P<0.05$).

EKA and iCHOL promoted spinogenesis and synaptogenesis

To determine the effects of EKA and iCHOL on dendrite spinogenesis, we measured filopodial growth (Fig. 2A and 2B) and spine formation (Fig. 2C and 2D). Formation of dendritic spines is preceded by development of filopodia. DiO staining was applied to neurons at DIV 12 to visualize dendritic filopodia. EKA (1 $\mu\text{g}/\text{mL}$) and iCHOL (10 $\mu\text{g}/\text{mL}$) increased the number of filopodia 2.4- and 2.2-fold higher, respectively, than the vehicle ($P<0.05$). To evaluate spine formation, neurons were stained with DiO at DIV 17. Spine formation was 1.7- and 1.4-fold higher following treatments with EKA and iCHOL, respectively, compared with the vehicle ($P<0.05$). These results suggest that both EKA and iCHOL promote spinogenesis of hippocampal cells.

The development of dendritic spines supports the occurrence of new synapses. Because excitatory synapses occur on dendritic spines, formation of new spines steadily generate functional synapses. The numbers of presynaptic (SV2 staining) and postsynaptic (PSD-95 staining) puncta and their colocalized puncta (synapses) were analyzed to assess synaptogenesis in cultured hippocampal neurons at DIV 17 (Fig. 3). Both EKA and iCHOL significantly improved the densities of pre- and postsyn-

aptic puncta ($P<0.05$) compared with the vehicle. EKA and iCHOL increased the number of presynaptic puncta 3.6- and 2.6-fold, respectively, compared with the vehicle ($P<0.05$). EKA treatment increased the number of presynaptic puncta 1.4-fold compared with the iCHOL group ($P<0.05$). Meanwhile, EKA and iCHOL treatments increased the numbers of postsynaptic puncta 2.5- and 2.9-fold, respectively, compared with the vehicle ($P<0.05$). Although the number of postsynaptic puncta was 1.2-fold higher following iCHOL treatment compared with EKA treatment, these treatments did not significantly differ. Similarly, treatments with EKA and iCHOL increased the number of colocalized puncta 3.8- and 3.0-fold compared with the vehicle, respectively ($P<0.05$), but EKA and iCHOL treatments did not statistically differ. These results show that both EKA and iCHOL significantly promote synaptogenesis by increasing the expression of pre- and postsynaptic puncta and synapse puncta. Further, 1 $\mu\text{g}/\text{mL}$ of the EKA containing several neurotrophic compounds showed similar levels of activity as 10 $\mu\text{g}/\text{mL}$ of the single cholesterol compound.

EKA and iCHOL improved functional presynaptic plasticity

During the synaptogenic phase, neurons were stained

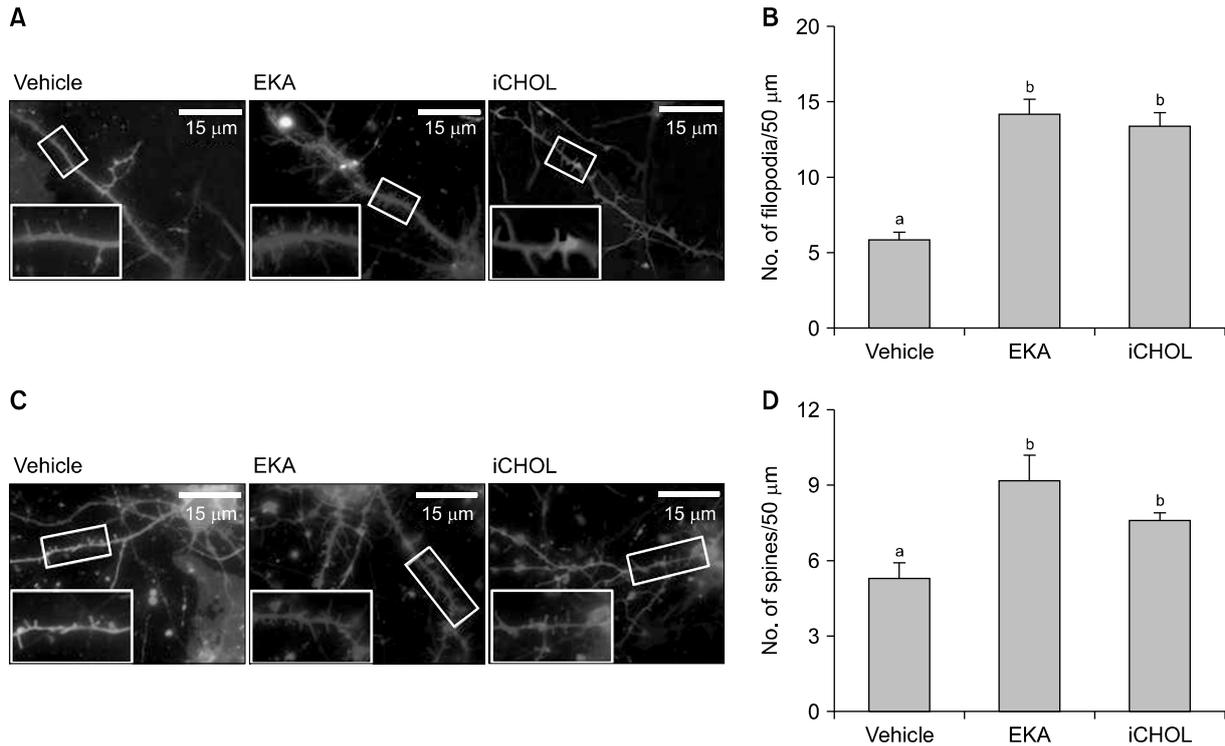


Fig. 2. Formation of filopodia and spines on *Kappaphycus alvarezii* ethanolic extract (EKA)- and isolated cholesterol (iCHOL)-treated neurons. (A) Representative fluorescent images of filopodia formation at days *in vitro* (DIV) 12. (B) Number of filopodia per 50 μm of dendritic segments. (C) Representative fluorescent images of spine formation at DIV 17. (D) Number of spines per 50 μm of dendritic segments. Different letters (a,b) indicate statistically significant differences between the means ($P<0.05$). Bars represent means \pm SE (n=25 neurons).

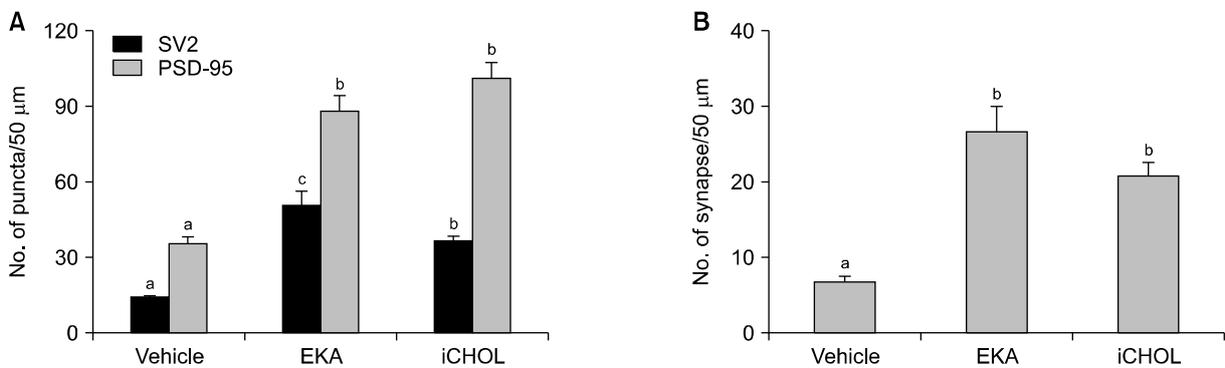


Fig. 3. Synaptogenesis of *Kappaphycus alvarezii* ethanolic extract (EKA)- and isolated cholesterol (iCHOL)-treated neurons at 17 days *in vitro*. (A) Number of puncta for synaptic vesicle protein 2 (gray bar) and postsynaptic density protein-95 (black bar) per 50 μm of dendritic segments. (B) Number of colocalized puncta (synapse) per 50 μm of dendritic segments. Different letters (a,b) indicate statistically significant differences between the means ($P<0.05$). Bars represent means \pm SE (n=25 neurons).

with FM1-43 on 2 different days (DIV 12 and DIV 17) to evaluate their functional presynaptic plasticity (Fig. 4). The intensities of stained puncta showed that both EKA- and iCHOL-treated cells had significantly higher intensities than neurons treated with the vehicle (1.5- and 1.4-fold, respectively; $P<0.05$) at DIV 12. Subsequently, at DIV 17, EKA and iCHOL treatments significantly increased the intensities of the stained puncta 1.6- and 1.4-fold, respectively, compared with the vehicle ($P<0.05$). Supplementation with EKA and iCHOL improved the activities of synaptic vesicles and transmission. At both DIV 12 and 17, EKA-treated cells showed signifi-

cantly higher puncta intensities than iCHOL-treated cells (1.1- and 1.2-fold, respectively; $P<0.05$). Thus, cholesterol was a representative sterol by its abundance in the EKA, and was one of the neurotrophically active compounds in the EKA and in *K. alvarezii*.

DISCUSSION

The red seaweed *K. alvarezii* is a well-known producer of carrageenan and other diverse biologically active substances. Our previous studies showed that *K. alvarezii* ex-

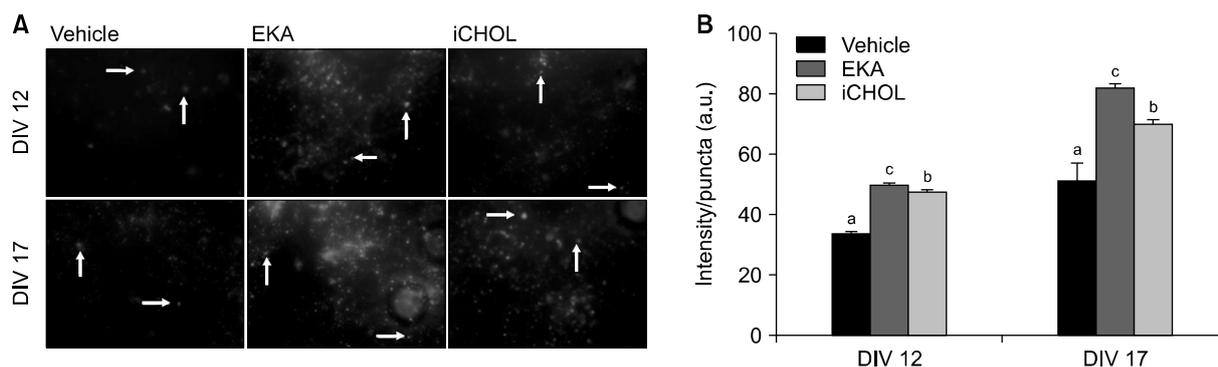


Fig. 4. Effects of *Kappaphycus alvarezii* ethanolic extract (EKA) and isolated cholesterol (iCHOL) on FM1-43-stained neurons at days *in vitro* (DIV) 12 and DIV 17. (A) Representative images of synaptic vesicles in vehicle-, EKA-, and iCHOL-treated cells. White arrows indicate the individual punctae. (B) Comparison of vehicle, EKA, and iCHOL on the intensity of FM1-43-labeled puncta. a.u., arbitrary unit. Different letters (a-c) indicate statistically significant differences between the means ($P < 0.05$). Data represent means \pm SE ($n = 300 \sim 350$ puncta).

hibited neurite outgrowth activity (Tirtawijaya et al., 2016) and that it contains sterol as one of the major compounds in the active lipophilic fractions (Tirtawijaya et al., 2018). We also identified the neurotrophic compounds ispronidine, oleic acid, stigmast-4-ene-3,6-dione, oxysterol, and campesterol by GC-MS. In this study, the most abundant active sterol compound isolated from *K. alvarezii* was identified as cholesterol by RP-HPLC, $^1\text{H-NMR}$, and $^{13}\text{C-NMR}$. Cholesterol is commonly found in humans and animals, of which approximately 25% is localized to the brain (Björkhem and Meaney, 2004). Several reports have shown that cholesterol is also present in the red seaweeds *Ahnfeltia plicata*, *Gigartina exasperate*, *Porphyra yezoensis* (Ilias et al., 1985), *Digenia simplex*, *Polysiphonia brodiaei*, *Prangos ferulavea*, *Laurencia paniculata*, *Laurencia papillosa*, *Chondria collinsiana*, *Spyridia filamentosa* (Al Easa et al., 1995), *Kappaphycus* sp (Rajasulochana et al., 2009), *Gracilaria salicornia*, and *Hypnea flagelliformis* (Nasir et al., 2011). For all the red seaweeds listed above, cholesterol is the dominant sterol, ranging from 1 ~ 450 mg/100 g dry weight of the seaweed powder. We found that both iCHOL and sCHOL showed similar effects on spinogenesis and synaptogenesis. These findings, in addition to results from NMR and HPLC analyses, confirmed that cholesterol is the most dominant sterol in *K. alvarezii*. Cholesterol is crucial for brain function. Dysregulation of cholesterol homeostasis in the brain is linked to chronic neurodegenerative disorders (Vance, 2012). A reduction of cholesterol prevents neurite growth, neuronal damage, and synapse loss (Pfrieger, 2003). Cholesterol is involved in neurotransmitter release and synaptogenesis. Saher et al. (2005) reported that cholesterol is also important for growth of myelin membranes and brain maturation.

In addition to promoting spinogenesis and synaptogenesis in neuronal cultures, both EKA and iCHOL enhanced the sizes of the reserve vesicle pool at presynaptic terminals of hippocampal neurons. Bhuiyan et al.

(2015) showed that the brown seaweed *Undaria pinnatifida* improved filopodia density; however, EKA and iCHOL increased filopodia density 1.4- and 1.3-fold greater than *U. pinnatifida*, respectively, at the same DIVs. Further, the red seaweed *Gelidium amansii* increased postsynaptic and synapse puncta (Hannan et al., 2014); however, compared with *G. amansii*, we showed that EKA and iCHOL induced higher postsynaptic puncta (1.7- and 1.9-fold, respectively) and synapse puncta (1.4- and 1.1-fold, respectively). Hence, EKA and its iCHOL are considered to possess potent spinogenesis and synaptogenesis activities. In general, EKA induced higher neurotrophic activities than iCHOL. This may be due to the presence of various neurotrophic compounds in EKA which provide synergistic effects. The practical synergistic effects of EKA compounds on spinogenesis and synaptogenesis have not yet been determined; however, our data suggest that the aquaculturable *K. alvarezii* may be reused for the health of the human brain after carrageenan polysaccharides are extracted.

We also measured the functionality of synaptic vesicles by FM1-43 staining. Functionality is an essential determinant of neurotransmission because the vesicles release synaptic transmitters during exocytosis and are reloaded after being regenerated from endocytosis (Iwabuchi et al., 2014). The increase of FM1-43 fluorescence in presynaptic terminals of EKA- and iCHOL-treated neurons represents the amount of synaptic vesicle recycling. Cholesterol in the plasma membrane and synaptic vesicles is a principle part of exocytosis and endocytosis (Petrov et al., 2010). The plasticity of functional synapses in hippocampal neurons is related to cognitive function (Neves et al., 2008). The high intensity of fluorescence observed in EKA- and iCHOL-treated neurons indicates high accumulation in presynaptic vesicles. The accumulation of synaptic vesicles in presynaptic sites suggests that synapse maturation may increase the formation of functional neurons.

In conclusion, we found that both EKA and iCHOL from *K. alvarezii* show potent effects on promoting spinogenesis and synaptogenesis by increasing dendritic filopodia, spine formations, presynaptic puncta, postsynaptic puncta, cocolonized puncta, and functional presynaptic plasticity. These results suggest that *K. alvarezii* may be used as a functional food source to facilitate neuronal development to help prevent brain aging and to reduce the incidence of neurodegenerative diseases by reconstructing partially damaged neuronal networks.

ACKNOWLEDGEMENTS

This research was supported by the International Research Collaboration and International Publication Grant, Ministry of Research, Technology, and Higher Education, Republic of Indonesia.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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