



Cytotoxic and pharmacokinetic studies of Indian seaweed polysaccharides for formulating raindrop synbiotic candy



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ABSTRACT

Gut microbiome evidenced as the assembling mode of action facilitates the relationship of environmental factors (such as diet and lifestyle) with colorectal cancer. The cytotoxic and anticancer studies of the enzymatically extracted polysaccharides from selected Indian seaweeds (such as *S. wightii*, *E. compressa*, and *A. spicifera*) on Raw 264.7 macrophage and HT-29 human colon cancer cell line were investigated. *E. compressa* showed nitric oxide production up to a concentration of $6.99 \pm 0.05 \mu\text{M}$. The polysaccharide extract of seaweed (PES), *A. spicifera* (100 µg/ml) had shown the highest *in-vitro* cytotoxicity effect on HT-29 cells up to $52.13 \pm 1.4\%$. Absorption, distribution, metabolism and excretion (ADME) predictions were performed for exploring the possibility of anti-cancer drug development. The formulated synbiotic candy exhibited post storage survivability of probiotic species *L. plantarum* NCIM 2083 up to 10^7 CFU/ml until three weeks and it could be an aesthetic functional food for treating colon cancer.

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1. Introduction

Cancer is a cluster of diseases with high mortality and morbidity rate. Colorectal cancer is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women [1]. As per GLOBOCAN (Global Cancer Observatory) report, more than 1.8 million cases of colorectal cancer has been reported to date [2]. The mortality rate of patients diagnosed with colorectal cancer remains nearly 40%, largely due to metastatic invasion of the liver. Despite the advancement in therapeutic medication, the affliction is believed to escalate by 60% with a further 2.2 million new cases and 1.1 million cancer deaths by 2030 [3].

Currently, the gut microbiome mediated immune system is receiving attention for the treatment of several health ailments by immunomodulation such as malnourishment, gastrointestinal disorders, diabetes and others [4]. Gut microbiome lodging with trillion of microbes play a significant role in nutrient processing, vitamin production and physiologically affect colon cancer development by metabolic mediated changes in immune response [3,5]. Colorectal cancer is enriched differentially with several microbes such as *Fusobacterium*

nucleatum and *Bacteroides fragilis* [6]. Beneficial gut microbes such as *Lactobacillus* sp. and *Bifidobacterium* sp. that assist in the production of short-chain fatty acids (SCFA) like butyrate and propionate are found depleted in the gut microbiome of the patients [7]. In order to eliminate carcinogens and tumor progression, beneficial gut microbes (probiotics) should be densely populated in the gut. Prebiotics, a dietary compound with the non-digestible, selective substrate and easily fermentable properties could particularly enhance the population of probiotics [8].

The enhanced incidence of colorectal cancer in developed and developing countries has provoked scientists worldwide to employ natural and synthesized compounds to suppress the proliferation of invasive cancer. Marine macroalgal seaweeds are significantly used as functional foods and medicinal compounds for several health ailments [9]. Dietary fiber or non-digestible polysaccharides from several brown, green and red seaweeds has been examined for their anticancer activity. Recent studies explored that novel compounds from seaweeds are found to be a promising source of human therapeutic agents [10]. Naturally obtained seaweed polysaccharides has been recently recognized as a reliable and trustworthy approach with immense potential for colon cancer treatment.

Sulfated seaweed polysaccharides are gaining central attention due to its extensive pharmacological activities. Fucoidan, a sulfated polysaccharide from brown seaweeds, has shown beneficiary therapeutic

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properties such as antitumor, immunomodulating, antioxidant, antiangiogenesis, anti-inflammatory and anticoagulant activities [10]. Green seaweeds such as *Enteromorpha*, *Ulva*, *Monostroma*, and *Caulerpa* species are found to synthesize enormous quantities of sulfated polysaccharides with anticancer and immunomodulating activities [11,12]. Carrageenans are sulfated polysaccharide of red seaweeds with basic structural units of carrabiose disaccharide consists of alternating β -1,3- and α -1,4-linked galactose residues that exhibit antitumor and immunoregulatory activities [13].

In this present study, the anticancer activity of seaweed polysaccharides was evaluated *in-vitro* using HT-29 human colon cancer cell line. Cytotoxicity effect of seaweed polysaccharides on immune cell Raw 264.7 macrophage cell line was also investigated. Molecular Docking Analysis for determining the binding affinity of the seaweed polysaccharides such as fucoidan, ulvan and carrageenan towards M3 Muscarinic Acetylcholine Receptor (4V6O) was executed. Absorption, distribution, metabolism and excretion (ADME) predictions were studied to determine the possibility of anti-cancer drug development. *In-vitro* gastrointestinal digestion and tolerance of symbiotic beads formed by the encapsulation technique were explored. Symbiotic candy was formulated, and the viability of probiotic species *Lactobacillus plantarum* NCIM 2083 over three weeks was also investigated.

2. Materials and methods

Analytical grade chemicals and reagents were procured from Sigma Aldrich (USA) and Himedia (India). Polysaccharide extraction, characterization and its *in-vitro* prebiotic activity has been reported earlier [14].

2.1. Proton nuclear magnetic resonance spectroscopy (^1H NMR spectra)

To identify the structure of seaweed polysaccharide, NMR (^1H NMR) was performed. Three milligrams of the water-soluble polysaccharide was dissolved in 0.5 ml of 99% deuterium oxide (D_2O). NMR spectra of the samples were recorded using Ultrashield Bruker 300 spectrometer at room temperature with a frequency of 400 MHz, an acquisition time of 5.29 s and a pulse duration of 11 ms.

2.2. Cytotoxicity effect on RAW 264.7 macrophage cell lines

Raw macrophage 264.7 cell lines were harvested from maintenance cultures and were counted by hemocytometer. Cells were cultured in a 96-well plate (7×10^3 cells/well) at 37 °C, and then exposed to different concentrations (25, 50 and 100 $\mu\text{g}/\text{ml}$) of PES for 48 h. A 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution of 4.5 mg/ml was added to each well at 3 h before the end of the experiment and incubated in the dark. The media was removed, and the resultant formazan crystals were dissolved in Dimethyl sulfoxide. The absorbance was measured by a microplate reader at 595 nm [15]. All the experiments were performed in triplicates, and the percentage of cell viability was calculated by comparing the absorbance of the control and treated cells.

2.3. Inhibition of nitric oxide production assay

Nitric oxide production assay was performed by photometric analysis. Macrophage cells were cultured in 96 well plates with 10^4 – 10^5 cells per well and incubated for 24 h at 37 °C. Bacterial lipopolysaccharides (1 $\mu\text{g}/\text{ml}$) was used as a control. Seaweed polysaccharides of 50 and 100 $\mu\text{g}/\text{ml}$ were added and incubated for 24 h at 37 °C. The collected supernatant (100 μl) was added with Griess reagent and incubated for 10 min. Absorbance was noted at 550 nm [16].

2.4. Anti-cancer activity on HT-29 colon cancer cells

2.4.1. MTT assay

HT-29 cells were harvested from maintenance cultures and counted by hemocytometer. Cells were cultured in a 96-well plate (7×10^3 cells/well) at a temperature of 37 °C, and then exposed to different PES concentrations (25, 50 and 100 $\mu\text{g}/\text{ml}$) for 48 h. A 20 μl of MTT solution (4.5 mg/ml) was added to each well at 3 h before the end of the experiment and incubated in the dark. The media was removed, and the resultant formazan crystals were dissolved in Dimethyl sulfoxide. The absorbance was measured by a microplate reader at 595 nm [15]. All experiments were performed in triplicates, and the percentage of cell viability was calculated by comparing the absorbance of the control and treated cells.

2.4.2. ROS generation assay

HT-29 cells were cultured in a 12 well plate (1×10^5) at 37 °C under 5% CO_2 in a humidified chamber. The cells were exposed to different concentrations (25, 50 and 100 $\mu\text{g}/\text{ml}$) of PES for 48 h. The cells were then incubated with 5 $\mu\text{M}/\text{ml}$ of dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min at dark. Before analysis with flow cytometry, the cells were washed with Phosphate-buffered saline (PBS) and dislodged by trypsin [17].

2.4.3. Apoptosis assay

HT-29 cells were cultured in a 24 well plate (5×10^4) at 37 °C under 5% CO_2 in a humidified chamber. The cells were exposed to different concentrations (25, 50 and 100 $\mu\text{g}/\text{ml}$) of PES for 48 h. Then the cells were fixed with 10% formalin for 10 min at room temperature followed by blocking and permeabilization. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) and subjected to immunofluorescence imaging [18].

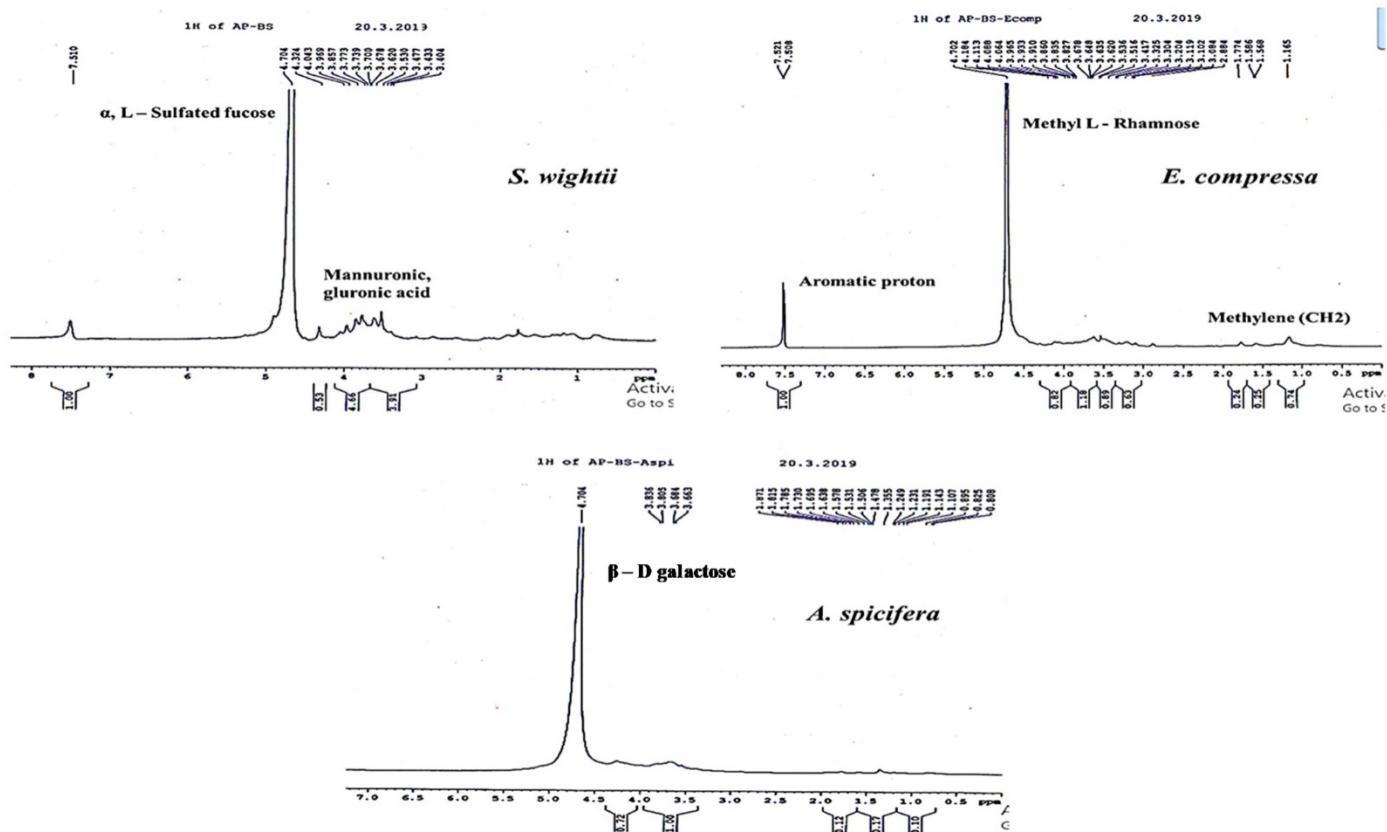
2.5. Molecular docking analysis and ADME predictions

The binding affinity of the polysaccharide compounds extracted from Indian macroalgal seaweeds towards M3 Muscarinic Acetylcholine Receptor (4V6O) was executed using a flexible docking protocol of Glide v.5.4 using Schrodinger 2019-1. M3 Muscarinic acetylcholine receptor structure was optimized and minimized at neutral pH. Grid box of $10 \times 10 \times 10$ Å was developed around the active site residues such as Tyr-148, Ser-151, Trp-199, Tyr-506, Asn-507, Tyr-529 and Cys-532. Glide flexible protocol was carried out such that all the three ligands were docked completely into the active site residues. Extra precision (XP) docking scores (XPG score) and binding free energies (ΔG) were calculated. The compounds retrieved with good interaction towards M3 Muscarinic acetylcholine receptor were submitted to QikProp v.6.5 of Maestro v.11.5 to calculate the molecular descriptors and physicochemical properties to ADME/T properties [19].

2.6. Candy formulation by an encapsulation technique

2.6.1. *In vitro* gastrointestinal digestion and tolerance of free cells

L. plantarum was grown in MRS broth for 48 h and was centrifuged at 10000 rpm for 10 min to obtain a cell pellet. The collected cell pellet was then added with 5 ml of saline. The initial CFU count of the cell suspension was enumerated by plating on MRS agar after appropriate serial dilution. The enzymatic solution for *in-vitro* gastrointestinal digestion was prepared by dissolving 0.02 g pepsin in 2 ml of 0.1 M HCl and 0.12 g of bile with 0.02 g of pancreatin in 10 ml of 0.1 M NaHCO_3 . Five milliliter of cell suspension was added to 100 ml MRS broth with initial pH adjusted to 2.0. Two milliliter of pepsin solution was then added to the mixture. Two hours after digestion, the cell load was calculated by withdrawing 2 ml spent broth and plating with appropriate dilutions on MRS agar plates. The pH of the digestion mixture was then adjusted to 6.0. Ten milliliter of bile/pancreatic solution was added to the digestion mixture. After 2 h, the viable cell count was calculated by the earlier mentioned methods.

Fig. 1. ¹H NMR spectra of the Indian seaweed polysaccharide extracts.

2.6.2. In vitro gastrointestinal digestion and tolerance of encapsulated cells

Sodium alginate (2%) and CaCl₂ (5%) solutions for bead encapsulation were prepared. Trisodium citrate (3%) solution for dissolving entrapped bacterial cells was prepared. Five milliliter of cell suspension was added to 50 ml of sodium alginate solution. The concentrated PES of *S. wightii*, *E. compressa* and *A. spicifera* was added to 2% sodium alginate at 2:1 ratio. Inulin was used as a positive control and sodium alginate without PES was used as a negative control. The cell suspension individually was loaded in a 31-gauge syringe and added in a dropwise manner to CaCl₂ solution through the needle. The symbiotic beads formed were filtered through Whatman filter paper #1 from the CaCl₂ solution and stored in the refrigerator at 4 °C till further use. The mean diameter of the collected symbiotic beads was measured using a ruler scale. The viable cell count of the symbiotic beads after digestion was calculated by dissolving them in a 10 ml trisodium citrate solution (pH 6.0) followed by plating on MRS agar with appropriate serial dilution. The

encapsulation efficiency (EE) reveals the effectiveness of the entrapment of combined microbes and enzymes and the viability of cells was calculated using Eq. (1).

$$EE = \left(\frac{N}{N_0} \right) * 100 \quad (1)$$

where *N* is the number of entrapped viable cells in symbiotic beads and *N*₀ is the initial free cells added to alginate mix.

2.6.3. Post-storage survivability of raindrop symbiotic candy

The alginate mix was prepared by adding PES at the ratio of 2:1. Five milliliter of cell suspension was then added to the alginate mixture. The alginate mixture was loaded in a 31-gauge syringe, and symbiotic beads were formed by adding alginate mix to CaCl₂ in a dropwise manner. The symbiotic beads formed were further hardened by placing them in the refrigerator at 4 °C for 30 min and then they were filtered with Whatman filter paper #1. Raindrop symbiotic candy was prepared by adding symbiotic beads (20) infused 2% alginate mixture in a mould and kept at the freezer of the refrigerator for 30 min. Then they were transferred to 5% CaCl₂ to form a symbiotic candy. Post-storage survivability (for three weeks) of the formulated candy placed at room temperature was studied by dissolving them at 3% trisodium citrate (pH 6.0) followed by MRS agar plating at appropriate serial dilution.

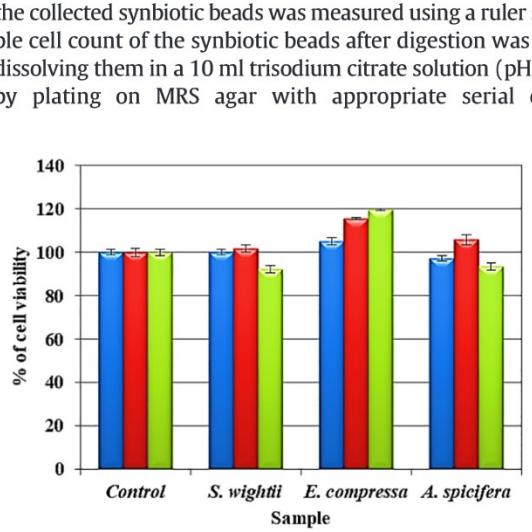


Fig. 2a. Effect of PES on the cell viability of RAW 264.7 macrophage cell.

Table 1

Inhibition of nitric oxide production by polysaccharide extract of Indian seaweeds.

Sample	LPS control	50 µg/ml	100 µg/ml
<i>S. wightii</i>	11.00 ± 0.29	4.63 ± 0.26	1.03 ± 0.29
<i>E. compressa</i>	11.00 ± 0.29	6.99 ± 0.05	5.28 ± 0.45
<i>A. spicifera</i>	11.00 ± 0.29	4.72 ± 0.22	2.97 ± 0.06

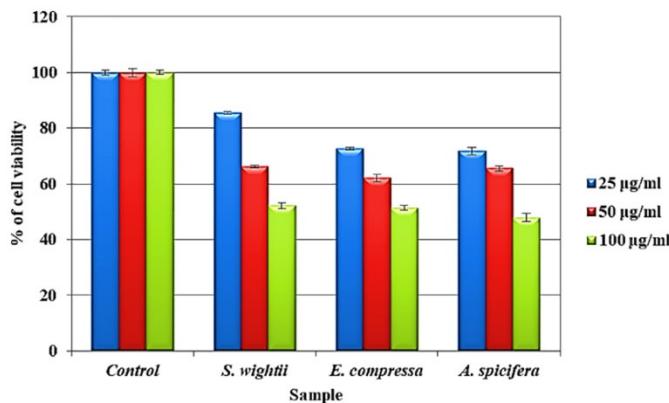


Fig. 2b. Effect of PES on the cell viability of HT-29 cell

2.6.4. Texture profile analysis of formulated symbiotic candy

Texture profile analysis (TPA) is a double compression test (two-bite test) for determining and quantifying multiple textural properties of pharmaceuticals, gels, and personal care. During a TPA test, samples were compressed twice using a texture analyzer to provide insight into how samples behave when chewed. The TPA for the formulated candy was carried out using TexturePro CT V1.7 Build 28 with trigger load of 0.07 N, test speed and return speed of 0.50 mm/s. The probe used was TA11/1000.

2.7. Statistical analysis

All experiments were performed in triplicates. Mean and standard deviations were calculated, and the data was reported in standard format with an error bar. Statistical calculations were made using the Microsoft Excel 2016 software, and the differences were analyzed.

3. Results and discussion

The structural characteristics of the obtained PES reported in an earlier study [14] confirms the semi-crystallinity and the presence of bioactive compounds using X-Ray Diffraction and UV-Visible spectral analysis respectively. The functional groups and compositional analyses were carried out by FTIR and HPLC. Further, H^1 NMR spectroscopy analysis was carried out to further confirm the polysaccharide content in it. The prebiotic score was found to be positive for all three seaweed samples considered in the study [14]. Prebiotic compounds should target

the growth of probiotic microbes and the production of fermented end products such as SCFA. The polysaccharides obtained from the selected Indian seaweeds were analyzed for their fermentability and digestibility. The cytotoxicity effect and nitric oxide productivity of the PES were evaluated on Raw macrophage cell lines. HT-29 colon cancer cells were used to study the anti-cancer effect of PES. The symbiotic candy (PES + *L. plantarum*) was formulated using the alginate spheroidization method.

3.1. Proton nuclear magnetic resonance spectroscopy (H^1 NMR spectra)

The H^1 spectra for PES (*S. wightii*) revealed a major peak at 4.704 ppm and chemical shift at 3.5–4.5 ppm which were found to be the corresponding peaks of α , L-sulfated fucose, glucuronic acid and mannosic acid. H^1 spectra for PES (*E. compressa*) revealed peaks at 4.702 ppm and 3.536 ppm, which were found to be the corresponding peaks of 4-linked L-rhamnose-3-sulfate and glucuronic acid. The peaks obtained at 1.165 ppm and 7.521 ppm are the relative peaks of methyl and aromatic protons [20]. Polysaccharides of *Ulva* sp. majorly constitute L-rhamnose, D-glucuronic acid, D-xylose and sulfate [21]. Sulfated glucuronorhamnopyran is a typical ulvan polysaccharide of the green alga species, whose backbone consists of an alternating sequence of 4-linked L-rhamnose-3-sulfate and D-xylose residues (ulvobiose) with monomeric D-glucuronic acid or D-glucuronic acid-3-sulfate on O-2 of some L-rhamnose-3-sulfate units as its side chains [22,23]. The obtained peaks are correlated well with previously reported data of other *Ulva* sp. [24]. H^1 spectra for PES (*A. spicifera*) at 4.704 ppm revealed the presence of β -D galactose linkage followed with multiple peaks of sulfate groups [25,26] (see Fig. 1).

3.2. Cytotoxicity effect on RAW 264.7 macrophage cells

The dose-dependent concentrations of extracted Indian seaweed polysaccharides were treated with RAW 264.7 macrophage cell line to determine their cytotoxic effect. As represented in Fig. 2a, the viability of the Raw 264.7 cell line was not considerably influenced by PES. Further, *E. compressa* had increased the viability to a lesser extent. The uniform dosage of PES (25, 50, 100 µg/ml) utilized in the present study allows the easy comparison of the results.

3.3. Inhibition of nitric oxide production assay

The dose-dependent concentration of PES leads to the limited release of nitric oxide that indicates the biocompatibility of the

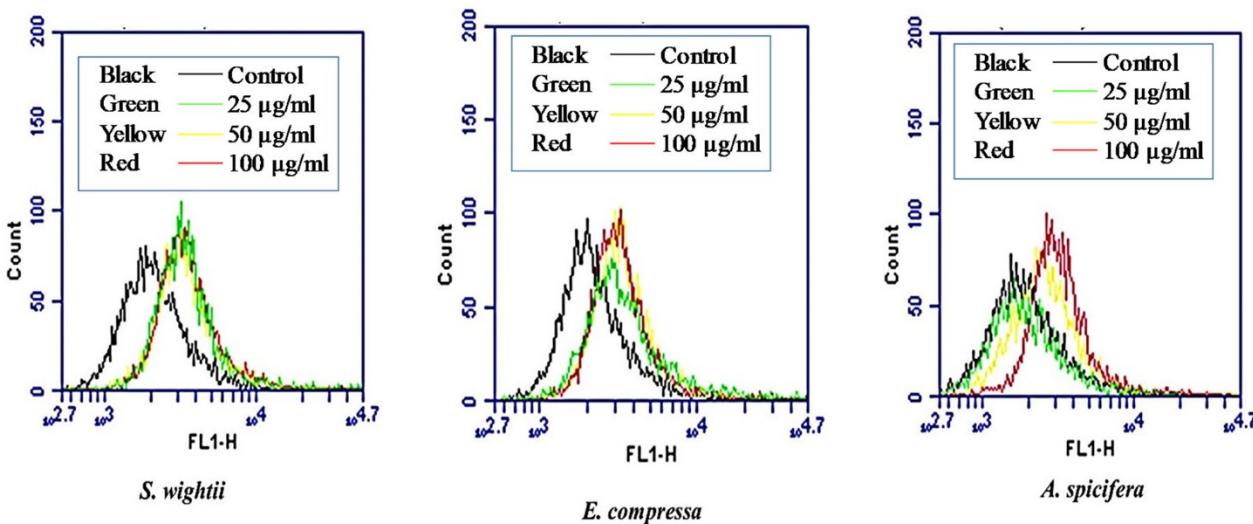


Fig. 3. ROS generation by PES on HT-29 colon cancer cells

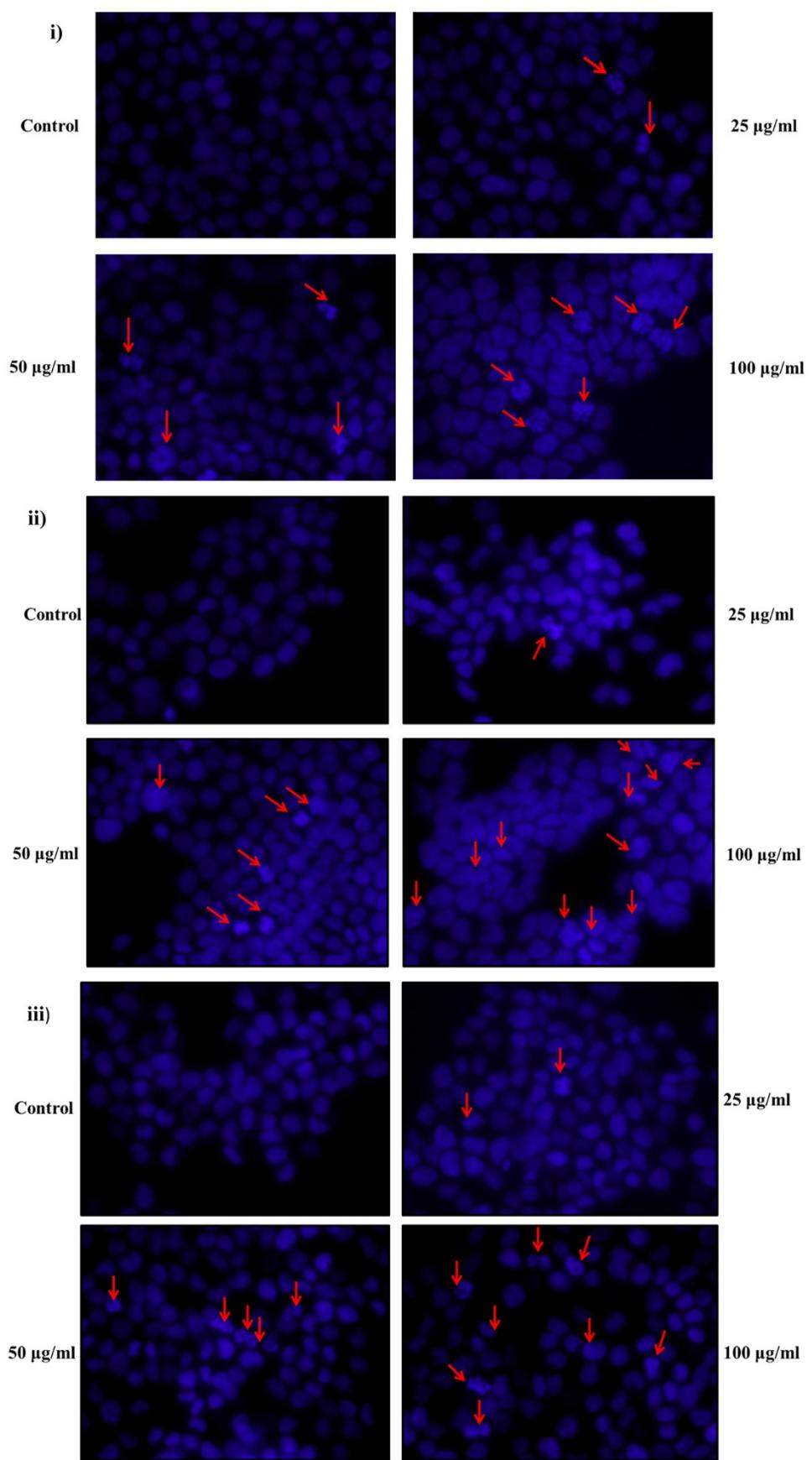


Fig. 4. Morphological changes of nuclear chromatin in apoptosis induced by i) *S. wightii*, ii) *E. compressa*, iii) *A. spicifera* observed under a fluorescent microscope.

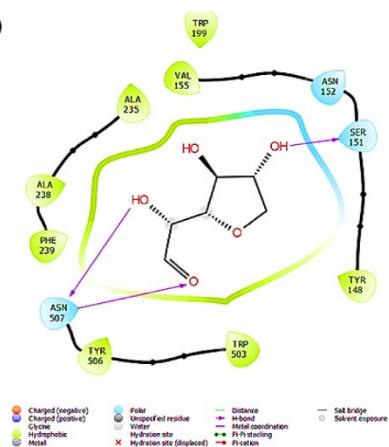
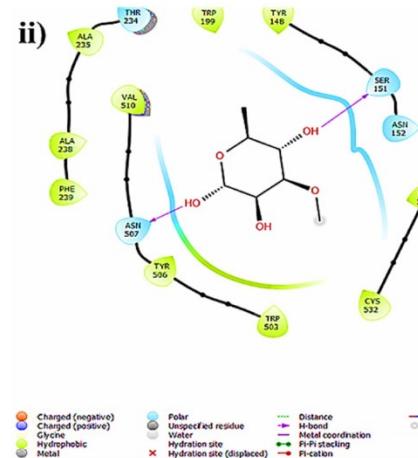
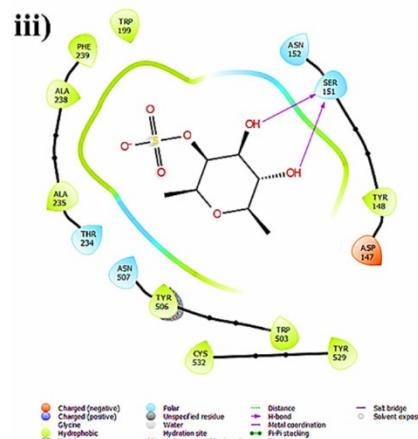
Active site residues of 4V6O: Tyr 148, SER 151, Trp 199, Tyr 506, Asn 507, Tyr 529, Cys 532**XPG score is Docking Score; ΔG is binding free energy****i)****Carrageenan: XPG score = -7.091 kcal/mol; ΔG score = -31.27 kcal/mol****ii)****Ulvan: XPG score = -6.911 kcal/mol; ΔG score = -30.22 kcal/mol****iii)****Fucoidan: XPG score = -6.554 kcal/mol; ΔG Score = -25.12 kcal/mol****Fig. 5.** Molecular docking study of polysaccharide compounds i) Carrageenan, ii) Ulvan iii) Fucoidan on M3 Muscarinic acetylcholine receptor.

Table 2a

Principle descriptors of three leads.

Leads	MW (g/mol)	Rotor	SASA	FOSA	WPSA	PISA	Volume	Donor HB	Acceptor HB	Glob
Carageenan	162.2	5	336.2	139.4	0.000	0.00	531.00	3.00	8.80	0.94
Ulvan	178.2	4	365.6	251.0	0.000	0.00	592.87	3.00	8.50	0.93
Fucoidan	242.2	5	424.6	226.5	2.160	0.00	697.88	3.00	9.10	0.89

Footnote: (range 95% of drugs).

MW: molecular weight (130.0/725.0).

Rotor: no. of rotatable bonds (0.0/15.0).

Dipole: dipole moment (1.0/12.5).

SASA: total solvent accessible surface area (300.0/1000.0).

FOSA: hydrophobic solvent accessible surface area (0.0/750.0).

FISA: hydrophilic solvent accessible surface area (7.0/330.0).

PISA: carbon Pi solvent accessible surface area (0.0/450.0).

WPSA: weakly polar solvent accessible surface area (0.0/175.0).

PSA: vdW polar surface area (7.0/200.0).

Volume: molecular volume (Å³) (500.0/2000.0).

Donor: donor - hydrogen bonds (0.0/6.0).

Acceptor HB: acceptor - hydrogen bonds (2.0/20.0).

IP (eV): ionization potential (7.9/10.5).

EA (eV): electron affinity (−0.9/1.7).

Glob: globularity (0.75/0.95).

The range for properties of 95% drug is given based on QikProp calculations.

polysaccharide compound. Nitric oxide is a key player of the host defence system and the signal transducer of the nervous and immune system. Nitric oxide is secreted by several immune cells such as macrophages, natural killer cells and neutrophils. The macrophage cell line was activated by bacterial lipopolysaccharide (1 µg/ml). *E. compressa* stimulated the production of nitric oxide up to a concentration of $6.99 \pm 0.05 \mu\text{M}$ (Table 1). Overproduction of nitric oxide leads to other inflammatory diseases [27]. The PES concentration (100 µg/ml) of *S. wightii* (1.03 ± 0.29) followed by *A. spicifera* and *E. compressa* inhibited the production of nitric oxide. These results indicated that sulfated polysaccharides are found to be biocompatible and could enhance anti-inflammatory activity by limiting the nitric oxide production. Thus, the PES on dose-dependent concentration could modulate the immune response based on the necessity of the host system.

3.4. Anti-cancer activity

3.4.1. Effect of PES on the viability of HT-29 colon cancer cells

The dose-dependent concentrations of extracted Indian seaweed polysaccharides were treated with HT-29 colon cancer cell line to determine their anticancer activity. The cell viability (%) decreased with an increased dose of PES. The IC₅₀ value was noted against HT-29 cells at PES concentration of 100 µg/ml. The PES (Fig. 2b) showed considerable growth-inhibiting property on HT-29 colon cancer cells. PES *A. spicifera* (100 µg/ml), comparatively reduced the cell viability of HT-29 cells to $47.87 \pm 1.4\%$ which was followed by *E. compressa* ($51.38 \pm 0.9\%$) and *S. wightii* ($52.13 \pm 0.9\%$).

3.4.2. ROS generation assay by DCFH-DA staining

The widely accepted technique for measuring the redox state of the cell was 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) because of its extreme sensitivity towards free radicals. Reactive oxygen species (ROS) are molecules containing radicals of the unpaired electron. DCFH-DA, a cell-permeable dye, was used as a ROS indicator. ROS generation in cancer cells was measured based on the intensity of the DCFH-DA by flow cytometry. Intracellular ROS was measured to find a possible association of ROS inducing cancer cell death. The marked shift towards the right side with greater fluorescent intensity (shown in Fig. 3) indicates the increased generation of ROS in PES loaded treatment as compared to control. The enhancement of ROS level leads to more frequent free radical attacks on cancer cell results in apoptosis [28].

3.4.3. Apoptosis assay by DAPI staining

Apoptosis activity of PES (*S. wightii*, *E. compressa*, *A. spicifera*) on the HT-29 colon cancer cell line was studied using DAPI staining because of its convenience upon fluorescent detection. The morphological changes were detected using high fluorescence and found to be intact nuclei and regular shape in normal cells, whereas in apoptotic cells, it denotes abnormal and condensed chromosome nature [29]. An increased apoptosis activity was observed with an increase in the concentration of PES. Among all the studied compounds of PES, *E. compressa* exhibited greater dose-dependent apoptosis activity followed by *S. wightii* and *A. spicifera*, which is indicated by arrows in Fig. 4. These results indicate that PES could induce apoptosis in colon cancer cells that can be formulated as a functional food for treating colon cancer. The commercial fucoidans

Table 2b

ADME properties of three compounds.

Leads	LogP o/w	Logs	ClogS	QLogBB	Rule of 5	Rule of 3	QLog KP	Log Khsa
Carageenan	−1.776	−0.493	−0.164	−1.212	0	0	−4.665	−0.994
Ulvan	−0.883	−0.864	−0.360	−0.554	0	0	−3.247	−0.912
Fucoidan	−0.668	−1.200	−1.011	−1.359	0	0	−4.650	−1.108

Footnote: (range 95% of drugs)

LogP o/w: log P for octano/water (−2.0/6.5).

Logs: log S for aqueous solubility (−6.5/0.5).

ClogS: log S – conformation independent (−6.5/0.5).

QLogBB: log BB for brain/blood (−3.0/1.2).

QLog KP: log KP for skin permeability (−8.0/−1.0).

Log Khsa: log Khsa serum protein binding (−2.5/1.5).

Lipinski rule of 5 violations (maximum is 4).

Jorgensen rule of 3 violations (maximum is 3).

Table 3

In-vitro gastrointestinal digestion and tolerance of encapsulated cells.

Sample	Initial cell count (log CFU/ml)	After 2 h (log CFU/ml)	EE (%)	After 4 h (log CFU/ml)	EE (%)
Free cells	13.5 ± 0.2	7.9 ± 0.1	—	0	—
Alginate	7.50 ± 0.1	7.30 ± 0.04	66.6	7.0 ± 0.06	33.3
Alginate + inulin	8.35 ± 0.2	8.20 ± 0.08	68.8	8.14 ± 0.2	63.6
Alginate + <i>S. wightii</i>	8.37 ± 0.04	8.11 ± 0.06	56.5	8.04 ± 0.1	47.8
Alginate + <i>E. compressa</i>	8.50 ± 0.2	8.37 ± 0.03	74.1	8.27 ± 0.06	61.3
Alginate + <i>A. spicifera</i>	8.46 ± 0.1	8.21 ± 0.06	55.1	8.14 ± 0.04	48.2

act on the HT-29 cell line by inducing apoptosis through mitochondrial and cell signalling mechanisms [30].

3.5. Docking studies

M3 Muscarinic acetylcholine receptor was reported to be overexpressed in colon cancer cells [31]. Based on NMR analysis, the compounds in PES were found to be fucoidan (*S. wightii*), glucuronorhamnoxyran- a typical ulvan (*E. compressa*), and carrageenan (*A. spicifera*). Docking studies were carried out to analyze the binding affinities between M3 Muscarinic acetylcholine receptor and ligand molecules present in PES, XP docked ligands were reassessed for binding free energy. Among the three leads, carrageenan exhibited three hydrogen bonds with the highest binding affinity with ΔG of -31.27 kcal/mol and XPG score of -7.091 kcal/mol. Ser-151 formed a hydrogen bond with the hydroxyl group of an oxolan moiety of carrageenan. Asn-507 formed two hydrogen bonds with oxygen and hydroxyl group of carrageenan. Residues such as Tyr-148, val-155, Trp-199, Ala-235, Ala-238, Phe-239, Trp-503 and Tyr-506 have entailed Vander Waals interactions with carrageenan.

Ulvan exhibited two hydrogen bonds with binding affinity as ΔG of -30.22 kcal/mol and XPG score of -6.911 kcal/mol. Ser-151 formed a hydrogen bond with a 5R hydroxyl group of an oxane-diol moiety of ulvan. Asn-507 formed a hydrogen bond with a 2R hydroxyl group of ulvan. Residues such as Tyr-148, Asn-152, Thr-234, Ala-235, Ala-238, Phe-239, Trp-503, Tyr-506, Val-510, Tyr-529 and Cys-532 were entailed van der Waals interactions with ulvan. Fucoidan exhibited two hydrogen bonds showing binding affinity with a ΔG score of -25.12 kcal/mol and XPG score of -6.554 kcal/mol. Ser-151 formed two hydrogen bonds with 3R and 4R hydroxyl group of fucoidan. Active site residues like Asp-147, Tyr-148, Asn-152, Trp-199, Thr-234, Ala-235, Ala-238, Phe-239, Tyr-503, Tyr-506, Asn-507, Tyr-529 and Cys-532 were entailed van der Waal's interactions with fucoidan. The molecular docking for all these PES components was shown in Fig. 5.

3.5.1. ADME predictions

Different principal descriptors and ADME properties of three proposed agonists and co-crystal ligands were calculated and presented in Tables 2a and 2b.

3.6. Candy formulation by an encapsulation technique

3.6.1. In-vitro gastrointestinal digestion and tolerance of free and encapsulated cells

The most important characteristic of the probiotics is their resistance towards stressful gastric pH 2.0, and tolerance towards digestive enzyme pepsin, pancreatin and bile acids [32]. The probiotic activity of *L. plantarum NCIM 2083* was studied by digesting them in both free and encapsulated form under gastrointestinal conditions. The encapsulated form had shown considerable resistance when compared to free form. Moumita et al. [33] reported that probiotic *L. plantarum* on encapsulation exhibited higher tolerance towards *in-vitro* gastrointestinal conditions. The decrease in encapsulation efficiency after 4 h was due to the porosity and sensitivity of sodium alginate gels towards higher pH. Hence alginate encapsulation confers both protection and release of the compounds. The inclusion of prebiotics in the encapsulated beads helps to enhance the viability of the probiotics [34]. *E. compressa* (61.3%) aided cell viability as well enhanced the *L. plantarum* as similar to that of commercial prebiotic compound inulin (63.6%), which was followed by *S. wightii* (47.8%) and *A. spicifera* (48.2%) as shown in Table 3.

3.6.2. Post-storage survivability of formulated raindrop symbiotic candy

Symbiotic candy was formulated by a double encapsulation technique to enhance and maintain its viability. The post-storage survivability of the formulated symbiotic candy was studied by placing the candy at room temperature in a sterile condition for 3 weeks. The viability count of the *L. plantarum NCIM 2083* in symbiotic candy with prebiotic compound *E. compressa* had decreased from a log value of 11.50 ± 0.2 to log value of 9.40 ± 0.1 (1st week), 9.14 ± 0.1 (2nd week), 8.92 ± 0.2 (3rd week) as shown in Table 4. Though the viability loss is to a greater extent, probiotic *L. plantarum NCIM 2083* CFU/ml was at a considerable range of 10^7 even after incubation for three weeks at room temperature without refrigeration. The shelf stability of the candy even after three weeks was due to the included prebiotic compound. The formulated symbiotic candy maintained at room temperature prevents the utilization of a refrigerator for storing. Yet, the viability of *L. plantarum* could be further stabilized if stored under optimal refrigerated conditions.

3.6.3. Texture profile analysis (TPA) of formulated symbiotic candy

The TPA of the symbiotic candy was studied to determine the important factors of the candy such as hardness, springiness, gumminess, chewiness, adhesiveness and cohesiveness. The load applied over the candy concerning distance (Fig. 6) and the complete texture profile of the formulated candy was shown in Table 5.

4. Conclusion

The symbiotic candy formulated using seaweed polysaccharides as the prebiotic source and *L. plantarum NCIM 2083* as probiotics could be a suitable functional food to treat colon cancer. The results from the present study demonstrated that sulfated polysaccharides from Indian seaweed, *E. compressa* had shown a reasonable result as a potential

Table 4

Post storage survivability of formulated raindrop symbiotic candy.

Sample	Initial cell count (log CFU/ml)	1st week (log CFU/ml)	2nd week (log CFU/ml)	3rd week (log CFU/ml)
Alginate	11.29 ± 0.1	8.41 ± 0.2	7.69 ± 0.2	—
Alginate + inulin	11.35 ± 0.2	10.25 ± 0.3	9.97 ± 0.2	7.60 ± 0.1
Alginate + <i>S. wightii</i>	11.37 ± 0.04	9.34 ± 0.1	9.00 ± 0.3	8.34 ± 0.1
Alginate + <i>E. compressa</i>	11.50 ± 0.2	9.40 ± 0.1	9.14 ± 0.1	8.92 ± 0.2
Alginate + <i>A. spicifera</i>	11.46 ± 0.1	9.29 ± 0.2	8.04 ± 0.3	7.60 ± 0.2

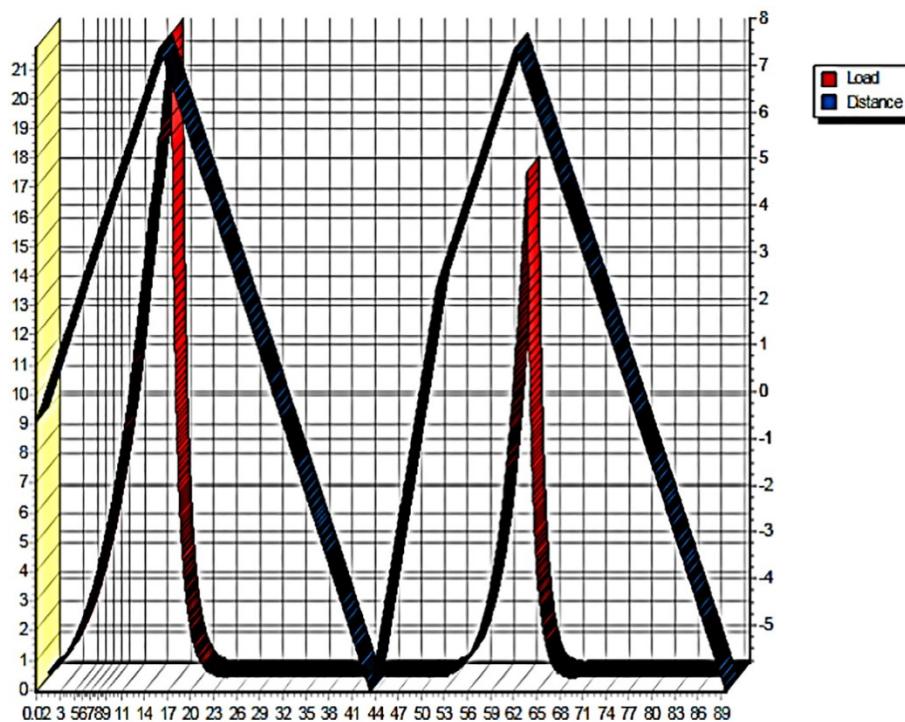


Fig. 6. The load applied over the candy with respect to distance.

prebiotic compound and also as an anti-tumor agent inhibiting the viability of HT-29 colon cancer cells. Since colorectal cancer is associated with gut microbiota and lifestyle, the formulated symbiotic candy could assist in maintaining a healthy microbiome. Further, *in-vivo*

studies using gnotobiotic animal models and human trials should be studied to unravel the pharmaceutical market of formulated symbiotic candy.

Table 5
Texture profile analysis of formulated raindrop symbiotic candy.

Texture profile	Units
Hardness cycle 1	21.75 N
Deformation at hardness	8.00 mm
Hardness work cycle 1	0.06 J
Recoverable deformation cycle 1	2.93 mm
Recoverable work cycle 1	0.01 J
Total work cycle 1	0.07 J
Rigidity 1	0.07 N
Load at target	21.75 N
Deformation at target	8.00 mm
Adhesive force	0.05 N
Adhesiveness	0.00 J
Resilience	0.14
Stringiness length	0.74 mm
Stringiness work done	0.00 J
Quantity of fractures	1.00 with 1% of load sensitivity
Fracturability	18.21 N
1st fracture load drop off	0.41 N
1st fracture work done	0.04 J
1st fracture deformation	7.18 mm
Hardness cycle 2	17.10 N
Hardness work cycle 2	0.02 J
Cohesiveness	0.39
Recoverable deformation cycle 2	2.38 mm
Recoverable work 2	0.01 J
Total work cycle 2	0.03 J
Springiness	5.08 mm
Springiness index	0.64
Gumminess	8.38 N
Chewiness	0.04 J
Chewiness index	5.37 N
Corrected cohesiveness	0.33
Corrected gumminess	7.11 N
Corrected chewiness	0.04 J

CRediT authorship contribution statement

M. Ajanth Praveen:Conceptualization, Methodology, Investigation, Writing - original draft.**K.R. Karthika Parvathy:**Conceptualization, Project administration, Funding acquisition, Writing - review & editing. **Srimanta Patra:**Resources, Data curation. **Imran Khan:**Resources, Data curation. **Pradeep Natarajan:**Formal analysis. **P. Balasubramanian:**Supervision, Writing - review & editing.

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Declaration of competing interest

The authors declare no conflict of interest.

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