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In vitro antioxidant and wound healing activity of *Sargassum polycystum* hydroethanolic extract in fibroblasts and keratinocytes

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ABSTRACT

Objective: To investigate the *in vitro* antioxidant and wound healing properties of the hydroethanolic extract of *Sargassum polycystum*, and elucidate the mechanism of its wound healing activity.

Methods: Human dermal fibroblast and HaCaT cells were used to evaluate the proliferation by sulforhodamine B and dsDNA assay after treatment with *Sargassum polycystum* extracts. Scratch wound healing and phalloidin-rhodamine staining were employed to observe migratory activity and filopodia formation, respectively. Western blot and real-time RT-PCR assays were performed to determine the protein and gene expressions related to wound healing activities.

Results: The phytochemical analysis found a higher level of flavonoid than phenolic compound in *Sargassum polycystum* extracts. In human dermal fibroblast cells, *Sargassum polycystum* extracts at 50 and 100 μg/mL significantly increased fibroblast proliferation and the gene expressions of hyaluronic acid synthase 1 (*HAS1*), *HAS2*, *HAS3*, collagen type 1 alpha 1 chain (*COL1A1*), collagen type 3 alpha 1 chain (*COL3A1*), and *elastin*. The phosphorylation of Akt, ERK1/2, and p38 MAPK was also significantly upregulated after treatment with *Sargassum polycystum* extracts. Additionally, 50 and 100 μg/mL of the extracts prominently enhanced the proliferation, migration, and filopodia formation of HaCaT cells, as well as the protein levels of pFAK/FAK, pSrc/Src, pAkt/Akt, pERK1/2/ERK1/2, Rac1 and Cdc42.

Conclusions: *Sargassum polycystum* extracts show promising wound healing activities in human dermal fibroblasts and keratinocytes.

KEYWORDS: Dermal fibroblast; *Sargassum polycystum*; Wound healing; Antioxidant; Proliferation

1. Introduction

The skin contains two main layers (the outer and inner layers), which are commonly known as the epidermis and dermis[1]. An *in vitro* evaluation of the epidermis and dermis layer has been mainly performed using human keratinocytes cell line (HaCaT) and human dermal fibroblasts cell line (HDF)[2]. Re-epithelization in cutaneous wound healing is associated with keratinocyte migration and proliferation to replace worn-out cells due to abrasion in the

Significance

Sargassum polycystum, a brown seaweed, is known as food and traditional medicine for alleviating many health conditions. However, the wound healing effect of *Sargassum polycystum* extract has not been explored yet. The current study found that the hydroethanolic extract of *Sargassum polycystum* increased the proliferation and migration of both human fibroblasts and keratinocytes by activating FAK, Src, Akt, and ERK1/2 upstream signaling pathway as well as Cdc42 and Rac1 downstream signaling pathway.

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epidermis[3]. In addition, fibroblasts are the initial cells in the wound area as the proliferation and migration are primary biological processes required in skin wound repair and essential in secreting collagen to control the structural integrity[4,5]. Wound healing is a fundamental biological process that repairs skin integrity and type I collagen has been well-established as a pivotal role during wound healing in the skin[5,6]. In addition, hyaluronic acid (HA) synthesis by HA synthase (HAS1, HAS2, and HAS3) and the biological response have been described in skin moisture and elasticity, and also play an important role in the proliferation, differentiation, and migration of skin cells during the wound repair[7,8].

Src, a non-receptor protein tyrosine kinase, has been involved in the proliferation and migration of cells[9,10]. Furthermore, many studies have proved that Src is needed in wound healing response and tissue regeneration in *Drosophila* and zebrafish[11,12]. Src and focal adhesion kinase (FAK) are cross-activating proteins and the activation of both Src and FAK is important in wound healing response in keratinocytes[13]. In addition, autophosphorylation of FAK (Y397) recruits Src and then causes FAK/Src complex activation to stimulate signaling downstream events, for example, ERK1/2, p38 MAPK, Akt and small GTPases (Rac1, Cdc42, and RhoA)[13,14]. Several studies have described that the activation of FAK, Src, MAPK, and Akt stimulates the wound healing process[13,14], whereas the FAK, Src, MAPK, and Akt inactivation inhibits wound closure[15].

For many centuries, marine seaweed has been reported as food and traditional medicine, especially brown seaweed. *Sargassum polycystum* (*S. polycystum*), one type of brown seaweed, is distributed generally in tropical areas including Thailand, China, Malaysia, and Japan[16]. In addition, *S. polycystum* has been regionally used as food and traditional medicine and is also noted to have potent biological activities, including anti-inflammatory, antioxidant, anti-cancer, anti-hyperglycemia, and hepatoprotective properties[17–20] with high phytochemical contents, such as sterols, saponins, flavonoids, phenolics, alkaloids, and steroids[21,22]. Flavonoids, a type of phenolic compound, are found in *S. polycystum*[21,22] and have been reported to possess the wound healing activity in both *in vitro* and *in vivo* studies[23]. However, no studies have been conducted on the wound healing activity of the crude extract of *S. polycystum*. Thus, the purpose of the current study was to assess the phytochemical composition and investigate the wound healing properties of *S. polycystum* hydroethanolic extract in HaCaT and HDF cells.

2. Materials and methods

2.1. Extraction of S. polycystum

S. polycystum adult-stage was collected from October to December 2019 from Trang, Thailand. *S. polycystum* was washed and preserved in a hot air oven (45℃) overnight before being powdered. The dried powder (1.2 kg) was incubated at room temperature in 70% ethanol for 72 h and then filtered with filter papers (Whatman®, No. 1). Thereafter, the solvent was evaporated under reduced pressure at 50℃ using an evaporator (Buchi, Switzerland). The yield value of the hydroethanolic extract of *S. polycystum* was 14.54% (*w/w*).

2.2. Chemical analysis

2.2.1. High–performance liquid chromatography (HPLC) with photodiode array detector analysis of C–phycocyanin and fucoidan

The hydroethanolic extract of *S. polycystum* (100 mg) was dissolved in 5 mL of 0.1% trifluoroacetic acid (TFA, Fisher Scientific, France) and filtered through a 0.45-µm PTFE syringe filter before being applied to the HPLC system. The extract was analyzed using HPLC with a photodiode array detector by Shimadzu LC-10ADvp series liquid chromatography equipped with Shim-pack GISS C18 column (4.6 mm × 150.0 mm, particle size 5 µm) (Shimadzu, Kyoto, Japan). The 0.1% TFA in Milli-Q water (Millipore, Billerica, MA, USA) was performed as a mobile phase and carried out at a flow rate of 1.5 mL/min under isocratic conditions for *C*-phycocyanin analysis in the extract. For fucoidan analysis from the hydroethanolic extract of *S. polycystum*, HPLC Shimadzu LC-10ADvp series LC with a refractive index detector (RID-6A, Shimadzu) equipped with a Shodex OHpak SB-803 HQ column (300.0 mm × 8.0 mm) and a Shodex OHpak SB-804 HQ column (300.0 mm × 8.0 mm) (Showa Denko K.K., Tokyo, Japan) was used and the flow rate was fixed at 1.0 mL/min using a Milli-Q water as a mobile phase. Chromatograms were detected at 210 nm. The calibration curves were used to quantify the amount of *C*-phycocyanin and fucoidan in the hydroethanolic extract of *S. polycystum*. The analysis of the samples was performed in triplicate. *C*-phycocyanin (Sigma-Aldrich, USA) and fucoidan (Sigma-Aldrich, USA), reference standards, were solubilized in 0.1% TFA.

2.2.2. Analysis of total phenolic and flavonoid contents of the hydroethanolic extract of S. polycystum

Folin-Ciocalteu assay was used to evaluate the total phenolic content of the hydroethanolic extract of *S. polycystum* according to the method described by Sukketsiri *et al.*[24] with some modifications. In brief, 20 µL of the hydroethanolic extract of *S. polycystum* was incubated with 100 µL of Folin-Ciocalteu reagent (10%) and 80 µL of sodium carbonate (7%) into a 96-well plate for 30 min in the dark. The phenolic content was measured by a microplate reader (Synergy™, Bio-tex, VT, USA) at 760 nm and represented as mg gallic acid equivalent/g extract. Aluminium chloride colorimetric method was utilized to measure total flavonoid content as previously described by Sukketsiri *et al.*[24] with some modifications. The hydroethanolic extract of *S. polycystum* (20 µL)

was mixed with aluminum chloride (15 μL), 10% sodium acetate (20 μL), and distilled water (145 μL) into a 96-well plate for 15 min in the dark. The content of flavonoid was detected at 430 nm and presented as mg quercetin equivalent/g extract.

2.3. Antioxidant activity of the hydroethanolic extract of S. polycystum

2.3.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging activity of the hydroethanolic extract of *S. polycystum* was carried out by DPPH assay[24]. The extract (5 mg/mL) was mixed with DPPH solution (0.1 mM) in methanol into a 96-well plate at room temperature for 30 min and determined using a microplate reader (Synergy™, Bio-tex, VT, USA) at 517 nm. The radical scavenging activity (%) was determined according to the formula: $(Abs_{control} - Abs_{extract})/Abs_{control} \times 100$.

2.3.2. Ferric reducing antioxidant power (FRAP) assay

FRAP was measured as described by Chotphruethipong *et al.*[25] with some modifications. The hydroethanolic extract of *S. polycystum* (300 μL) was incubated with 2.2 mL FRAP reagent (0.3 mol/L acetate buffer pH 3.6, 10 mmol/L of 2,4,6-tripyridyl-S-triazine in 0.04 mol/L HCl and 0.02 mol/L $FeCl_3 \cdot 6H_2O$) for 15 min in a water bath shaker at 37℃. Then, the absorbance was detected at 593 nm and the result was represented as μM Fe^{2+}/g sample.

2.3.3. Metal chelating activity (MCA) assay

MCA was measured as described by Chotphruethipong *et al.*[25] with some modifications. The hydroethanolic extract of *S. polycystum* (1 mL) was mixed with 0.05 mL of 2.0 mmol/L $FeCl_2$, 0.2 mL of 5 mmol/L ferrozine, and 2.75 mL of distilled water at room temperature for 10 min in the dark. The absorbance was detected at 562 nm by a spectrophotometer and expressed as μM EDTA equivalents/g extract.

2.4. Cell culture and cell viability

The viability of HaCaT and HDF cells treated with the hydroethanolic extract of *S. polycystum* was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HaCaT (CLS cell line service, Heidelberg, Germany) and HDF cells (Sigma-Aldrich, USA) at a density of 1×10^4 cells/well were cultured in a completed medium (DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% *L*-glutamine) overnight and then treated with the hydroethanolic extract of *S. polycystum* at the concentration of 10-1 000 μg/mL for 24, 48, and 72 h. After incubation, the viability of both cells was determined by MTT (Thermo Fisher, USA) method. After 2 h of 500 μg/mL MTT incubation, the absorbance was measured at 570 nm by a microplate reader (Synergy™, Bio-tex, VT, USA). Cell viability treated with the extracts was compared to the untreated cells (% of control).

2.5. Evaluation of cell proliferation by total cellular protein content and dsDNA assay in HaCaT and HDF cells

For total cellular protein content, HaCaT and HDF (1×10^4 cells/well) cells were treated with the hydroethanolic extract of *S. polycystum* at 10, 50, and 100 μg/mL in 96-well plates for 24 h. Both cells were collected and incubated for 60 min with sulforhodamine B (0.04%; *w/v*) (Sigma-Aldrich, St. Louis, MO, USA) as previously described by Woonnoi *et al*[13]. After 60 min of incubation, the cells were rinsed in acetic acid (1%; *v/v*), added with the tris base (0.01 M), and finally read by a microplate reader (Synergy™ H, Bio-tex, VT, USA) at 510 nm. The cell proliferation (%) was determined according to the formula: $Abs_{extract}/Abs_{control} \times 100$.

For the dsDNA assay, HaCaT and HDF cells (1×10^6 cells/well) were treated with the hydroethanolic extract of *S. polycystum* at 10, 50, and 100 μg/mL for 24 h in a 6-well plate. Then, the dsDNA concentration was determined by a dsDNA assay kit (Invitrogen, USA) followed by using a fluorescence microplate reader (Synergy™, BioTek, VT, USA) at emission 485 nm and excitation 535 nm to measure the fluorescence intensity in each well plates. The cell proliferation (%) was calculated according to the formula: (DNA concentration of extract treatment/DNA concentration of the control) × 100.

2.6. Wound healing activity in HaCaT cells by scratch wound healing assay

HaCaT cells (0.2×10^6 cells/well) were seeded in a completed DMEM medium in a 6-well plate until the cells reached approximately 100% confluence. After confluence, the wound space was produced by a sterile 200 μL pipette tip, rinsed with phosphate buffered saline, and finally treated with the extract at 10, 50, and 100 μg/mL under 1% fetal bovine serum. The wound area was taken under a phase-contrast microscope at 0 and 24 h. The percentage of wound area was calculated according to the formula: (average area at 24 h/average area at 0 h) × 100.

2.7. Filopodia formation in HaCaT cells by phalloidin rhodamine staining

The phalloidin rhodamine staining was used to evaluate the formation of filopodia as described by Woonnoi *et al*[13]. After treatment with the hydroethanolic extract of *S. polycystum* at concentrations of 10, 50, and 100 μg/mL for 24 h, HaCaT cells (5×10^4 cells/well) were fixed for 20 min in paraformaldehyde (4%;

w/v) and permeabilized for 10 min at room temperature in Triton-X 100 (0.1%; *v/v*). The fixed cells were blocked for 1.5 h with bovine serum albumin (2%; *w/v*), washed with phosphate buffered saline, and then incubated for 30 min at room temperature with phalloidin rhodamine and Hoechst 33342. The filopodia were captured under a fluorescence microscope (Olympus IX70 with DP50). The filopodia formation (%) was calculated using the formula: (average number of filopodia/total number of cells) × 100.

2.8. Protein expression by Western blotting analysis

To examine the expression of Cdc42, Rac1, RhoA, p38, ERK1/2, Akt, Src, and FAK protein, HaCaT cells (3×10^6 cells/plate) were treated with the hydroethanolic extract of *S. polycystum* (10, 50, and 100 μg/mL) for 24 h. Then, the protein was extracted using RIPA buffer containing protease inhibitor at 4℃ for 30 min, homogenized, and centrifuged at 14 000 rpm for 20 min. The amount of total protein in the cells was measured using Bradford reagent and 75 μg of proteins were loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and then moved onto PVDF membranes. The membranes were blocked using 5% skim milk at room temperature for 2 h, washed, and incubated at 4℃ overnight with primary antibodies to Cdc42 (ab187643), Rac1 (ab155938), RhoA (ab54835), p38 (ab31828), p-p38 (ab4822), ERK1/2 (ab36991), pERK1/2 (ab50011), Src (ab109381) (Abcam, Cambridge, UK; 1∶1 000), Akt (SC-81434), pAkt (SC-514032), pSrc (SC-81521), FAK (SC-271126), pFAK (SC-81493) (Santa Cruz Biotechnology, Dallas, USA; 1∶1 000), and β-actin (Thermo Scientific, Waltham, MA, USA; 1∶1 000). Subsequently, the secondary antibody conjugated with HRP was incubated at room temperature for 2 h and the specific bands of protein were observed with chemiluminescence (Merck Millipore, USA). Densitometry of specific protein bands was determined using Image J 1.42q software (National Institutes of Health) and normalized with β-actin.

2.9. mRNA expression by real–time reverse transcriptase–polymerase chain reaction (RT–PCR) analysis

HDF cells were treated with the hydroethanolic extract of *S. polycystum* (50 and 100 μg/mL) for 24 h and harvested in TRIzol reagent (Thermo Fisher Scientific, USA). The amount of RNA was measured using a NanoDrop spectrophotometer (Eppendorf, Germany). Then, cDNA was synthesized using SuperScript® VILO™ cDNA synthesis kit (Thermo Fisher Scientific, USA), and the hyaluronic acid synthase 1 (*HAS1*), *HAS2*, *HAS3*, collagen type 1 alpha 1 chain (*COL1A1*), collagen type 3 alpha 1 chain (*COL3A1*), and *elastin* mRNA expressions were quantified by real-time RT-PCR using SensiFAST SYBR No-ROX kit (Bioline, UK) using specific primer sequences (Table 1). All data were analyzed by normalized gene expression using the $2^{-\Delta\Delta CT}$ method.

2.10. Statistical analysis

The values were presented as mean ± standard error of the mean (n = 4). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by LSD *post hoc* test. $P<0.05$ was considered statistically significant.

3. Results

3.1. HPLC profiles and phytochemical contents in the hydroethanolic extract of S. polycystum

Phytochemical screening of the hydroethanolic extract of *S. polycystum* displayed the highest concentration of flavonoid (200.91 ± 2.71) mg quercetin equivalent/g extract, followed by phenolic compound (18.39 ± 1.03) mg gallic acid equivalent/g extract. In addition, the HPLC chromatogram of *C*-phycocyanin and fucoidan was shown in Figure 1. *C*-phycocyanin and fucoidan peaks were presented at a retention time of (13.41 ± 0.15) min and (11.12 ± 0.02) min, respectively. The hydroethanolic extract of *S. polycystum* contained approximately (1.29 ± 0.01)% *w/w* of *C*-phycocyanin and (0.48 ± 0.01)% *w/w* of fucoidan.

3.2. In vitro antioxidant activity of the hydroethanolic extract of S. polycystum

The antioxidant activity of the hydroethanolic extract of *S. polycystum* was investigated using DPPH, FRAP, and MCA assays.

Table 1. Primer sequences used for real-time RT-PCR analysis.

Genes	Forward	Reverse
HAS1	CCACCCAGTACAGCGTCAAC	CATGGTGCTTCTGTCGCTCT
HAS2	GTCCCTACCGAGTCTCTTCT	TTTTTAAGTTTCCGCTTCTG
HAS3	GGTTGGACCTACAAGGAGGC	GGTTCATGCTGGTGTCCTCA
COL1A1	AGACATCCCACCAATCACCTG	GGCAGTTCTTGGTCTCGTCAC
COL3A1	GGACCTCCTGGTGCTATAGGT	CGGGTCTACCTGATTCTCCAT
Elastin	CACTGGGGTATCCCATCAAG	GTGGTGTAGGGCAGTCCATAG
β–actin	GATCATTGCTCCTCCTGAGC	ACTCCTGCTTGCTGATCCCAC

HAS: hyaluronic acid synthase, COL1A1: collagen type 1 alpha 1 chain, COL3A1: collagen type 3 alpha 1 chain.

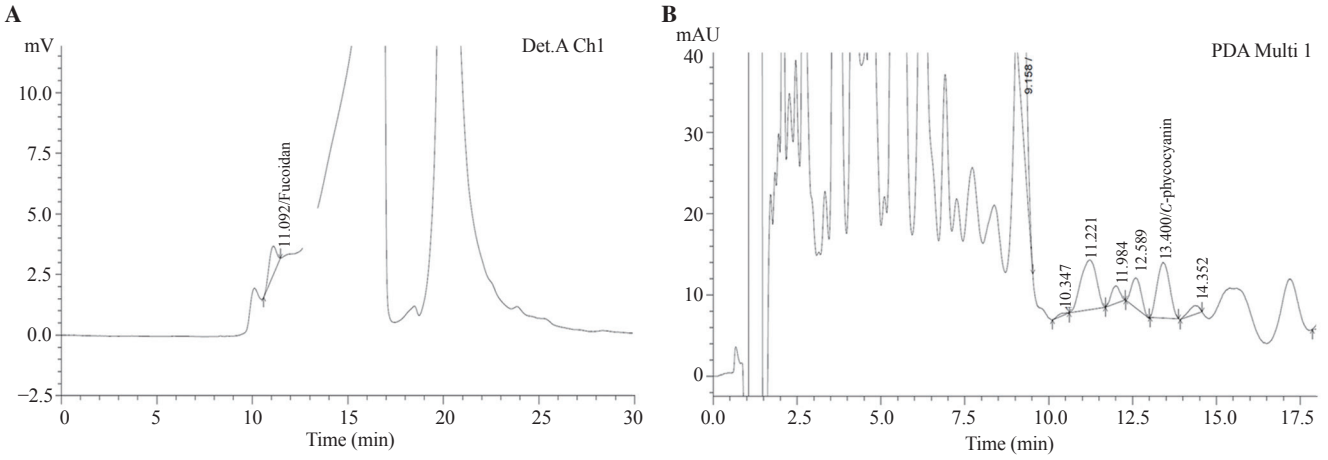

Figure 1. High-performance liquid chromatography chromatograms of (A) fucoidan and (B) *C*-phycocyanin.

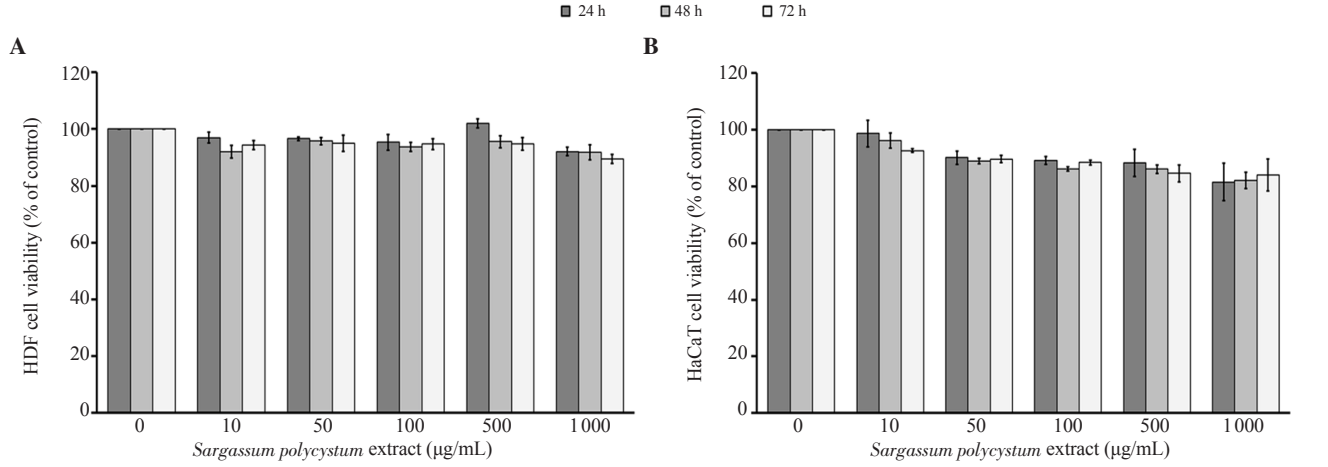

Figure 2. Cytotoxicity of the hydroethanolic extract of *Sargassum polycystum* in human dermal fibroblasts (HDF) (A) and HaCaT human keratinocytes (B). Both cells were treated with the hydroethanolic extract of *Sargassum polycystum* at the concentration of 10-1 000 μg/mL for 24, 48 and 72 h and the viability was detected by MTT assay. The values are expressed as mean ± standard error of mean (n=4).

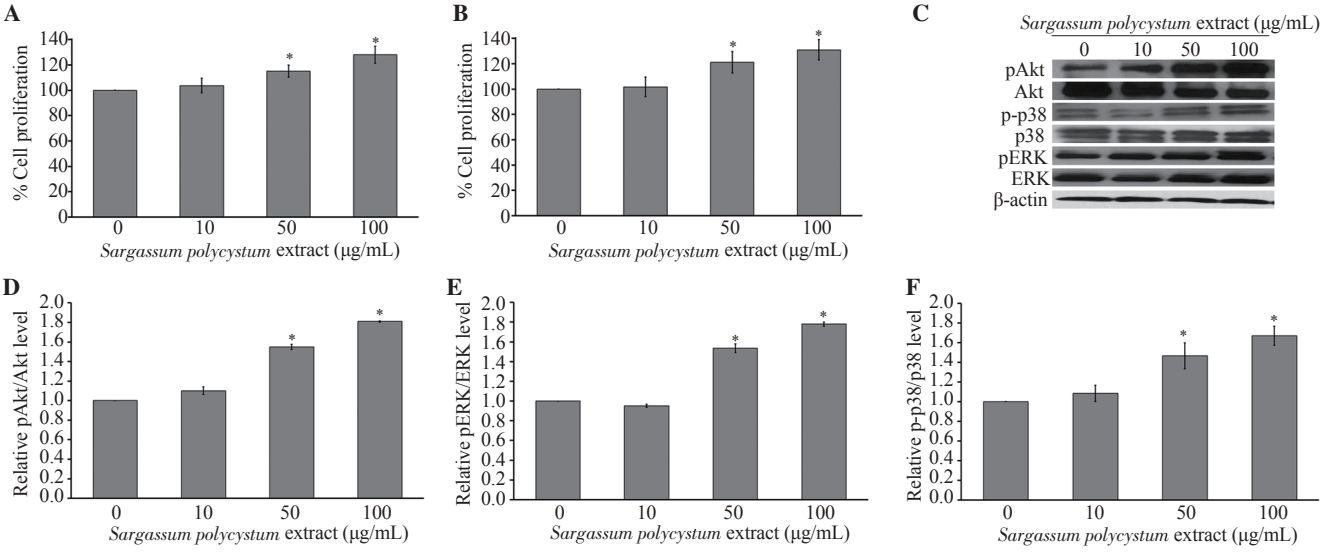

Figure 3. Effect of the hydroethanolic extract of *Sargassum polycystum* on the proliferation of HDF cells. HDF cells were treated with the extracts at 10-100 μg/mL for 24 h. (A-B) % cell proliferation determined by dsDNA assay kit and sulforhodamine B assay, (C) representative protein bands of Akt, ERK1/2 and p38 MAPK, (D) relative protein levels of pAkt/Akt, (E) relative protein levels of pERK/ERK, and (F) relative protein levels of p-p38/p38. The values are expressed as mean ± standard error of mean (n=4). $^*P$<0.05 compared with the control.

The quantification of antioxidant activity by DPPH was (57.10 ± 0.82)% for the hydroethanolic extract of *S. polycystum* and (92.62 ± 0.05)% for vitamin C. FRAP and MCA in the hydroethanolic extract of *S. polycystum* were (18.52 ± 1.27) µM Fe^{2+}/g sample and (0.27 ± 0.01) µM EDTA/g sample, respectively.

3.3. Cytotoxicity activity of the hydroethanolic extract of S. polycystum in HDF and HaCaT cells

Figure 2A and 2B revealed that the hydroethanolic extract of *S. polycystum* did not significantly increase the cytotoxicity in both cell types. The cell viability was above 95% at all concentrations.

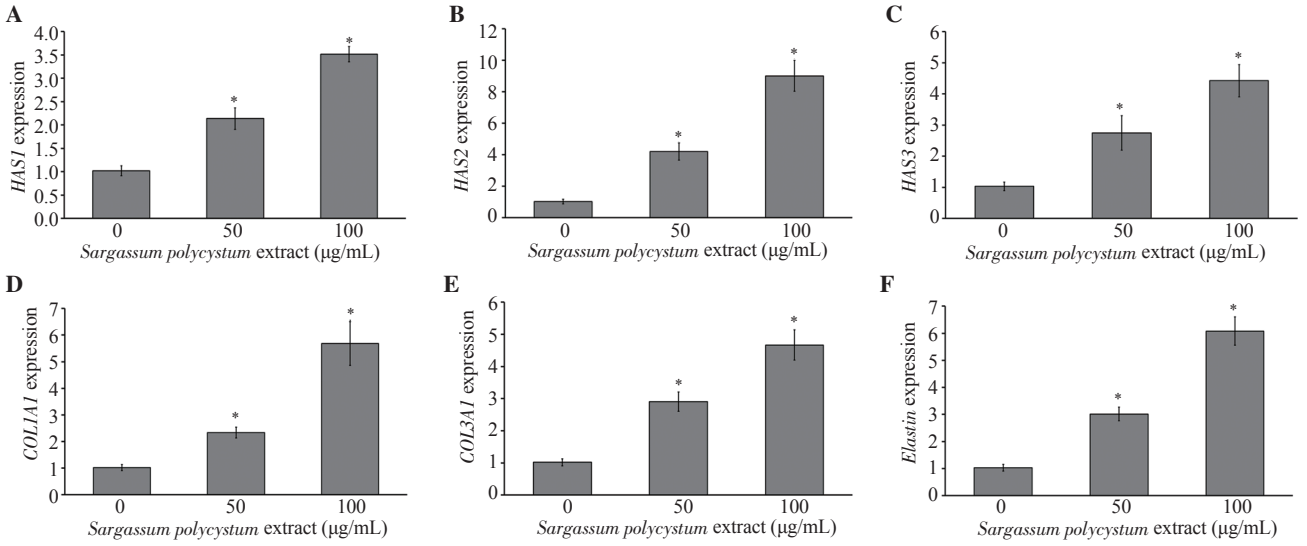

Figure 4. Effect of the hydroethanolic extract of *Sargassum polycystum* on the gene expressions of (A) *HAS1*, (B) *HAS2*, (C) *HAS3*, (D) *COL1A1*, (E) *COL3A1*, and (F) *elastin* in HDF cells. HDF cells were treated with the extracts at 50-100 µg/mL for 24 h. The values are expressed as mean ± standard error of mean (n=4). *P<0.05 compared with the control.

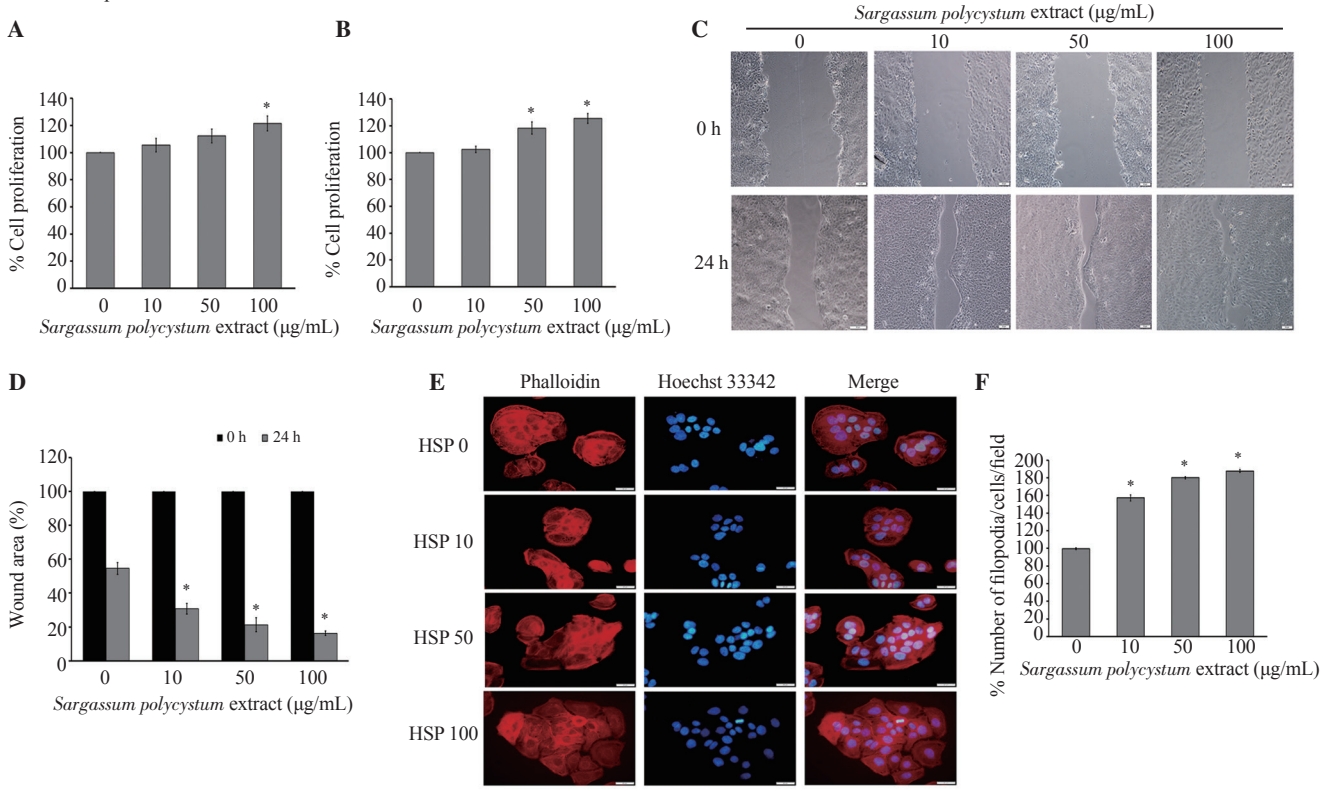

Figure 5. Effect of the hydroethanolic extract of *Sargassum polycystum* on the proliferation, migration, and filopodia formation of HaCaT cells. HaCaT cells were treated with the extracts at 10-100 µg/mL for 24 h. (A-B) % cell proliferation determined by dsDNA assay kit and sulforhodamine B assay, (C) representative images of wound areas (scale bar = 50 µm), (D) the percentage of wound area, (E) representative images of filopodia formation by phalloidin-rhodamine staining (scale bar = 20 µm), and (F) the number of filopodia per cell was calculated as a percentage. The values are expressed as mean ± standard error of mean (n=3-4). *P<0.05 compared with the control. HSP: hydroethanolic extract of *Sargassum polycystum*.

3.4. Hydroethanolic extract of S. polycystum stimulates the proliferation of HDF cells by activating the p38, ERK1/2, and Akt pathway

The proliferation of HDF cells is an important step in the process of wound repair. Therefore, the proliferation of HDF cells was investigated in this study by dsDNA assay and sulforhodamine B assay. The proliferation of HDF cells was enhanced with increasing concentrations in both dsDNA assay (Figure 3A) and sulforhodamine B assay (Figure 3B). The highest proliferation was observed at the concentration of 100 μg/mL with values of (127.98 ± 6.87)% by dsDNA assay and (130.48 ± 7.94)% by sulforhodamine B assay (Figure 3A and 3B). After 24 h of treatment, the protein expressions of Akt, ERK1/2, and p38 MAPK were determined using Western blotting analysis. The phosphorylation of Akt, p38, and ERK1/2 was significantly increased (P<0.05) after administration with 50 and 100 μg/mL of the hydroethanolic extract of *S. polycystum* compared with the control (Figure 3C-F). These findings implied that the hydroethanolic extract of *S. polycystum* promoted HDF proliferation by Akt, ERK1/2, and p38 MAPK activation.

3.5. Hydroethanolic extract of S. polycystum increases the gene expression in HDF cells

The effects of the hydroethanolic extract of *S. polycystum* on *HAS1*, *HAS2*, *HAS3*, *COL1A1*, *COL3A1*, and *elastin* gene expressions in HDF cells are presented in Figure 4. The hydroethanolic extract of *S. polycystum* at 50 and 100 μg/mL significantly upregulated the expression levels of *HAS1* by 2.14 ± 0.23 and 3.52 ± 0.16 (Figure 4A), *HAS2* by 4.21 ± 0.55 and 9.00 ± 0.98 (Figure 4B), *HAS3* by 2.75 ± 0.55 and 4.42 ± 0.52 (Figure 4C), *COL1A1* by 2.33 ± 0.20 and 5.68 ± 0.82 (Figure 4D), and *COL3A1* by 2.91 ± 0.30 and 4.67 ± 0.47 (Figure 4E), respectively. In addition, the extract (50 and 100 μg/mL) enhanced the *elastin* mRNA expression compared to the control group (P<0.05) (Figure 4F).

3.6. Hydroethanolic extract of S. polycystum enhances the proliferation, migration, and filopodia formation of HaCaT cells

The proliferation and migration of skin keratinocytes to the wound site is a major step in the wound-healing process. Similar to HDF cells, the proliferation of HaCaT cells was also increased by treatment with the hydroethanolic extract of *S. polycystum*. The maximum proliferation was observed at 100 μg/mL with (121.56 ± 5.56)% in dsDNA assay (Figure 5A) and (125.13 ± 3.69)% in the sulforhodamine B assay (Figure 5B). The scratch wounds closed more quickly in HaCaT cells treated with 50 and 100 μg/mL of the hydroethanolic extract of *S. polycystum* than those in the untreated group (Figure 5C and 5D). Treatment with 10, 50, and 100 μg/mL of the hydroethanolic extract of *S. polycystum* enhanced the scratch closure rate to 69.17%, 78.69%, and 83.72% in HaCaT cells after 24 h compared to the untreated group (45.42%) (Figure 5C and 5D). Moreover, rhodamine-labeled phalloidin was mainly stained polymeric F-actin like filopodia and lamellipodia. As presented in Figure 5E and 5F, the hydroethanolic extract of *S. polycystum* at 10, 50, and 100 μg/mL caused an increase in the reorganization of cytoskeletal actin stress fibers in HaCaT cells with the percentage of filopodia number increased by 157.28%, 180.07%, and 187.71%, respectively compared with the control group (P<0.05).

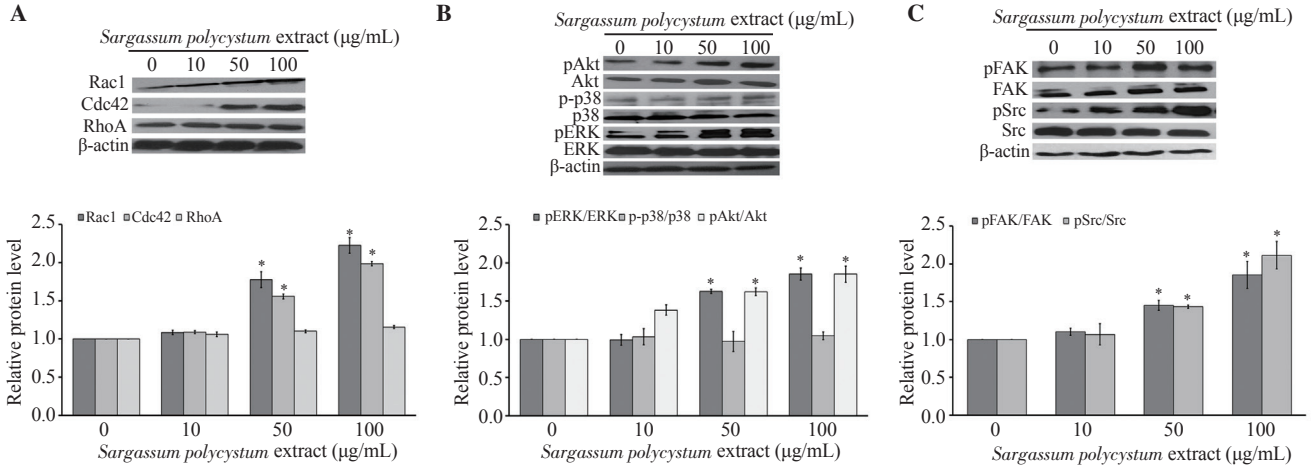

Figure 6. Effect of the hydroethanolic extract of *Sargassum polycystum* on the protein expressions of Rac1, Cdc42, RhoA, pAkt/Akt, p-p38/p38, pERK/ERK, pFAK/FAK and pSrc/Src in HaCaT cells. HaCaT cells were treated with the extracts at 10-100 μg/mL for 24 h. The values are expressed as mean ± standard error of mean (n=4). $^*P$<0.05 compared with the control.

3.7. Hydroethanolic extract of S. polycystum upregulates upstream and downstream proteins related to proliferation and migration in HaCaT cells

As presented in Figure 6A, the level of Cdc42 and Rac1 proteins was significantly upregulated in HaCaT cells treated with 50 and 100 μg/mL of the hydroethanolic extract of *S. polycystum* (P<0.05). However, the hydroethanolic extract of *S. polycystum* at all concentrations did not change the expression of RhoA protein compared with the control (Figure 6A). In addition, the phosphorylation of FAK, Src, Akt, and ERK1/2 was significantly increased (P<0.05) after administration with 50 and 100 μg/mL of the hydroethanolic extract of *S. polycystum* compared with the control (Figure 6B and C). In contrast, the expression of p38 MAPK protein did not change (Figure 6B). Taken together, this finding implies that the hydroethanolic extract of *S. polycystum* improves wound repair by increasing the keratinocyte proliferation, migration, and filopodia formation by activating FAK, Src, Akt, and ERK1/2 upstream signaling pathway, which contributes to stimulating Cdc42 and Rac1 downstream signaling pathway.

4. Discussion

Chronic skin wounds significantly impact public health services and decrease the patient's quality of life[6]. The process of wound closure and tissue healing is complex, involving various processes, for example, inflammation, re-epithelialization, forming of granulation tissue, and wound contraction. The development of pathological conditions, such as chronic inflammation, infections, and pathological scarring can occur during the wound healing processes[26]. In this study, we evaluated the effect of the hydroethanolic extract of *S. polycystum* on the viability, proliferation, and migration of HDF and HaCaT cells. In addition, the mechanisms of the extracts on the induction of cell proliferation and migration were also investigated.

Phytochemical screening of the hydroethanolic extract of *S. polycystum* showed the presence of the highest concentration of flavonoids, followed by a phenolic compound which might be accountable for the antioxidant activities and wound healing properties of these extracts. Several studies established that flavonoids and phenolic compounds possess wound healing properties[27,28]. In addition, flavonoids compound in this study was consistent with the phytochemical characterization in previous reports which might be related to the antioxidant activities presented in *S. polycystum*[20,29]. HPLC analysis of the hydroethanolic extract of *S. polycystum* also found the presence of *C*-phycocyanin and fucoidan which was similar to the previous reports[30,31]. Both *C*-phycocyanin and fucoidan have been reported to have antioxidant activities[31,32]. Furthermore, the MTT assay revealed that the hydroethanolic extract of *S. polycystum* did not cause cytotoxic effect in both HDF and HaCaT cells. Previous studies stated that the crude *S. polycystum* extracts and fucoidan isolated from *S. polycystum* showed cytotoxicity on cancer cells such as HeLa cervical cancer cells, HL-60, and MCF-7 cells[30,32,33]. However, the cytotoxicity of crude *S. polycystum* extracts and bioactive compounds against normal cells was not reported before. Based on our present findings, it can be concluded that the hydroethanolic extract of *S. polycystum* is safe and can be used for further experiments.

The main cells found in the granulated wound tissues are fibroblasts which are important for the synthesis of collagen and the extracellular matrix (ECM) component arrangement[5]. In wound healing, fibroblasts play a vital role by secretion of several growth factors that promote the deposition of matrix and angiogenesis[3]. The proliferation and migration of dermal fibroblasts is a crucial process and a rate-limiting step during wound healing especially in the granulation tissue formation[5,34]. Our finding found that *S. polycystum* extracts at a high concentration cause an increase in the proliferation of HDF cells. In addition, the expressions of pAkt/Akt, pERK/ERK, and p-p38/p38 MAPK were significantly upregulated after treatment with 50 and 100 μg/mL *S. polycystum* extracts. Several studies reported that the activation of ERK, p38MAPK, and Akt signaling pathways is essential in the proliferation and differentiation of fibroblasts in the wound repair process[35,36]. Moreover, Rognoni *et al.*[37] demonstrated that an initial phase of dermal fibroblast proliferation as well as migration followed by high ECM deposition, which regulates fibroblast proliferation, plays an improtant role in successful wound healing. In this study, *S. polycystum* extracts increased the expressions of *HAS1*, *HAS2*, *HAS3*, *COL1A1*, *COL3A1*, and *elastin* mRNA in HDF cells, which were mediated by ERK, p38 MAPK, and Akt signaling pathway. Type Ⅰ and Ⅲ collagen, HAS1, HAS2, and HAS3 were found to increase the healing and repair sites in the skin and other tissues which contribute to regulating cellular proliferation and differentiation and have a crucial role in skin hydration[38,39]. Baicalein, or 5,6,7-trihydroxy flavone, a flavonoid found in *S. polycystum* extract with 70% ethanol, has been noted to accelerate tendon-bone healing[20,40]. In addition, fucoidan, a marine-derived algal polysaccharide, increases collagen deposition and accelerates wound healing[41]. Our findings implied that *S. polycystum* extracts stimulated the proliferation of dermal fibroblasts in the wound closure process and might improve skin moisture by enhancing the content of hyaluronic acid in the dermal ECM. However, there are some limitations in this study. The protein expressions of HAS1, HAS2, HAS3, COL1A1, COL3A1, and elastin should be detected for further experiments.

The proliferation and migration of keratinocytes is an important step

in the initial wound healing process that leads to successful wound healing[13]. Re-epithelialization in the wound repair process involves the migration and proliferation of keratinocytes into the zone of the skin basement membrane[3]. In this study, *S. polycystum* possesses skin wound healing capabilities by enhancing the proliferation and migration of keratinocytes. Keratinocyte proliferation, migration, extension, and cytoskeleton contraction have been regulated by various signaling, for example, FAK, Src, Akt, MAPKs, and small GTPases that are involved in the keratinocyte migration and proliferation[13,14,33]. Small GTPases (Rac1, Cdc42, and RhoA) have been reported to control the cytoskeletal reorganization, filopodia, and lamellipodia formation that are associated with the migration of cells[13,14]. The formation of filopodia and lamellipodia has been regulated through Rac1 and Cdc42, leading to the rearrangement of the cytoskeleton that is related to the migration of keratinocytes and the wound repair process[13,14]. Our finding observed that the F-actin and filopodia formation was significantly increased after treatment with the extracts in HaCaT cells. Furthermore, the protein expression of Cdc42 and Rac1 was significantly upregulated in HaCaT cells treated with *S. polycystum* extracts, leading to an increase in filopodia formation and migration of keratinocytes. The cellular proliferation and re-epithelialization process during wound repair was regulated by the FAK-Src complex[9,10,13]. Moreover, the stimulation of the FAK-Src complex activates small GTPases (Rac1, Cdc42, and RhoA), leading to the production of lamellipodia and filopodia which induces keratinocyte migration[13]. In this study, treatment with *S. polycystum* extracts upregulated the phosphorylation of FAK (Tyr397) and Src (Tyr418). Moreover, the extracts significantly increased the phosphorylation of Akt and ERK1/2, suggesting that the stimulation of keratinocyte proliferation and migration is regulated by Akt and ERK1/2 pathway. ERK1/2 and Akt pathways are also important signalings in regulating the proliferation and migration of cells and also controlling the wound healing process[36]. Several studies revealed that the suppression of FAK, Src, ERK1/2, and Akt signaling pathways inhibits cell proliferation and migration, therefore slowing down the wound repair process[15]. Additionally, fucoidan was found to possess wound healing activities in HaCaT cells by accelerating anti-inflammatory and antioxidant activities[42,43]. Taken together, *S. polycystum* extracts significantly stimulated keratinocyte proliferation and migration by regulating the FAK, Src, Akt, and ERK1/2 signaling pathways.

In conclusion, *S. polycystum* extracts increased the proliferation and the gene expression of *HAS1*, *HAS2*, *HAS3*, *COL1A1*, *COL3A1*, and *elastin* in HDF cells by inducing Akt, ERK1/2, and p38 MAPK pathways. Furthermore, *S. polycystum* extracts stimulated the FAK, Src, Akt, ERK1/2, Rac1 and Cdc42 signaling pathways leading to an increase in the migration of HaCaT cells and thus contributing to wound closure. However, an *in vivo* study needs to be carried out as well as other mechanisms that control the wound healing process should be investigated in a future study to further verify the wound healing activity of *S. polycystum* extracts.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Authors' contributions

WS and ST contributed to the design of the work, critical revision of the article and final approval of the version to be published. Both WW and FM performed the data collection, data analysis and interpretation. NK, CA and WS contributed to drafting the article. WS supervised the project and funding acquisition.

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