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Enhancement Peripheral Regeneration as a Target of Potential Diabetic Neuropathy Treatment from *Lumbricus rubellus* Fraction DLBS1033N: The Role of Cell Viability and Migration

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ABSTRACT

Diabetic Peripheral Neuropathy (DPN) is highly prevalent in diabetic patients, significantly diminishes the patient's quality of life, and has no definitive therapy currently. Based on the basic pathology of DPN, it's critical to restrict neuronal loss while also promoting neuron regeneration. As a peripheral regenerating cell, diabetic Schwann cells (SC) tend to have a low rate of cell proliferation and regeneration capacity. DLBS1033N, a protein hydrolysate obtained from *Lumbricus rubellus*, has been confirmed to promote Schwann cell line RSC96 growth and survival by inducting Nerve Growth Factor (NGF) expression via phosphatidylinositol-3-kinase (PI3K) pathway. This pathway primarily contributes to SC proliferation and migration during the peripheral regeneration process. Herein, the contribution of DLBS1033N to peripheral regeneration in high-glucose (50mM)-induced rat Schwann cell line RSC96 injury, a well-known DPN in vitro cell model, was evaluated. RSC96 were treated with high glucose (50mM) with or without DLBS1033N 25, 50, and 100 µg/mL for 24, 48, and 72 h. MTS assay kit were used to evaluate cell viability. The results showed that DLBS1033N significantly improved cell proliferation in 48 h incubation time in a *dose-dependent* manner ($p < 0.05$). Furthermore, the scratch assay confirmed that DLBS1033N significantly increased cell migration at a concentration of 100 µg/ml by 19% for 48 H incubation ($p < 0.05$). In conclusion, DLBS1033N enhanced peripheral regeneration which can be used as an effective and promising DPN treatment.

Keywords: diabetic neuropathy, *Lumbricus rubellus*, Schwann cell, peripheral regeneration

INTRODUCTION

Indonesia is the seventh-highest percentage of diabetic patients globally (IDF, 2021). This metabolic disease may develop serious microvascular complications such as diabetic peripheral neuropathy (DPN), diabetic nephropathy, and diabetic retinopathy (Harding et al., 2019). Approximately 50% of patients with chronic diabetes report DPN symptoms such as signal conduction abnormalities, axonal loss, and demyelination that are associated with high morbidity and mortality (Gonçalves et al., 2017). Meanwhile, current DPN therapy is simply symptomatic relief (Dewanjee et al., 2018) and prevention strategies such as lifestyle modification and glucose level control (Pop-Busui et al., 2017) instead of developing other strategies for axonal regeneration.

Several studies have demonstrated how this metabolic disease affects peripheral nerve injury following the failure of axonal regeneration (Sango et al., 2017). In particular, diabetes alters the molecular function and morphology of Schwann cells (SC) (Gonçalves et al., 2017). SCs are glial cell in the peripheral system that provides axon ensheathment to facilitate impulse propagation

and secret abundant neurotrophic factors, especially Nerve Growth Factor (NGF) as protection and regeneration sources (Jessen & Mirsky, 2019). This regeneration capacity appeared in the early development of diabetes since DPN prevalence is closely linked to the duration and severity of the disease (Sango et al., 2017). SC will proliferate and migrate into the injured lesion of nerve to support the axonal regrowth process (Cheng et al., 2021). However, disease progression ultimately leads to the failure of its regeneration function as well as massive programmed SC death (Liu et al., 2020). Due to the main contribution of SC to peripheral function and protection, *Schwannopaty* awareness becomes a critical aspect of DPN (Gonçalves et al., 2017).

A previous study reported that down-regulation of NGF levels plays a significant role in the pathogenesis of DPN. High-glucose levels significantly reduced NGF secretion from cultured immortalized mouse Schwann cells, implicating the inhibition of neurite outgrowth (Tosaki et al., 2008). Cellular NGF depletion causes inhibition of PI3K/Akt pathway, which impedes cell proliferation, survival, and induction of apoptosis (Dewanjee et al., 2018). Exogen NGF administration could improve sensory disorders in a diabetic rat model. However, clinical studies of recombinant human NGF have not been successfully carried out (Sango & Yamauchi, 2014). An elevated level of NGF also correlates with the enhancement of SC migration, which is a critical requirement for peripheral regeneration following injury (Qin et al., 2016).

Therapeutic proteins have rapid improvement after the insulin therapy discovery. Earthworms are the natural source of protein therapeutics. These species contain up to 65% of the total protein content (Rodrigues et al., 2017). DLBS1033N protein fraction of *Lumbricus rubellus* was developed by hydrolysis technique of DLBS1033 (Trisina et al., 2011) which is supposed to produce low molecular weight protein. This new form was expected to contain high active peptides and free amino acids. A previous study confirmed DLBS1033N activity as a therapeutic candidate for nerve regeneration treatments by promoting Schwann cell line RSC96 growth and survival. This mechanism of action involves the induction NGF expression through PI3K signaling pathway interaction (Karsono et al., 2018). Due to the depletion of NGF expression in DPN, it is critical to carry out a study investigating the effect of DLBS1033N linking the aspect of diabetic degeneration with induction of NGF expression.

MATERIALS AND METHODS

DLBS1033N preparations and characterization

DLBS1033N was prepared as mentioned in the previous experiment (Karsono et al., 2018). The total protein content of DLBS1033N was estimated using Bradford Assay. A total of 1 mg/mL of bovine serum albumin (BSA) was dissolved in purified water as a standard curve. 100 µL of 100 and 200 mg/mL DLBS1033N in purified water were mixed with 2 mL of Bradford reagent and were incubated for 10 min. The absorbance was measured at 595 nm.

Since DLBS1033N is a protein hydrolysate of DLBS1033, the protein pattern was visualized using Tricine SDS-PAGE to compare each protein profile. The process was carried out with 10% and 16% separating gel 10%, 4% stacking gel, 10 µL DLBS1033N, DLBS1033, and ULMW protein as a ladder.

Cell Culture and treatment

Schwann cell line of *Rattus norvegicus* RSC96 (ATCC® CRL2765™) was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Thermofisher 12800017, containing 4.500 mg/L or 25mM D-glucose) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientifics, Inc.) and maintained in incubator 37^{o}C and 5% CO_2. Cells were trypsinized and plated in 96-well plates ($1x10^4$ cells/well) for viability assay and 6-well plates ($5x10^5$ cells/well) for migration assay after the cells reached approximately 70% of confluence. The cells were allowed overnight and cultured in a serum-free medium within 4 h. The association of osmolarity as a neurotoxicity factor was not considered (Wu et al., 2012).

Cell viability assay

Cell viability was determined using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS Assay) (Promega). RSC96 was treated by D-glucose (Merck) exposure at different concentrations of 50, 100, and 150 mM for 0, 24, 48, and 72 h. After the desired period of incubation time, 20 µL of MTS reagent was added to each well and was incubated for 1.5 h at 37^{o}C. The absorbance was recorded at 490 nm. The 50 mM high glucose for 48 h was enough to cause obvious injury on SC and could maintain that effect

until 72h compared to untreated control. Thus, 50 mM glucose concentration was deemed appropriate for future experiments. The protection effect of DLB1033N at concentrations 25, 50, and 100 µg/mL (Karsono et al., 2018) was determined with the presence of 50 mM glucose.

Migration assay

An artificial straight-line scratch was created on a monolayer of RSC96 cells in 6-well plates using p200 yellow pipette tip. RSC96 is an adherent cell, hence, additional agents were not needed for this process. The cells were then treated with complete and serum-free media as positive and negative controls, respectively. Migration activity induction of DLBS1033N was determined at 100 µg/mL in serum-free conditions. After treatment, cells were captured at 0, 24, and 48 h incubation in the same specific area.

Statistical Analysis

Data was collected and presented as the mean ± SD. Statistical differences were determined by using One-Way ANOVA test with Tukey multiple comparison test. A *p*-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Protein estimation of DLBS1033N by Bradford method

The total protein value obtained from DLBS1033N was 11.45 mg/g (Table I). The standard curve of BSA was established as follows: y = 1.3688x + 0.1332 (R^2 = 0.991) (Figure 1).

Table I. Total protein of DLBS1033N using Bradford method

Sample (mg/mL)	Abs	Concentration (mg/g)
100	0.45	11.58
200	0.75	11.32
Concentration average (mg/g)		**11.45**

Abs: Absorbance at 595 nm

Protein profile of DLBS1033N by SDS-PAGE

The SDS-PAGE showed that DLBS1033N has a unique protein pattern that is different from the unhydrolyzed compound (DLBS1033). DLBS1033N possessed two new bands with molecular weight of approximately 16-19 kDa. The other band appeared at 6.5 kDa with a protein intensity elevation of 14 kDa (Figure 2).

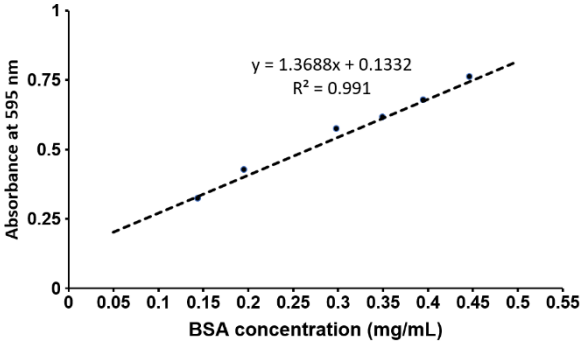

Figure 1. Bovine Serum Albumin (BSA) standard graph for protein concentration determination

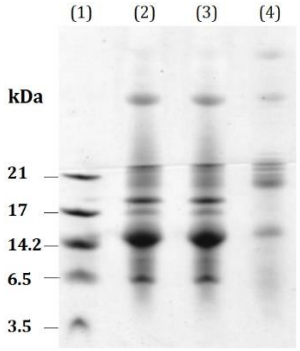

Figure 2. SDS-PAGE analysis of samples. Lane 1: standard protein marker ULMW (Ultra low molecular weight); Lane 2 and 3: DLBS1033N 100mg/ml; Lane 4: DLBS1033 100mg/ml.

DLBS1033N effects on cell proliferation in high-glucose-treated RSC96

Prior to evaluation of DLBS1033N activities on SC, the glucose concentration was optimized as a diabetic neuropathy model *in vitro*. RSC96 cells were cultured in DMEM as control group and various high-glucose concentration group (50, 100, and 150 mM) for 0, 24, 48 dan 72 h. There was no significant cell viability inhibition during 24 h incubation due to the low vulnerability of cells to oxidative stress on short-term high-glucose exposure (Ding, 2012). Cell proliferation decreased significantly after 48 h of exposure to high glucose (Figure 3) ($p < 0.05$). In 72 h, the cell viability of 50 mM glucose exposure was not significant compared to 100 and 150 mM concentrations. Therefore, the DPN model was established by 50 mM glucose exposure.

The viability of RSC96 was increased in a dose-dependent manner in 48h DLBS1033N-treated cells (Figure 4). These protective effects on RSC96 showed an increase of 24%, 49%, and 71% at 25, 50, and 100µg/mL of DLBS1033N, respectively.

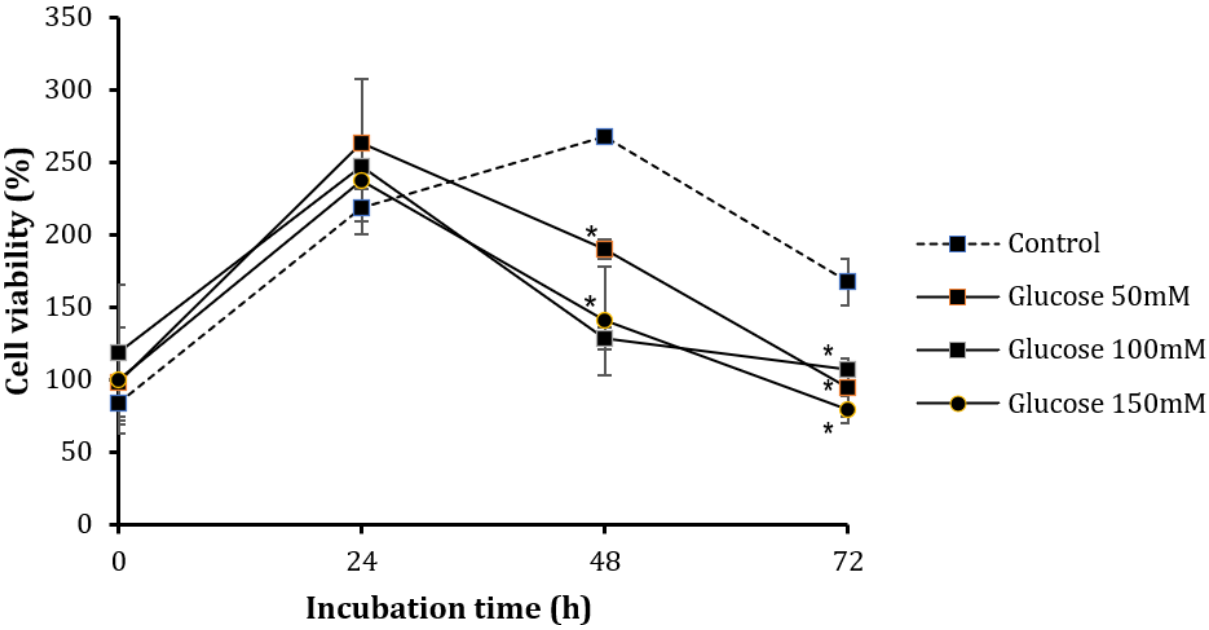

Figure 3. Glucose concentration optimization to obtained diabetic neuropathy model in RSC96. Cell viability was evaluated by MTS reagent after being treated with 50, 100, and 150mM of glucose for 0, 24, 48, and 72H. The results are expressed in a percentage of 0 H average value ± SD of three independent experiments. *p < 0.05 compared to the control group.

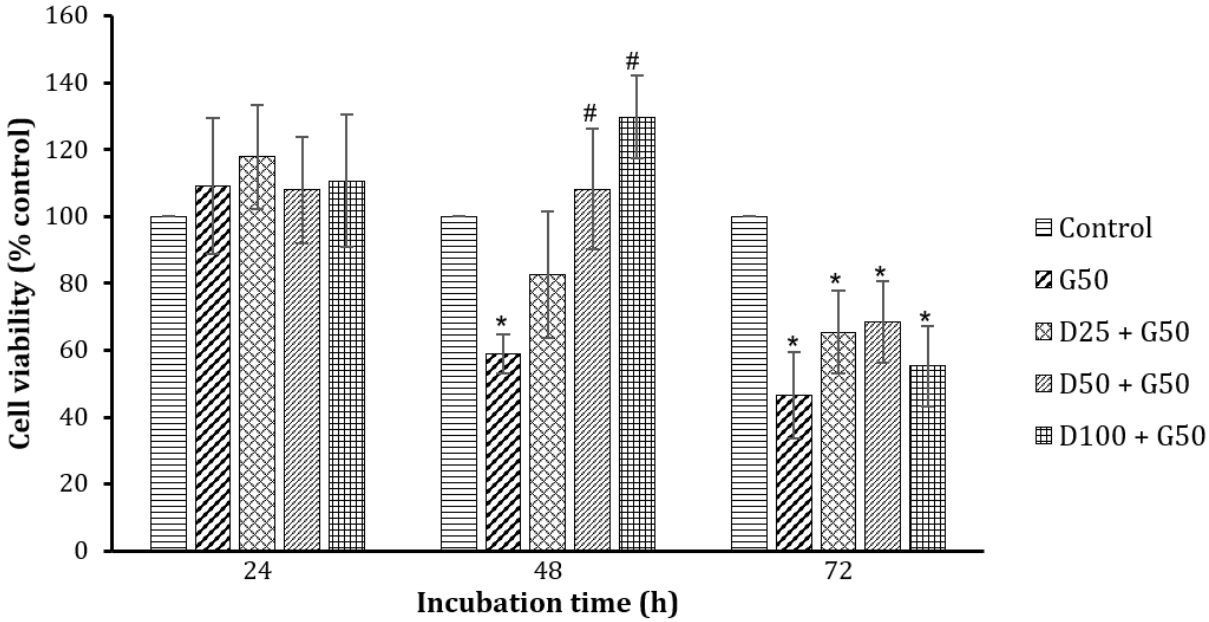

Figure 4. Effects of DLBS1033N 25, 50 and 100 µg/ml in high glucose condition (50mM) for 23, 48, and 72H incubation. The results are expressed in percentage of control (100%) based on respective incubation time. G50: Glucose 50mM group; D25: DLBS1033N 25 µg/ml; D50: DLBS1033N 50 µg/ml; D100: DLBS1033N 100 µg/ml. Data are mean ± SD of three independent experiments. * p < 0.05 compared to control group, # p < 0.05 compared to glucose group.

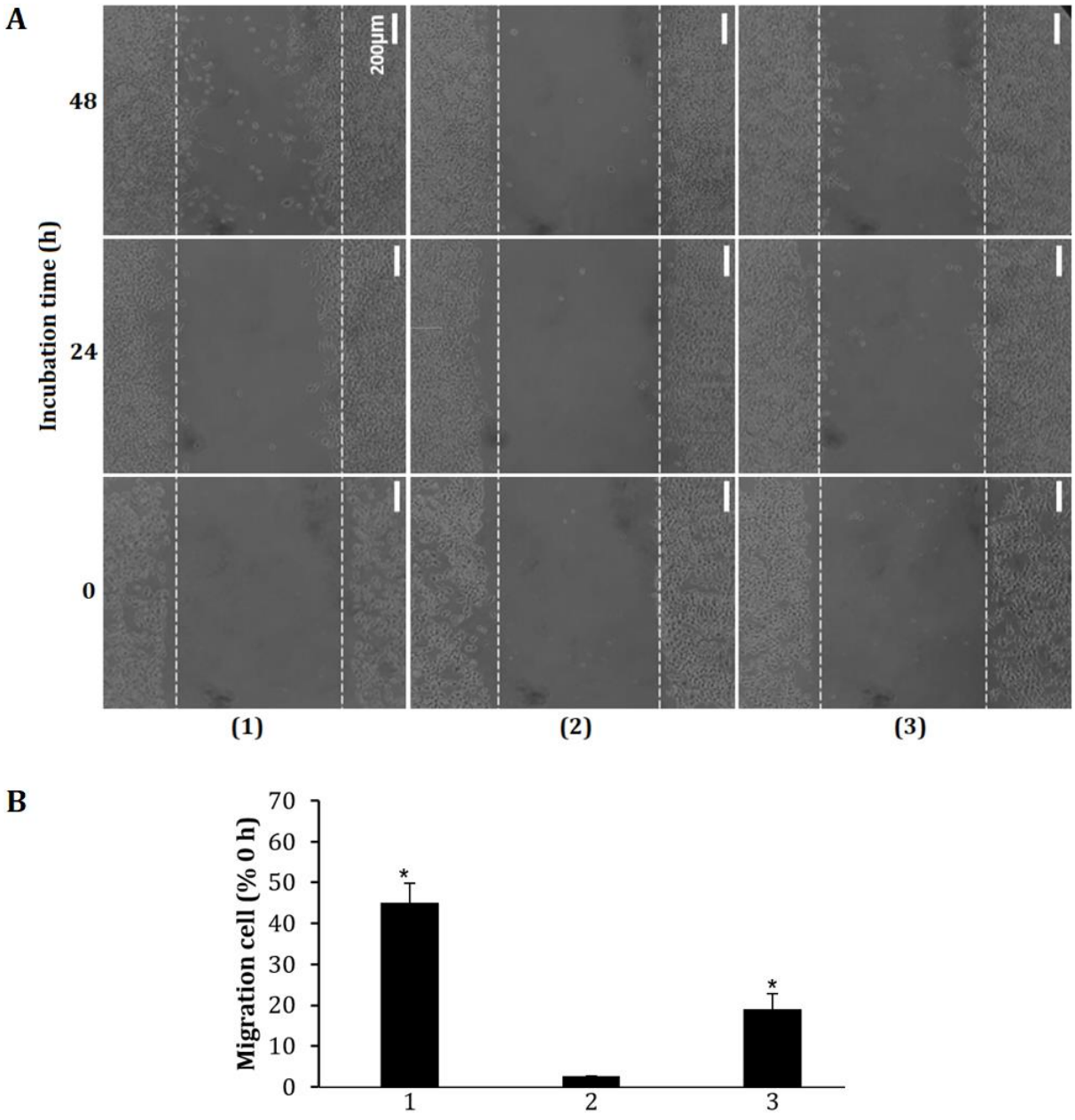

Figure 5. DLBS1033N effect on RSC96 migration activity. A: Cell migration quantified by scratch assay, B: Image-J semi-quantification of migrative cell from three independent quantification ± SD. (1): DMEM + FBS 10%; (2): DMEM serum-free; (3) DLBS1033N 100µg/ml (serum-free). *p < 0.05 compared to DMEM serum-free group.

However, 25 µg/mL ($p > 0.05$) was not statistically significant to improve the cell viability of SC compared to the glucose group. Both 50 and 100 µg/mL of DLBS1033N ($p < 0.05$) were significant compared to glucose group. The highest improvement was observed at a concentration of 100 µg/mL, which was then used to evaluate the migration effects.

The activity of DLBS1033N in increasing cell proliferation was not observed at 72 h incubation. All DLBS1033N variation doses were statistically significant to the control group ($p < 0.05$) even though cell viability was not lower than glucose group. The results show that the cut-off of proliferation enhancement is occurred at 48 h incubation.

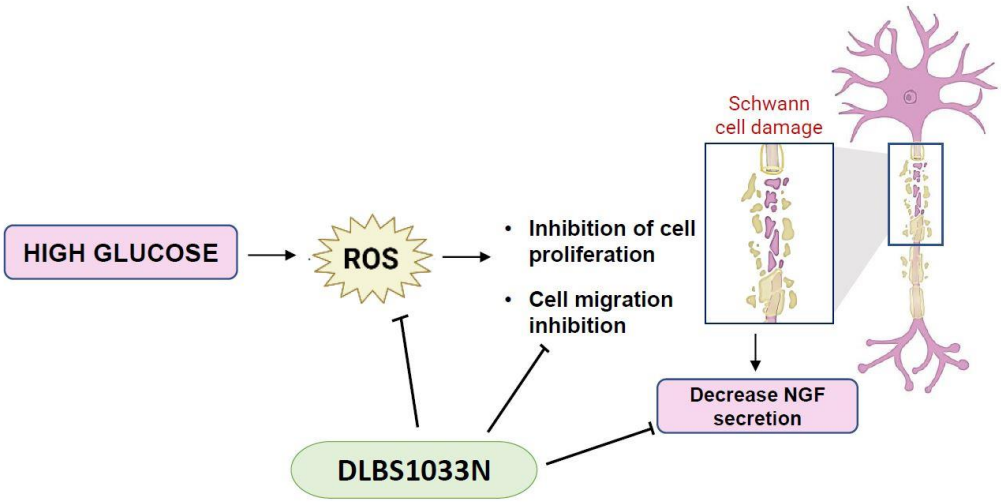

Figure 6. DLBS1033N mechanism was confirmed to promote cell viability in high-glucose conditions and has potential to induce cell migration. Radical scavenging by phenolic compound (Aldarraji, 2013) and induction of NGF secretion (Karsono et al. 2018) contribute to DLBS1033N potential therapy of DPN.

DLBS1033N effects on RSC96 migration activity

A scratch assay was carried out to evaluate the activity of DLBS1033N in promoting RSC96 migration. Since high-glucose induced inhibition of SC migration is predominantly owing to a reduction in proliferation (Gumy et al., 2008), the migration assay was evaluated without high-glucose condition. Therefore, the assay specifically evaluates the potential of DLBS1033N for enhancing migratory activity, which was not interfered by the death cell caused by high glucose exposure. The complete absence of fetal bovine serum in cell culture is used in order to inhibit cell proliferation in migration assay (serum deprivation) (Almeida et al., 2019). The serum-free media group only performed a 3% migration enhancement. DLBS1033N at 100 µg/mL in serum-free media ($p < 0.05$) for 48 h significantly enhanced RSC96 migratory activity compared to serum-free group, it was 19%. A 45% migration of DMEM with 10% serum ($p < 0.05$) that was significant compared to the serum-free group, as shown in Figure 5. These findings show that DLBS1033N can stimulate RSC96 cell proliferation and migration, which may contribute to neuron regeneration process.

L. rubellus has been widely used as a traditional remedy due to its high protein content (Hidayat et al., 2021). Based on biotechnology innovation, hydrolyzation is used as a technique to produce bioactive peptides with health benefits and enrichment potential (Abeer et al., 2021). A protein hydrolysate from *L. rubellus*, DLBS1033N, was confirmed to have a potential therapeutic for peripheral nerve regeneration. This exploration was based on the novel protein band discovery on the SDS-PAGE profile. DLBS1033 (an unhydrolyzed protein fraction), an established antithrombotic and thrombolytic agent (Trisina et al., 2011) has a different protein profile from DLBS1033N. The hydrolysate protein has new proteins below 20 kDa. It was presumed that different activities will be gained because the antithrombotic properties were performed by the serine protease group with molecular masses of 23-30 kDa (Zhao et al., 2010). DLBS1033N was confirmed to promote RSC96 growth and survival in the hypoxia-ischemic model through PI3k/Akt signaling activation. Moreover, NGF expression, an essential neurotrophic factor in the regeneration process, was significantly increased by DLBS1033N (Karsono et al., 2018).

NGF depletion was clear in the pathogenesis of DPN (Pittenger & Vinik, 2003). Soon after nerve injury, Schwann cells in the distal nerve began to dedifferentiate (downregulate pro-myelinating genes) into the immature stage of SC (Boerboom et al., 2017). During Wallerian degeneration, SCs active to proliferate and recruit the macrophages to phagocytose the axonal and myelin debris. After the microenvironment is cleared of debris, Schwann cells secrete neurotrophic factors, such as NGF and BDNF, and form the phenotype of the

Büngner Schwann cell. The repair cell starts to migrate to the injury site and align themselves to form a band of Büngner as a guidance cue for regenerating axons (Gimble et al., 2020). After axonal regeneration, SCs readily exit the cell cycle and differentiate again into myelinating or non-myelinating SC to support the complete functional recovery (Boerboom et al., 2017).

The biological alterations of axonal and SC under diabetic conditions are likely to affect peripheral regeneration. One of the strongly associated reasons with impaired axonal regeneration is the delay in the Wallerian degeneration process (Sango et al., 2017). This biochemical pathway led to SC function retardation caused by oxidative stress excessive production (Zenker et al., 2013). Therefore, reactive oxygen species (ROS) neutralizing was performed in several potential DPN treatment agents (Cheng et al., 2020; Jiang et al., 2020; Tiong et al., 2019; Wang et al., 2019; Wu et al., 2012). *L. rubellus* was confirmed to contain great phenolic constituents (Aldarraji, 2013). This aspect contributes to the protection activity of DLBS1033N in hyperglycemic SC.

Moreover, NGF expression induced by DLBS1033N (Karsono et al., 2018) is required to improve cell ability to resist apoptosis (Pittenger & Vinik, 2003) and suppress reticulum endoplasmic stress (RES). Both SC apoptosis and RES suppression are essential pathways in the onset and development of DPN (Li et al., 2017).

Peripheral nerve regeneration is dependent on the activation of PI3K/AKT pathway, because of the involvement of this pathway in SC proliferation and migration (Takaku et al., 2021). Thus, the states of the previous study about PI3K/AKT activation by DLBS1033N (Karsono et al., 2018), were confirmed in this study through the increased proliferation and migration. Furthermore, PI3K/Akt activation was performed by fibroblast growth factor-2 (FGF-2) which was suppressed in hyperglycemia conditions (Vasko et al., 2009). A previous study reported that earthworm extract significantly increased FGF-2 expression (Grdisa et al., 2004). This activity increased the SC migration along with urokinase plasminogen activator (uPA) activation. uPA expression could promote SC migration by the matrix molecular degradation. This activity was performed by Dilong, an earthworm extract that promotes SC migration (Chang et al., 2011). Therefore, specific signaling related to the migration of SC in high-glucose conditions to reach the highest potential of DLBS1033N to enhance peripheral regeneration is required to explore.

From this experiment, we revealed that DLBS1033N has different activities after undergoing hydrolysis. DLBS1033N was confirmed to promote cell viability in high-glucose conditions and has potential to induce cell migration. This activity was obtained from the ability of free radical scavenging by phenolic compounds (Aldarraji, 2013) and increased secretion of NGF (Karsono et al., 2018) (Figure 6). Further, studies on the signaling mechanism(s) and the development of the model of axonal dan Schwann cell interaction both in vitro and in vivo diabetes are needed to confirm the potential activity of DLBS1033N as a peripheral regeneration enhancer in diabetic neuropathy.

CONCLUSION

Based on the results, DLBS1033N was found to promote the viability in high-glucose conditions and migration of RSC96 Schwann cell line. These activities serve as a potential mechanism to enhance peripheral regeneration developing novel diabetic neuropathy treatment.

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