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In vitro antileishmanial, antibacterial, antifungal and anticancer activity of fucoidan from *Undaria pinnatifida*

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Abstract

Traditional approaches have been reported since a long time for curing and treatment of various ailments. Sulphated polysaccharides from brown seaweeds such as fucoidan have been reported to possess significant antiinflammatory, antioxidant, antimicrobial, antiviral and antitumor activities. Herein, the antileishmanial, antimicrobial and anticancer activity of fucoidan from *Undaria pinnatifida* were investigated. The antileishmanial activity was evaluated by MTT assay. The antimicrobial activities were determined by using the agar disc diffusion method. While anticancer activity was determined by using the SRB colorimetric method. Results showed that fucoidan effectively inhibited the growth of *Leishmania tropica* promastigotes showing mortality rates ranging from 4.2-73.5% with LD_{50} value of 31.72 µg/ml. The antibacterial and antifungal activities of fucoidanat the concentration of 30µg/disc were tested against gram-positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*),gram-negative bacteria (*Salmonella typhimurium*)and fungal strains (*Aspergillus flavus*, *Aspergillus fumigatus,* Mucor species). Zones of inhibition obtained were compared with that of different standards cefixime for antibacterial activity and clotrimazole for antifungal activity. The results showed the maximum zone of inhibition of the bacterial growth against *S. aureus* (15.67±0.76 mm)and fungal growth against *A. fumigatus* (11.83±1.01) among test organisms. Fucoidan has shown considerable anticancer potential against human liver cancer (HepG2) cells (LD_{50}, 18.01±1.2 µg/ml). Based on these results, it can be concluded that fucoidan as natural products, may serve as leads for the development of new pharmaceuticals having diverse therapeutic potential.

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Introduction

Traditional medicines (TM) are health care practices and treatments that are indigenous to the culture and historically operated predominantly outside the state-funded healthcare system. TM is an important, common element of health seeking and treatment for big number of people in low to middle-income countries (Suswardany *et al.*, 2015; Oyebode *et al.*, 2016). Recently, many studies have focused on marine creatures, such as seaweeds, in the pursuit of novel drugs from natural products (Romano *et al.*, 2017). Numerous seaweed species have been used as traditional medicines, foods, and nutraceuticals in various parts of the world. *Undaria pinnatifida* is mainly found in temperate coastal regions of the Northeast Pacific, including Japan, Korea, and Northern China (Phull *et al.*, 2017) and an economically important food source in these countries. However, it can be found in temperate regions of the world as an invasive species (Kang *et al.*, 2016). *U. pinnatifida* belongs to the family *Alariaceae* and breeds on rocks and reefs to a depth of 1–10 m in Korea, Japan and China. *U. pinnatifida* is used in traditional medicine and mainly applied to treat urination problems, fever, lumps, swelling and as a dietary supplement for post-childbirth women (Fitton, 2003). In China, as an herbal medicine, it has been employed to treat dropsy and urinary diseases. Ishihara *et al.* (1998) isolated 18:4 n-3 fatty acid from *U. pinnatifida* that inhibits leukotriene production in inflammation. Therefore, many of these effects are directly or indirectly associated with the anti-inflammatory and anti-oxidant potential of the seaweed (Phull *et al.*, 2017).

Seaweed derived fucoidan possess diverse pharmacological activities (Phull and Kim, 2017) such as therapeutic potential in surgery, anti-inflammatory, gastric protective effects, antioxidant, antithrombotic, anticomplementary properties, and activity against renalpathy, uropathy and hepatopathy (Li *et al.*, 2008). Despite of manifold studies carried out for the biological activities of fucoidan, it is necessitating to explore other bio-effects such as antileishmanial. To further evaluate the medicinal potential of fucoidan isolated from *U. pinnatifida*; a rich species with huge aquaculture potential, anti-leishmanial, antibacterial, and anticancer were investigated in the current study. Materials and Methods.

Fucoidan was purchased from Haewon Biotech, Inc. Republic of Korea. M199, penicillin and streptomycin, were purchased from Sigma-Aldrich (USA). Fetal bovine serum was purchased from PAA Laboratories GmbH. Sabouraud dextrose agar and broth, Nutrient agar and broth and buffered peptone water were purchased from Difco, Sparks, MD, USA. Microtiter plates were obtained from SPL Life Sciences, Republic of Korea. All other chemicals were obtained from Sigma-Aldrich unless otherwise mentioned.

Antileishmanial activity

Antileishmanial assay was performed according to the MTT colorimetric procedure previously described with slight modifications (Ahmed *et al.*, 2017). Initially, about one week cultured *Leishmania tropica* promastigotes were grown in Medium 199 supplemented with foetal bovine serum (10%), streptomycin sulphate (100 µg/ml) and penicillin G (100 IU/ml) at 25°C. An aliquot of 20 µl of test sample and 1.8 × 10^5promastigotes were transferred in each well 96-well plate with a final volume of 200 µl per well. Amphotericin B and PBS were used as positive and negative controls respectively. The culture were grown for 3 days at 24°C followed by the addition of 20 µl MTT solution (4000ppm) and the plate was again incubated for 4 hours until formazan crystal formation. Subsequently, the supernatant was removed carefully and 100 µl of DMSO was added for dissolving formazan crystals. Optical density was recorded at 540 nm using a microplate reader. The data obtained was analysed by using Graph Pad Prism (Graphpad Prism software Version 5.0, Graph-Pad software Inc, CA, USA).

Antibacterial assay

The antibacterial activity potential of fucoidan was evaluated against Gram positive (*M. luteus* ATCC-10240, *S. aureus* ATCC-6538) and Gram negative (*S. typhimurium* ATCC-14028) bacterial strains through disc diffusion method (Phull *et al.*, 2016; Ali *et al.*, 2016).

Each bacterial strain was refreshed in nutrient broth and 100 µl of refreshed culture (10^6 colony forming units/ml) were transferred and evenly distributed on a nutrient agar plate. An aliquot of 5 µl of sample (6 mg/ml DMSO) was loaded on sterile filter paper discs (6mm in diameter). Cefixime, an antibacterial drug and DMSO were used as positive and negative controls respectively. Thereafter, plates were incubated at 37°C for 24 h and next day growth inhibition zones (in mm) around disc were measured. The experiment was repeated three times.

Antifungal assay

The antifungal potential of fucoidan was investigated against *Aspergillus flavus* (FCBP-0064), *Aspergillus fumigatus* (FCBP- 66), and *Mucor*species (FCBP-0300) fungal strains according to the procedure with some modifications (Phull *et al.*, 2016). Spores of these strains were suspended in 0.02% Tween 20 solution turbidity was compared with McFarland 0.5 turbidity standard. Later on 100 µl of suspension of each fungal strain were transferred and homogeneously swabbed on sterile sabouraud dextrose agar (SDA) plates. An aliquot of 5 µlof sample solution (6 mg/ml DMSO) was loaded on sterile paper discs (6 mm in diameter) and placed in their respective position on the SDA agar plate. Clotrimazole as positive control and DMSO was used as negative control. Plates were incubated for 24-36 hours at 28°C and the average growth inhibition (in mm) around discs were recorded as inhibition zones. The experiment was performed in three individual experiments.

Anticancer activity

In vitro anticancer potential of fucoidan on human liver cancer cells (HepG2, RBRC-RCB1648) was investigated through previously described SRB colorimetric assay method with slight modification (Ahmed *et al.*, 2017). HepG2 cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) growth medium containing heat inactivated Fetal Bovine Serum (10%), streptomycin sulphate (100 µg/ml), penicillin G sodium (100 IU/ml), amphotericin B (0.25 µg/ml) and pH 7.4. The cells were grown in a CO_2 incubator in humidifies condition (5% CO_2, 95% air) at 37°C for 72 huntil the confluence reached ~75% and subsequently medium was replaced, cells trypsonised. Initially, 190 µl of cell suspension (1 x 10^5 cells/ml)were seeded per well in 96-well plate along with 10 µlof sample (0-100µg/ml) in respective labelled wells and subjected to incubate for 3 days in CO_2 incubator. Then, cells were fixed at 4°C with 50 µl of cold TCA solution (20% w/v) for 1 h, followed by thrice washing with distilled water, air drying and staining with 50 µl SRB solution (0.057% w/v in 1% acetic acid) for half an h at 25°C. Wells were again thrice washed with 1% v/v acetic acid and dried for 12 h at room temperature. Finally, 200 µl of Tris base (10 mM, pH 10) was added for 1 h to solubilize the bounded dye. The absorbance was recorded at 515nm by using a micro-plate reader (Biotech USA, microplate reader Elx 800) and % inhibition was calculated. DMSO and doxorubicin were used as negative and positive controls, respectively. An experiment was performed in triplicate and LD_{50} was calculated using GraphPad Prism (Graphpad Prism software Version 5.0, Graph-Pad software Inc, CA, USA).

Statistical analysis

All the experiments were performed in three independent experiments, and results are expressed as mean ± standard deviation (SD). The statistical analysis was performed by one way ANOVA followed by Dunnett's test in sigma plot 12.0 (Systat software Inc., CA, USA). The results were considered significant at the levels of $p<0.05$.

Result and discussion

Brown seaweed species contains enormous quantities bioactive macromolecules likefucoidan, fucoxanthin, xylofucoglycuronans and glycuronogalactofucans (Ahmadi *et al.*, 2015). Among these fucoidan are important polysaccharide having sulphate ester, L-fucose groups, other monomers such as proteins uronic acid and monosaccharides and possessing a variety of medicinal properties (Phull *et al.*, 2017; Phull and Kim, 2017).Although, fucoidan has complex structure but the structural backbone hasbeen elucidated and basic structure of fucoidan from *U. pinnatifida*is presented in Fig. 1.

Variety of bioactivities of fucoidan supports its use as functional food for health beneficial effects, along with prevention and management of different diseases (Vo *et al.*, 2012).

Moreover, multifunctional activities, making fucoidan, a potential substance in therapeutical, cosmeceutical and nutraceutical industries (Wijesingheand Jeon, 2012).

Table 1. Antimicrobial activity of fucoidan against different bacterial and fungal strains.

Bacteria strains	Zone of inhibitions (mm)			Fungal strains	Zone of inhibitions (mm)		
	NC	FU	PC		NC	FU	PC
M. luteus	--	13.83±1.04[a]	23.17±2.3[b]	*A. flavus*	--	8.5±0.87[a]	19±2.18[b]
S. aureus	--	15.67±0.76[a]	24.3±0.76[b]	*A. fumigatus*	--	11.83±1.01[a]	20.3±1.0[b]
S. typhimurium	--	8±0.51[a]	21.5±1.0[b]	*Mucor species*	--	7.54±0.4[a]	18.17±1.3[b]

NC: negative control, Fu: Fucoidan, PC: positive control (cefixime for antibacterial activity and clotrimazole for antifungal activity), -- = not detected, letters were given according to increasing means value, same letters represents no significant difference in same row ($P<0.05$).

Antileishmanial potential

Leishmaniasis is the protozoal disease caused by *Leishmania* parasite. More than 300 million people are threatened from this disease around the globe and around 1-2 million cases are appearing every year (Oliveira *et al.*, 2009). Pentavalent antimonial sodium stibogluconate (Pentostam) and meglumineantimoniate (Glucantime) have been used for the treatment of leishmaniasis, however, these drugs showed side effects due to their prolonged parenteral consumption (Javed *et al.*, 2015). In recent days, amphotericin B and pentamidine are also used for the treatment which also possesses lethal effects in some cases (Hepburn *et al.*, 1994; Santos *et al.*, 2008). Natural products are being explored for the treatment of leishmaniasis, due to virulent effects (Khan *et al.*, 2015), resistance of parasites (Al Nasr and Ahmed, 2017; Légaré and Ouellette, 2017) and high cost of current drugs for leishmaniasis (Desjeux, 2004; Zulfiqar *et al.*, 2017).

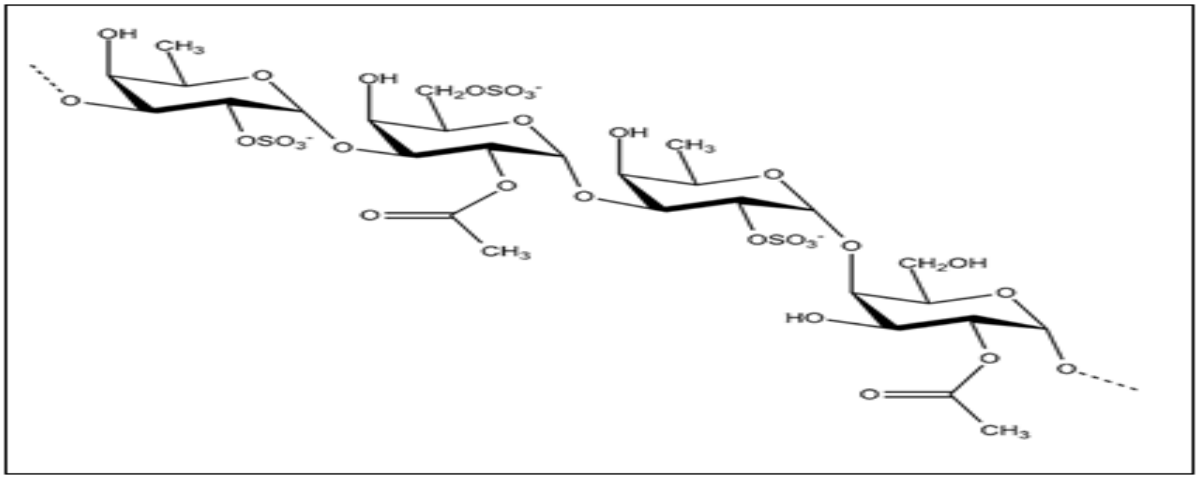

Fig. 1. Basic structure of fucoidan from *Undariapinnatifida*.

Brown algae and medicinal plants are rich depositories of therapeutic constituents. In the current study, antileishmanial activity was performed to explore the potential of fucoidan by inhibiting and retarding multiplication of leishmanial parasite. Fig. 2 shows the percent mortality of *L. tropica* strain caused by fucoidan. It was shown that antileishmanial activity is concentration dependent and activity was directly proportional to dose of test sample. At lowest concentrations (1.56, 3.13 and 6.25 ppm), about 4.2, 6.45 to 10.63 % mortality rate was recorded, respectively.

At the maximum concentration (100 µg/ml), highest mortality rate (73.69%) was recorded. The LD_{50} value of fucoidanfor antileishmanial activity was calculated as 31.72 µg/ml. Where as, LD_{50} of positive control (amphotericin B) was observed as 0.056 ± 0.002 µg/ml.

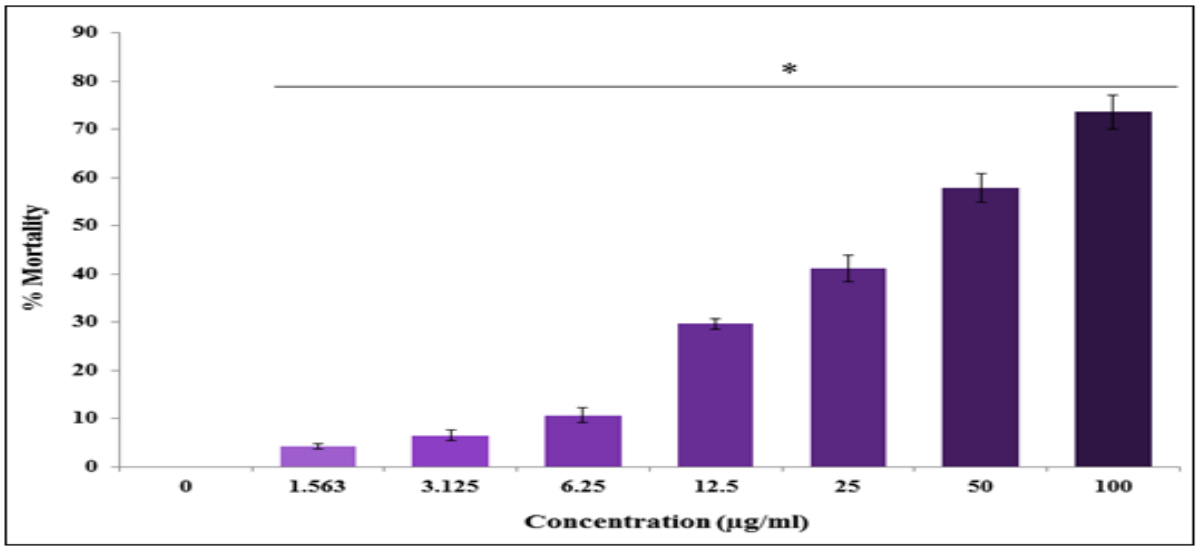

Fig. 2. Antileishmanial effectiveness of fucoidan against *L. tropica* strain. Results are the mean ± standard deviation of three independent experiments. Amphotericin B (0.056 ± 0.002 µg/ml.) was used as positive controls. Significance differences were considered when *P<0.05.

Antimicrobial potential

In this study, antibacterial and antifungal activity of fucoidan was assessed against different pathogenic microbial strains through disc diffusion method as shown in Table 1. The highest antibacterial activity of fucoidan was observed against *S. aureus* with 15.67 ± 0.76 mm zone of inhibition followed by *M. luteus* (13.83 ± 1.04 mm). The lowest activity was observed for *S. Typhimurium* with 8.5 ± 0.05 mm zone of inhibition.

Antibacterial activity fucoidan from *Sargassum wightii* derived fucoidan against human bacterial pathogens, including *Salmonella typhi*, *Vibrio cholerae*, *Shigella sonnie, Klebsiella, Pseudomonas aeruginosa*, *Proteus proteus*, *Escherichia coli*, *Klebsiella pneumoniae* species (Marudhupandi and Kumar, 2013). Recently, Pérez *et al.*, (2016) have reviewed the antimicrobial activity potential of different active molecules isolated from seaweeds against the pathogens e.g., *S. aureus* and *P. aeruginosa* that commonly cause infections in humans. Moreover, *S. aureus*is one of the most common foodborne pathogen and its control is important for food industry (Khan *et al.*, 2016).

As shown in Table 1, fucoidan were also found active against fungi and recorded highest zone of inhibition for *A. fumigatus* (11.83 ± 1.0 mm) followed by *A. flavus* (8.5 ± 0.87 mm). Mucor species were resistant to fucoidan and has shown lowest activity among tested strains. Various studies are being carried out to explore the natural substances that could be used against Methicillin-resistant *S. aureus* (MRSA) for combating the therapeutic problems related to *S. aureus* (Gibbons *et al.*, 2003; Lee *et al.*, 2014; Pérez *et al.*, 2016). These studies support the present results of antibacterial activity and further it is suggested to elucidate the molecular mechanism of the activity. Gram positive bacterial strains are more susceptible to the algal extract due the variation in cell wall structure and composition (Yamashita *et al.*, 2001). Whereas the cell wall of gram negative strains acts as a barrier for different antibiotics and environmental conditions (Masschelein *et al.*, 2015). Fucoidan are a bioactive polysaccharide present in the cell walls of numerous brown algae species such as *U. pinnatifida* (Phull *et al.*, 2017). These fucoidan fractions of *L. japonica*, *Sargassum fulvellum*, *Eisenia bicyclis*, *L. angustata*, *Ecklonia cava*, *S. kjellmanianum* and *L. angustata* have been reported to have antimicrobial properties (Ale *et al.*, 2011; Choi *et al.*, 2015).

Anticancer potential

Rapidly growing population, aging causes variation in lifestyle which results in amplified contact with main cancer risk factors such as sedentary lifestyle, smoking, and unhealthy diet that ultimately increases the worldwide burden of cancer with 8.2 million cancer related deaths and 14.1 million cancer cases in 2012 (Tervonen *et al.*, 2017). Liver cancer most often occurs in men, it is second and sixth leading cause of cancer deaths in men of low developed and more developed countries, respectively. A global estimated liver cancer related deaths is 745,500 and 782,500 new cases occur in 2012 and more than 70% of which are hepatocellular carcinoma (Torre *et al.*, 2015).

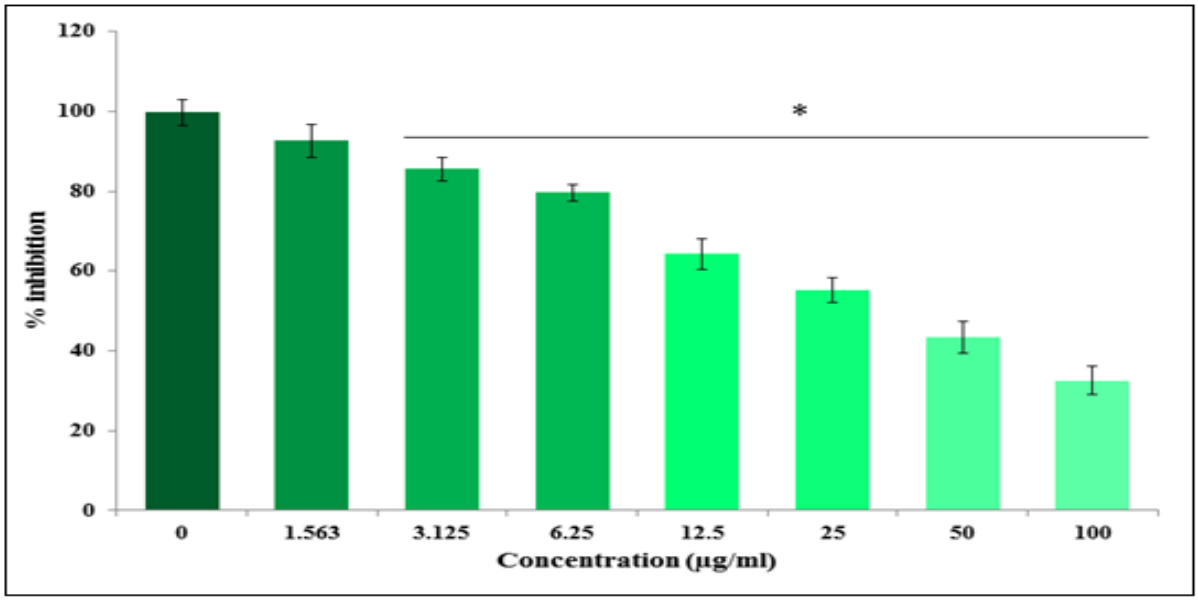

Fig. 3. Different concentrations of fucoidan were used for anticancer activity against HepG2 cell. Doxorubicin (LC_{50}5.81 ± 0.34 µg/ml) was used as positive controls and DMSO as negative control. The results were considered statistically significant at level of * P<0.05.

The anticancer activity of fucoidan was investigated against human liver cancer (HepG2) cells. Inthis study concentration dependant anticancer activity was observed in fucoidan exposed HepG2 cells, at the dose of 1.563-100 µg/ml and growth inhibition of 7.3-67.4% with an LD_{50} of 18.01±1.2µg/ml. While, doxorubicin used as standard drug showed anticancer activity with $LD_{50}$of 5.81±0.34 µg/ml. The anticancer results are presented in fig. 3. Liver cancer is one of the leading widespread cancers. Moreover, hepatocellular damage occurs via oxidative stress and chronic inflammation (Machana *et al.*, 2012).

We have previously reported the significant *in vitro* and *in vivo* antioxidant and anti-inflammatory activity of this molecule (Phull *et al.*, 2017; Phull and Kim, 2017). Fucoidan isolated from brown seaweed *Turbinaria conoides* effectively inhibited the growth of A549 (human lung cancer) cells in a dose-dependent manner and potent anticancer activities were 24.9-73.5% in the concentrations of 31.25-500 µg/ml (Marudhupandi *et al.*, 2015). Xue *et al.* (2012) have reported the effectiveness of crude fucoidan on mouse breast cancer *in vitro* and *in vivo* and the results showed that crude fucoidan inhibited mouse breast cancer growth due to increased apoptosis induction, suppressed lung metastasis and decreased angiogenesis. These data suggest that fucoidan may serve as a potential therapeutic agent for cancer.

Conclusion

In the current study, *in vitro* growth inhibitory potential of fucoidan from *Undaria pinnatifida* was investigated on *Leishmania tropica* promastigotes and human liver cancer cells (HepG2) through MTT colorimetric and SRB procedures, respectively. In addition, antimicrobial activity was also evaluated by using disc diffusion method. The result showed the

significant antileishmanial and anticancer activities of fucoidan. Furthermore, it also inhibited the growth of fungal strains and displayed broad spectrum antibacterial potential. The results obtained in the current study can be supportive data for future investigations that will lead to the use of fucoidan in therapeutical formulation. Additionally, detailed investigations are needed to evaluate the mode of actions of these activities.

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