

Mushroom Polysaccharides-Alginate/ κ -Carrageenan Microcapsules Trigger NK Cells-Cytotoxic Activity Against Colon Cancer: Induction of Kappa B- Alpha Inflammatory Pathway

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Abstract

Purpose

The main objective of this study is to explore the effects of the extracted mushroom polysaccharides in the form of polysaccharides-Alginate (Alg.)/kappa carrageenan (El-Aassar MR) microcapsules to activate natural killer cells (NK) against colon cancer.

Methods

Water soluble polysaccharides were extracted from nine wild isolates of Egyptian Mushroom, and their safety patterns were checked on peripheral blood mononuclear cells (PBMC). The extracted *Agaricus bisporus* MH751906 polysaccharide was microcapsulated in Alg/κ-carrageenan microcapsules. The morphology and swelling behavior of Alg/κ-carrageenan microcapsules, Alg/κ-carrageenan*polysaccharide microcapsules was determined. Also, the *in vitro* release of Polysaccharide from Alg/κ-carrageenan*polysaccharide microcapsules was analyzed. The Effects of Alg/κ-carrageenan*polysaccharide microcapsules on activating NK cells against colon cancer was evaluated at both cellular and molecular levels.

Results

The results showed that, the extracted polysaccharide from the isolate 8 (submitted to the Gen Bank as *Agaricusbisporus* MH751906) was the safest sample on PBMCs even at 5mg/ml. The microencapsulated polysaccharides in Alg/κ-carrageenan*polysaccharide formula showed better thermal stability at high temperature with higher hydrogel swelling rates in alkaline pH. Upon NK cells activation with microcapsules (^ANK cells), a significant decrease in CD11b and CD16⁻CD56⁻ and an increase in CD16⁺CD56⁺ NK cell populations were recorded. These activated NK cells showed 74.09% cytotoxic effects against CaCO-2cellswith an increase of cancer cell populations in the G0/G1 phase. Furthermore, the ^ANK cells-CaCo-2treated cells recorded down regulations both Bcl2 and TGF genes and up regulation of IkappaB-α expression.

Conclusion

A novel polysaccharides-alginate/κ-carrageenan microcapsules preparation to enhance NK cell cytotoxic effects against colon cancer cells and this inhibitory effect is associated with the regulatory effects of inflammatory pathway through the induction of IkappaB- alpha.

1. Introduction

In recent years, biopolymers are one of the most promising biomaterials, and widely used for producing hydrogels used in tissue engineering and controlled drug release system (El-Aassar MR 2014), natural polymers such as κ-carrageenan; Sodium alginate, Hyaluronic acid, and Chitosan based hydrogels have attracted considerable attention from researchers due to several advantages such as great

biodegradability, biocompatibility, non-toxicity, renewability, and low cost (Wei W 2017). Carrageenan is a linear sulfated polymer polysaccharide produced by certain species of red seaweeds which classified according to the presence of the sulfate content and substitutions (Yang B 2009). They are three groups including Kappa, Iota (ι), and Lambda (λ) carrageenan; Most importantly, significant properties of κ -carrageenan are biocompatibility, high gelling capacity and strong bonding with other biopolymers for preparation of microbeads and films (El-Aassar MR 2015). κ -carrageenan based hydrogels are gaining interest on drug delivery studies and effective drug delivery systems using κ -carrageenan hydrogels. Sodium alginate has a high content of carboxyl groups derived from brown seaweed or Sargasso (Shim J 2014), alginate are extensively used in biomedical application as desirable encapsulation material due to its biocompatibility, pH sensitivity, biodegradability, nontoxicity, gelling property, and the stability of sodium alginate, has a unique combination of mass transfer resistance (Gurikov et al. 2015). The microbeads form alginate and κ -carrageenan is formed with high number of hydrogen bonds between the two compounds, and the network crosslinking through hydrogen bonding and ionic interaction (Grdadolnik et al. 2017; Li H 2018). So, the combination of κ -carrageenan and alginate with other biopolymers for preparation of beads, microcapsules, microspheres, nanofibers and films have been widely used in biomedical applications, especially in release of drugs or proteins because of its biocompatibility, biodegradability. The edible fungi are usually favored in the individual diet because they are poor in fat and rich in proteins, vitamins and fibers (Carrieri R 2017). Mushrooms showed history of conventional use to improve health and wellness and for various illnesses treatment (Zhang et al. 2017). Mushroom dried matter contains total carbohydrate with percentages 50 to 65%, 19 to 35% proteins and 2 to 6% fats (Rathore et al. 2017). These substances make Mushrooms exhibit significant biological actions against different pathological diseases involving cancer (Friedman 2016). Different studies reported a promising antitumor and immunomodulatory activity to various non-toxic polysaccharides isolated from natural sources including Mushrooms (Zong A 2012). In addition, mushrooms showed promising antifungal, anti-inflammatory, antitumor, antiviral, hepatoprotective, anti-diabetic, hypolipidemic, and hypotensive actions (Wasser SP 1999). According to recent reports, in the ecosystem, Mushrooms comprise at least 12,000 species and 2,000 species are considered as edible (Rathore et al. 2017). About 35 edible Mushroom species are commercially cultivated and nearly 200 species were wild collected strains and could be used for medicinal purposes (Rathore et al. 2017). In mushrooms there are many compounds that influence immunity via activating innate immunity components, as NK cells, macrophages, lymphocytes, or neutrophils. Upon activation, the immune system component started to secrete the pro-inflammatory cytokines such as TNF- α (tumor necrosis factor-alpha), IFN- γ (interferon gamma), IL-1 β (interleukin -1 beta), IL-10 (interleukin 10) or IL-12 (interleukin 12). These cytokines activate the adaptive immune system via B cells promotion to produce antibody that internally stimulate T cell differentiation to T helper Th1 and Th2 cells, which facilitate cell and humoral immunities (El Enshasy HA 2013). Furthermore, Mushrooms with their bioactive composites may also exert the activation effect via promoting of the most important antigen-presenting cells maturation and function; dendritic cells (DCs) (Zhong M 2017). There are different mushroom species that showed immunomodulatory properties as *Agaricus Blazei* Murrill, *G. lucidum*, *Grifola Frondosa* (Dicks.) Gray and *Hericium Erinaceus* (Bull.) Persoon. Polysaccharides, terpenes, terpenoids and lectins are the main

classes of immunomodulatory compounds (Guggenheim et al. 2014). In this current work, we formulate Alg/κ-carrageenan*polysaccharide microcapsules. This combination therapy enhances anti-tumors and may be efficient as preventive agents in some cancer diseases, and enhances NK cell cytotoxic effects against cancer cells

2. Material And Methods

I. Isolation and safety pattern of wild mushroom polysaccharides.

- Isolation and purification of wild mushroom strains.

Wild mushrooms growing species (9 isolates; I1-I9) were collected from Bahira, Egypt in sterile plastic bags. In a sterile hood, the gills of the fruiting bodies were cut into small pieces and transferred into Potato Dextrose Agar medium (PDA) plates. The inoculated plates were incubated at 25°C for 5 days; fungal purification processes were performed on PDA media by subsequently subcultures then, the purified strains were stored in 40% glycerol at -80°C.

- Extraction of mushroom polysaccharides.

Mushroom polysaccharides samples (I1-I9) were extracted according to Wu method (Wu 2017), the filamentous hyphae of the purified fungal strains were cultured on Potato Dextrose broth medium at 27°C for 8 days. After the formation of mycelial beads, the culture supernatants were collected by centrifugation at 4000 rpm for 15 min. The collected supernatants were boiled at 90°C for 1 hour, and the polysaccharide samples were overnight precipitated using absolute ethanol at 4°C. The crude polysaccharides were recovered by centrifugation at 5000 rpm for 30 min, and finally the resulted powder samples were dried in oven at 50°C for 48 hrs.

- Safety assay of the extracted polysaccharides.

For the determination of polysaccharides (I1-I9) nontoxic concentration, the cytotoxic assay was performed on peripheral blood mononuclear cells (PBMCs) using MTs assay Kit. Briefly, PBMC were isolated from blood samples of healthy volunteers using Ficoll-Hypaque density gradient centrifugation protocols, the recovered cells **were seeded at concentration 6×10⁴ cells/ml in 96-well plates (100 µl cell suspension per well)**. After 24 hours, 100 µl of the polysaccharide concentrations were incubated with PBMC for additional 48 hours. At the end of incubation, PBMCs cellular viability was quantified using BioVision's MTS Cell Proliferation Assay Kit. The mushroom strain that produced the safest polysaccharide sample was selected for molecular identification.

The polysaccharides samples which showed the lowest toxic pattern on PBMCs was selected for molecular identification, microencapsulation and testing its activation effects on NK cells.

- Genotypic Identification for the selected strain using 18S rRNA sequence.

Fungal DNA was isolated using Fungal DNA/RNA/Protein Kit (Qiagen) according to the instruction protocols. Two universal primers, forward primer (5'-ATCTGGTTGATCCTGCCAGT-3'), and reverse primer (5'-GATCCTTCCGCAGGTTTAC3') were used for the amplification of DNA regions of the mushrooms18S rRNA gene. The purified PCR product was subjected to DNA sequencing using forward primer in the sequence reaction. Sequencing was performed using Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained DNA nucleotide sequences were analyzed using NCBI-BLAST for confirming the identity of the obtained sequences (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequences alignment of the sequences and the other published ones were performed using ClustalW (1.83)(Thompson JD 1994). The amino acid sequences were used for comparison using MEGA 4 (Tamura K 2007), and phylogeny was tested with bootstrap method with 2,000 replications. The phylogenetic tree was analyzed and generated based on UPGMA statistical method.

II. Micro-encapsulation efficacy of the selected polysaccharides of the identified wild mushroom strain.

- Micro-encapsulation process of the selected polysaccharide

Microencapsulation process of the selected mushroom polysaccharide was performed using the method of Mohamadnia et al. (Tamura K). Sodium alginate (Alg; with a viscosity of 15,000–20,000 centipoises (cps)), κ -carrageenan, and calcium chloride anhydrous were purchased from Sigma-Aldrich (Schnelldorf, Germany). Alg and κ -carrageenan solutions were prepared separately by dissolving the biopolymer in distilled water followed by heating at 70°C and 80°C for Alg κ -carrageenan, respectively for 30 min. Polysaccharides (100 mg) were dissolved in distilled water (2.5 ml) and mixed with 5 ml of 0.5 % Alg and 2.5 ml of 1 % κ -carrageenan solution (pre-sterilized at 121°C for 15 minutes). An Inotech Encapsulator IER-50 (Switzerland; equipped with a 100 μ m/300 μ m concentric nozzle and 20 ml syringes to pump the Alg/ κ -carrageenan*polysaccharide solution) was used to produce microcapsules in voltage range 400–1700; schematically illustrated by Mahdavinia et al. (Mahdavinia et al. 2004) (**Fig. 1**). Microcapsules were produced at optimum solution flow rates, vibration parameters and electrostatic dispersion settings. The resulted microcapsules were incubated in a hardening solution of 3% CaCl₂ with continuous stirring for 30 min. Finally, the Alg/ κ -carrageenan*polysaccharide microbeads were rinsed twice with water and dried on tissue paper.

- Characterization of polysaccharide and Alg/ κ -carrageenan*polysaccharide microcapsules.

The morphology of polysaccharide, Alg/ κ -carrageenan microcapsules, Alg/ κ -carrageenan*polysaccharide microcapsules were investigated by SEM “JEOL, JSM-6360LA, Japan” at an acceleration voltage of 10.0 KV at different magnification powers. The FT-IR spectroscopy was measured via Shimadzu FTIR-8400 S Fourier transform spectrometer. Thermo-gravimetric analyses (TGA) for all formulations were carried out at a temperature range from 25 to 600°C under nitrogen atmosphere with heating rate of 10°C /min using Shimadzu TGA-50, Japan. Raman spectra of the samples were obtained

via Raman Microscope (Bruker, Senterrall, Germany) with the following parameters: power, 200–250 mW; scans, 100–1500; resolution, 4 cm⁻¹; crack, 5 mm; and wavelength, 1064 nm.

Polysaccharide *in vitro* release from Alg/κ-carrageenan*polysaccharide microcapsules.

In vitro polysaccharide release from the 100 μm microcapsules was carried out in the simulated gastrointestinal fluid at pH 1.2, phosphate buffer saline (PBS) pH 7.2, and acetate buffer solutions pH 3.6, 4.6 and 5.6. The Alg/κ-carrageenan*polysaccharide microcapsules were suspended in 50 ml of dissolution media at different pH values. These dissolution media were stirred at 120 rpm in a horizontal laboratory shaking water bath at 37 ± 0.5°C at different time intervals. Aliquots of the used buffer were withdrawn (replaced with same volume of fresh buffer to maintain the constant volume) to calculate the concentration of the released polysaccharide using phenol sulfuric acid assay (Cuesta G 2003). All release tests were performed in triplicate.

- Swelling behavior of Alg/κ-carrageenan*polysaccharide microcapsules.

Swelling behavior of the Alg/κ-carrageenan*polysaccharide microcapsules were evaluated at pH 7.4. The microbeads were accurately weighed and suspended in 10 ml of buffer solution. After specific time interval, the swollen beads were recovered and weighed again. The swelling ratio was calculated by the following equation (El-Aassar MR):

$$\text{Swelling ratio (\%)} = (W_s - W_d) / W_d \times 100 \dots \dots \dots (\text{El-Aassar MR})$$

Where W_s and W_d are the weights of swollen and dry beads, respectively.

III. The Effects of Alg/κ-carrageenan*polysaccharide microcapsules on activating NK cells against colon cancer.

- Natural killer cells

Natural killer cells were isolated from blood samples of health volunteers using RosetteSep™ Human NK Cell Enrichment Cocktail Kit. The Kit used targets non-NK cells for removal with antibodies recognizing specific cell surface markers.

- NK cells activation using Alg/κ-carrageenan*polysaccharide microcapsules.

After NK cells isolation, the recovered cells were culturing in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1 % penicillin-streptomycin solution **at 37°C in 5% CO₂ for 24 hours with prepared monocytes as feeder cells (1:2 ratio; NK: feeder cells)** (El-Deeb NM 2019). **After incubation, the exhausted old medium was discarded and replaced with Alg/κ-carrageenan*polysaccharide microcapsules (1 ml) suspensions or RPMI medium (as a negative control), cells were incubated for 36 hrs for activation.**

-NK cell- CD activation markers quantification after Alg/ κ -carrageenan*polysaccharide microcapsules treatment.

After NK activation with Alg/ κ -carrageenan*polysaccharide microcapsules (A NK cells), cells were collected for the activation marker quantification using monoclonal antibodies against CD56, CD16 and CD 11b (BD bioscience) at 1:400 in FACS blocking buffer, the data were acquired using calibur flow cytometry.

IV. Anticancer effect of Alg/ κ -carrageenan*polysaccharide microcapsules-activated NK cells (A NK cells) against colon cancer.

- The effect of activated NK cells on CaCO-2 Cell Lines

The anticancer effects of the A NK cells on colon carcinoma cells (CaCO-2cells) were quantified by using MTS assay and compared with NK cells that activated with polysaccharides (5 mg/ml). Both activated NK cells were incubated with CaCO-2cells in a co-culture model at 37°C in 5% CO₂ for 2 days with ratio (1:2, CaCO-2: NK cells). After 3 days, the exhausted culture medium was discarded, and the cytotoxic inhibition percentages on cellular viability were quantified by BioVision's MTS Cell Proliferation Assay Kit.

- Cell cycle analysis

The cell cycle pattern of CaCO-2- A NK treated cells was determined using Propidium Iodide assay. Aliquot of 6×10^4 cells/ml CaCO-2 cell suspension was cultured in 6 well cell plates that were incubated for 24 hours at 5% CO₂ and 37°C. After semi confluency, the exhausted media was removed and replaced with 2 ml of A NK cells at ration 1:2. The treated cells were incubated at the same previous conditions for 24 hours. After incubation, cells were collected, fixed in ethanol and washed twice in ice cold PBS, then resuspend as 200 μ L of 2×10^6 of cells in 300-500 μ l PI/ triton X 100 staining solution (1000 μ l of 0.1 % triton + 40 μ l PI + 20 μ l RNase). After that, the cell suspension was incubated at 37°C for 15 minutes then transfer to ice. The acquire cell cycle data was recorded using calibur flow cytometry.

- Molecular Mode of anticancer action of A NK cells against colon cancer cells

The anticancer effects of the activated A NK cells against CaCO-2 cancer cells were molecularly studied. To that end, the expression patterns of CaCO-2cells oncogenes and tumor suppressor genes (Ikap α , BCL2, Survivn and TGF) were quantified using designed primers that listed in **Table (El-Aassar MR)**. CaCO-2cells were cultured in 12 well plates (6×10^3 cell/ml) for 2 days with A NK cells or polysaccharide (5mg/ml)-activated NK cells. After incubation, cells were subjected to RNA extraction using QIAamp RNA kit, the first-strand cDNA was synthesized using oligo-dT primer and the M-MuLV reverse transcriptase (Vivantis technologies, Malaysia). The house-keeping β - Actin gene was used as internal controls for standardization of the PCR product and the RT-PCR was done with the cDNA using the Eva Green dye and Light Cycler fluorimeter (BIO RAD S1000 Tm thermal cyler).

V. Statistical analysis

The experimental results are expressed as mean \pm SD (standard deviation) for three replicates. The statistical analyses were carried out using graph pad prism8. Data obtained were analyzed statistically to determine the degree of significance between treatments using one- and two-way analysis of variance (ANOVA) at $P \leq 0.005$. Also, IC₅₀ values were carried out using GraphPad prism 8.

VI. Ethical statement

All experimental protocol which involved human samples in the current study was approved in accordance with guidelines of the ethical committee of City of Scientific Research and Technological Applications; In addition to the ethical committee of Al-Azhar University, Cairo, Egypt.

3. Results

I. Isolation, identification and safety pattern of wild mushroom polysaccharides

Safety patterns of Mushroom polysaccharides

In order to determine the nontoxic doses of extracted polysaccharides on the PBMC, cytotoxicity assay was done. The cytotoxicity results on PBMC of the polysaccharides of different isolated wild mushroom strains indicated that, the polysaccharides that extracted from isolate no.8 (I8) was the safest one and did not show any IC₅₀ value even at the concentration of (5mg/ml). At that concentration, the cellular viability inhibition percentages on PBMC reached 15.7%. On the other hand, polysaccharide extracts of the isolates (I 6, 2, 9 and 3) exhibited higher cytotoxicity with percentages 62.65, 57.17, 57.19 and 55.2, respectively (**Fig. 2**). The concluded results revealed that, polysaccharide samples which isolated from wild mushroom exert different safety pattern on PBMC with priority to isolate no. 8 that showed the safest effects. This strain was selected and molecularly identified by 18S rRNA gene sequencing. The mycelial culture genomic DNA, fragments of the rDNA region was amplified using universal primers and subjected to nucleotide sequencing. Our results showed that the amplified product size was 840 base pairs. The obtained 16S-rRNA gene sequence was registered in the gene bank as *Agaricus bisporus* (Egyptian isolate with an accession number of MH751906). A phylogenetic tree was developed for the candidate isolate along with the sequences collected from the GenBank database as shown in **Fig. (El-Aassar MR)**. Based on Distance matrix table, it was observed that the wild mushroom has homology score value of 88% with *Agaricus bisporus* H97 (CP015465), *Agaricus bisporus* AFTOL-ID 448 (AY787216), *Agaricus bisporus* (L36658), *Agaricus bisporus* Ab-12 (FJ869172) and *Agaricus bisporus* (U23724A).

II. Micro-encapsulation efficacy of the selected polysaccharides of the identified wild mushroom strain.

Characterization of the formed Alg/ κ -carrageenan*polysaccharide microcapsules

The extracted *Agaricus bisporus*MH751906 polysaccharide was microcapsulated in Alg/ κ -carrageenan microcapsules, all physicochemical character results of the formed microbeads and polysaccharides are

designated as the following:

-Morphology of the polysaccharide and Alg/ κ -carrageenan*polysaccharide microcapsules.

The scanning electron microscopy was carried out to characterize the morphology of the polysaccharide, Alg/ κ -carrageenan microcapsules, and Alg/ κ -carrageenan*polysaccharide microcapsules (**Fig. 4**) at different magnification power. The surface image of mushroom polysaccharides indicated the presence of rough and aggregates of irregular and angular particles surfaces shapes (**Fig. 4A**), while the Alg/ κ -carrageenan microcapsules showed a structure with a smooth and homogenous surface and neat edges (**Fig. 4B**). The morphology of Alg/ κ -carrageenan microcapsules surface before mixing with polysaccharides exhibited smooth and homogenous structures, which have been changed to small granules with irregular particle surface with highly porous nature after grafting process (**Fig. 4C**). This change confirmed the interaction network formation between the Alg/ κ -carrageenan*polysaccharide in the microcapsules. The changes of microcapsules surface are attributed to the presence of polysaccharide, and the interconnection between Alg/ κ -carrageenan polymer and polysaccharide.

-FTIR analysis of the polysaccharide and Alg/ κ -carrageenan*polysaccharide microcapsules.

The FTIR spectra of polysaccharide, Alg/ κ -carrageenan microcapsules, and Alg/ κ -carrageenan*polysaccharide microcapsules are shown in **Fig. 5A**. The IR spectrum of polysaccharide showed intense band at 1070-1040 cm^{-1} which is common to all sugar molecules. It is mainly due to, the coupling of the C–O or C–C stretching modes with the C–O–H bending modes. Also, a peak at 2933 cm^{-1} was recorded which represent the stretching vibration of C–H in the sugar ring. In addition, a peak at 1642 cm^{-1} appeared which is due to water molecule binding. Also, another peak was formed at 1640 cm^{-1} , this peak was attributed to amides, indicating polysaccharide conjugated proteins. Also, the formed band at 1000–1200 cm^{-1} suggested the pyrene monomers in polysaccharide structure. In the other hand, Alg/ κ -carrageenan microcapsules spectrum showed peaks at 915, 1024 and 1227 cm^{-1} which could be attributed to 3,6-anhydro-D-galactose, glycosidic linkage and ester sulfate stretching of κ -carrageenan backbone, respectively. Also, Alg/ κ -carrageenan*polysaccharide microcapsules spectrum showed absorption peaks at 1600 and 1400 cm^{-1} that were related to the symmetrical vibration of carboxylate ions on alginate and blend. All these data confirmed the physical characterization of the polysaccharide on Alg/ κ -carrageenan backbone.

-Raman Spectroscopy Analysis of the polysaccharide and Alg/ κ -carrageenan*polysaccharide microcapsules.

Raman spectra of polysaccharide, Alg/ κ -carrageenan microcapsules, and Alg/ κ -carrageenan*polysaccharide microcapsules are represented in **Fig. 5B**. The spectrum of the Alg/ κ -carrageenan microcapsules could be clearly assigned at approximately 1350 cm^{-1} . The following peaks were detected in the spectrum: the peak at around 1455 (stretch) which is corresponded to carboxyl ion of the Alginate hydrogel. Another peak at about 1160 cm^{-1} represented the alternation patterns of guluronic

and mannuronic units. Additionally, a spectral band at ($850\text{--}800\text{ cm}^{-1}$) was detected which represented the C-C groups of the Alg skeleton and α -configuration of the G-units in Alginic acid (Salomonsen et al. 2008). The spectrum of polysaccharides particles with high signal indicated high amount of galacturonic acid in molecules. Also, the marker band of pectin polysaccharides was centered at 852 cm^{-1} which was due to the vibrations of α -glycosidic bonds. Also, sets of many peaks were observed at $800\text{--}1150\text{ cm}^{-1}$ region. Alginate exhibits two characteristic peaks at 1055 cm^{-1} and 1085 cm^{-1} related to C-C, C-O and C-O-C observed in this region. These characteristic bands are also seen in the spectra of Alg/ κ -carrageenan*polysaccharide microcapsules. On the other hand, the Raman spectra of Alg/ κ -carrageenan*polysaccharide microcapsules showed the peaks located at 818.76 cm^{-1} which seems to be characteristic of mannuronic acid are commonly observed in this region, the peak around 1085 cm^{-1} related to C-O-C in Alg/ κ -carrageenan microcapsules. Whereas there were no peaks were appeared in the Raman spectra of polysaccharide which mean changes of band intensity and positions with the most pronounced band due to Alg/ κ -carrageenan interaction with polysaccharides. These results confirmed the interactions of Alg/ κ -carrageenan with polysaccharide and successful microcapsulation.

-Thermogravimetric (TGA) analysis

As shown in Fig. 5C, TGA profiles displayed that, Alg/ κ -carrageenan microcapsules, and Alg/ κ -carrageenan*polysaccharide microcapsules had three degradation stages. The first recorded dynamic weight loss could be resulted from the evaporation of water content of the polymer and polysaccharide structure. Alg/ κ -carrageenan microcapsules had higher weight loss (126.46°C , 30 %) than the Alg/ κ -carrageenan*polysaccharide microcapsules (118.13°C , 22%) which could be attributed to the nature of the sample and the drying process during the microcapsulation formulation. At the second stage, further 5% weight loss was observed in Alg/ κ -carrageenan microcapsules at approximately $126\text{--}193^\circ\text{C}$, and 4% in Alg/ κ -carrageenan*polysaccharide microcapsules at $118\text{--}184^\circ\text{C}$. The third stage was recorded at $193\text{--}300^\circ\text{C}$ which could be due to the decomposition of κ -carrageenan (Yong et al. 2019). At this point, approximately 50 % of the sample still remained and corresponds to intermediate carbonaceous char material. According to the thermograms, Alg/ κ -carrageenan*polysaccharide microcapsules was more thermally stable than Alg/ κ -carrageenan microcapsules. Finally, the Alg/ κ -carrageenan*polysaccharide microcapsules had lost approximately 5% of their mass between 350 and 600°C , which is recognized to the decomposition of polysaccharides backbones. In contrast, Alg/ κ -carrageenan microcapsules had lost approximately 15% of their mass observed at 350 and 600°C . So, the improved thermal stability of microcapsules could be due to the incorporation of polysaccharide particles could slightly affect the weight loss rate of Alg/ κ -carrageenan microcapsules.

1- In vitro polysaccharide release studies

As reported from our previous work (Mohamed et al. 2018), the *in vitro* polysaccharide release profile of polysaccharide from Alg/ κ -carrageenan microcapsules were performed at pH 1.2, pH 3.6, pH 4.5, pH 5.6, and pH 7.4 after 6 h at 37°C , respectively (Fig. 6 A). In acidic conditions, pH 1.2 (gastric pH conditions), the Alg/ κ -carrageenan microcapsules released about 5% of the encapsulated polysaccharide within 2 h

of incubation. On the other hands, at pH 7.4, the Alg/ κ -carrageenan microcapsules released the highest content of the encapsulated polysaccharide (25%) within 6 h of incubation. The release rate of encapsulated polysaccharide was fast in the first hour with 17.91% of final release rate after digestion. The higher amount of released polysaccharide from Alg/ κ -carrageenan microcapsules could be attributed to the higher swelling ability of Alg/ κ -carrageenan microcapsules in alkaline pH condition, and the diffusion of polysaccharide from microcapsules large pore size (Niamlang et al. 2011). So, we can conclude that, mushroom polysaccharide was successfully incorporated into the Alg/ κ -carrageenan microcapsules, and the encapsulation process with Alg/ κ -carrageenan significantly reduced the drug release rate in the acidic medium.

2- The swelling behavior

Among various factors, the swelling degrees of the Alg/ κ -carrageenan*polysaccharide microcapsules was studied to gain a better understanding of microcapsules sensitivity to media, and their influences on the drug release from microbeads. Our results indicated that (Fig. 6 B), the microbeads swelling rates increased over the time. In basic solution, pH 7.4, it is apparent that, the Alg/ κ -carrageenan microcapsules comprising polysaccharide showed the higher swelling rates. This could be due to the strong bonds between carboxylate and sulfate groups onto sodium alginate and κ -carrageenan. However, in basic solution pH 7.4, the carboxylate and sulfate groups became ionized which enhanced the electrostatic repulsion between alginate and κ -carrageenan that led to a significantly swell of the hydrogels.

III. NK cells activation using mushroom polysaccharides and polysaccharides-Alg/k carr microcapsules against cancer cells

- Flow cytometry assay of activated NK CD markers

The flowcytometry analyses of the A NK cells are represented in **Fig. 7, A, B and C**. The data indicated that, upon NK cells activation with encapsulated mushroom polysaccharides for three days, a significant decrease in CD11b and CD16⁻CD56⁻ markers was recorded comparing with the un-activated NK cells; while a significant increase in the CD16⁺ CD56⁺ NK (increase in CD11b⁺ CD56^{bright} CD16^{dim} populations; mature NK^{reg} cells) cells populations was detected in A NK cells comparing with the control one.

IV. Anticancer effect of Alg/ κ -carrageenan*polysaccharide microcapsules-activated NK cells (A NK cells) against colon cancer

- Effect of A NK cells on CaCO-2 cell line

The cytotoxic effects of the activated NK cells against CaCO-2cells were quantified using MTS assay method. The results indicated that, NK cells that activated with polysaccharide alone and 2ml beads showed cytotoxicity effects against CaCO-2 cells with percentages 60.69 and 74.09, respectively,

compared with 27.8 inhibition percentage in CaCO-2 cellular viability in positive control cells (co-incubated with un-activated NK cells; **Fig. 8 A**). The morphological features of CaCo-2 cells after ^ANK cells treatment indicated the presence of apoptotic cells with fewer viable and attached cells that characterized by shrinking and blebbing of cellular membrane (**Fig. 8 B**).

- The mode of action of the activated NK cells on CaCO-2 cells

CaCO-2 cell cycle analysis

To understand the mode of action of ^ANK cells against CaCO-2 cells, the cell cycle analysis by Propidium iodide (Léonce et al.) stain was performed using flow cytometry. As shown in **Fig. 9A**, following 48 h co-incubation of CaCO-2 with ^ANK cells, the results indicated an increase in cell population in sub G0/G1 stage; apoptotic cells from 3.5 to 12.19) also, a significant increase in cell populations of the G0/G1 phase (32.08 % compared to 5.33 % in untreated cells) was recorded, this mean cell cycle arrest in G0/G1 phase. Also, the cell populations in the S phase of treated cells significantly decreased (24.9 % compared to 70.4 %) with also dramatically decreased in G2/M phase cell populations (0.01 % compared to 2.12 %).

- Molecular Mode of action of NK cells activated by polysaccharide and capsulated polysaccharide on CaCO-2 cells

The relative expression levels of apoptosis regulating genes (Ikappa, BCL2, Survivin, and TGF) were analyzed. The possible mechanisms of ^ANK cells-mediated cell death via the apoptotic regulators have been measured via gene expression patterns (**Fig. 9B**). ^ANK cells activated by beads and polysaccharide showed potentialities to down regulate the expression of TGF gene. While polysaccharide down regulated the gene expression with folding rate higher than that in beads treated cells. Also, polysaccharide showed ability to down regulate BCL2 gene expression in CaCo-2 treated cells, while ^ANK cells upregulated the expressions of BCL2 gene in CaCo-2 treated cells. The overall results indicated that NK cells activated by beads induced cellular apoptosis in human epithelial colorectal adeno-carcinoma cells via down regulation the expression of both survivin and TGF genes and upregulating the expression of IkBa gene. While treatment with NK cells activated with polysaccharide down regulated the expression of both Bcl2 and TGF genes and increased the expression of ikappa.

4. Discussion

Most of cancer treatments options such as surgery, radiotherapy or chemotherapy directly attack the cancer itself. But most of these treatments also attack not only attach to the cancer cells, but the most of these treatments don't have selectivity targeting to cancer cells, they also attack normal cells causing side effects. There are many recent studies which clearly explained the idea of the safety usage of various medicinal plants and natural products in controlling tumor growth and metastasis via activating immune

system with low toxicity for normal tissues (El-Deeb NM 2019; Han SS 2009; Harada M 1995; Ohnishi Y 1998). Mushroom as an example of natural product, it showed positive effects in controlling tumor growth by enhancing immune function and promote health with a limited cytotoxicity effect. Our results indicated that, the polysaccharides which extracted from isolate no.8 was the safest one and did not show any IC_{50} value even at the concentration of (5mg/ml). A previous In vitro study confirmed the anticancer effects of polysaccharide that extracted from edible *A. bisporus* with a significant effect against Hela cells (cervical cancer cells) at the high concentration of 200 μ g/ml (Jeong SC 2015), this concentration is the recorded safe one. Comparing with this published result of the *A. bisporus* safety assays, our nontoxic dose of *A. bisporus* (wild strain)- polysaccharides was 5 mg/ml, that mean a higher margin of safety.

In 1960s, the scientists discovered a type of T cells of a naturally occurring cytotoxic lymphocyte with intrinsic and innate anti-tumor properties (Harada M 1995; Lee SJ 2003). These types of cells today known as natural killer (El-Aassar MR) and do not require antigen exposure to mediate their anti-tumor toxicity cells (Abel AM 2018). Many studies indicated that, mushroom polysaccharides could stimulate NK cells-defendant immune response against different cancer type (El-Deeb NM 2019; Ng ML 2002). These immunomodulatory effects give the mushroom its superior actions as prophylaxis, prevention, and co-treatment agent with chemotherapy against various tumor models (Deng G 2005). Regarding to NK cells surface expression density of CD56 and the CD16 antigens, NK cells can divide in two subsets; cytotoxic (NK cells with dominant activating signals, target cells with a high expression of pressure stimulus-induced ligand) $CD56^{dim} CD16^{bright}$ and the immune regulatory $CD56^{bright} CD16^{dim}$ subsets (NK cells with dominant activating signals, target cells with a high expression of inflammatory molecules) (Cooper MA 2001; Fu B 2014). Upon NK activation with *A. bisporus* polysaccharides we observed an increase in $CD11b^{+} CD56^{bright} CD16^{dim}$ populations (mature NK^{reg} cells) that in turns mean an increase in cytotoxic NK cell populations via inflammatory molecules not perforin or granzymes. Another research study indicated that, dietary supplementation with white button mushrooms (*A. bisporus*) significantly enhances the activity of NK cells to lyse the target tumor cells in a dose-dependent manner (Wu D 2007). This effect is due to an increase in its toxic effects not its number (the splenic NK percentages not altered by mushroom supplementation) (Wu D 2007). So, based on all these findings, we could confirm that, *A. bisporus* encapsulated polysaccharides increased the activity of NK^{reg} cells against cancer cells via inflammatory pathway not by altering NK cells percentages.

Also, many reports explained that, cellular differentiation degrees were predictive of their sensitivity to NK cell mediated cytotoxicity, so, NF κ B blocking in the cells may de-differentiate and subsequently return the cells to more of undifferentiated phenotype, resulting in increased their sensitivity to NK cell mediated cytotoxicity (Abel AM 2018; Lee SJ 2003). Similarly, NF κ B inhibition by Sulindac increased the functional activation of NK and enhanced its cytotoxic effects against tumor cells (Abel AM 2018; Lee SJ 2003). A recent study explained that NF κ B inhibition signaling with a specific inhibitor (TPCK) significantly attenuated IFN-gamma production induced by these phyllanthusmin derivatives in human NK cells and reduce its cytotoxic effects (Chen et al. 2015). In the same context, our results showed that, up on

treatment with polysaccharide activated NK cells, the inhibition in NF κ B inflammatory pathway via up regulation of I κ B α expression (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α) was accompanied with an increase in NK cytotoxic effects against colon cancer cells. In addition, it was reported that TGF- β expression could reduce expanded NK cells cytotoxic function against colon cancer (HCT 116 and HT29) and myeloid leukemia (HL60) cells by rate 11-78% via decreasing its killing efficiency, activating receptors (CD16 and NKG2D) expression and TNF- α , IFN- γ , perforin and granzyme B production (Chen et al. 2015). So, upon treatment with *A. bisporu* polysaccharides, the expression levels of TGF- β gene were down-regulated in colon cancer treated cell, that resulted in the enhancement of NK cells-cytotoxic effects. These all data confirmed the dual function of *Absorbs* polysaccharides by activating NK cells and down regulating cancer cells TGF- β and NF κ B genes that amplify NK cells cytotoxic effects against cancer cell.

In recent years, material chemists have fabricated superior medications with improved biological, magnetic, and electrical features (10071826. 1999). Over the past few years, large attention has been focused on carrageenan-based bio nanocomposites due to their multilayered properties like biodegradability, biocompatibility, and nontoxicity. Because of its biocompatibility and consolidation behavior, CG is widely used by pharmaceutical scientists to improve drug formulation properties, especially to prolong drug release (Maderuelo C 2011). Particularly, CG based formulations are used for prolonged drug release, that is, for many hours or days (Grenha A 2010; Pavli et al. 2010). Some interesting features of CG like its adhesiveness and positive surface charge provide extra advantage in prolonging drug release in mucosal/epithelial tissues (Kianfar F 2012). In addition, κ -CG was also explored as the matrix of controlled release tablets. Using microcrystalline cellulose as the filler and theophylline monohydrate as model drug (20%), the effect of κ -CG content on drug release was studied. It was observed that 20% (v/v) κ -CG resulted in fast drug release. Slower release was observed at 30% (v/v) κ -CG content while at 70% (v/v) κ -CG, drug release followed zero-order kinetic (10071826.). Our results indicated the efficacy of Alg/ κ -carrageenan to microencapsulate mushroom polysaccharides to be used as immunomodulatory agent against cancer. Our polysaccharides released results indicated that, the less amount of polysaccharide release was detected because of the polysaccharide exhibited low solubility and dissolution rate under acidic conditions, and Alg/ κ -carrageenan microcapsules underwent shrinkage at pH 1.2 can be interpreted according to the protonation of functional groups, and difference in the pK_a values of functional groups on biopolymers and polysaccharide (Mahdavinia et al. 2004). The higher amount of released polysaccharide from Alg/ κ -carrageenan microcapsules can be attributed to the higher swelling ability of Alg/ κ -carrageenan microcapsules in alkaline pH condition, and the diffusion of polysaccharide from larger pore size inside microcapsules, and consequently, leading to the fast diffusion of polysaccharide from microcapsules (Niamlang et al. 2011). Lastly concluded that, the polysaccharide has successfully incorporated into the Alg/ κ -carrageenan microcapsules, and the encapsulation with Alg/ κ -carrageenan microcapsules significantly reduced the drug release rate in the acidic medium.

5. Conclusion

In conclusion, the providing data can enrich the existing comprehensive data of mushroom immunomodulatory behavior by using a novel polysaccharides-alginate/ κ -carrageenan microcapsules preparation to enhance NK cell cytotoxic effects against colon cancer cells. The inhibitory effect of polysaccharides- alginate/ κ -carrageenan microcapsules on colon cancer is associated with the regulatory effects of inflammatory pathway through the induction of $\text{I}\kappa\text{B}$ - α .

Declarations

Data availability statements

All data generated or analyzed during this study are included in this published article.

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Conflict of interest

The authors declare that they have no conflict of interest. All authors declare that, there is no competing of interests.

Author contributions

N.M.E.D., M.R.E.A., and M.M.S.F. sharing the study idea generation. N.M.E.D manage the NK activation and anticancer practical parts. N.M.E.D., M.R.E.A., M.M.S.F., A.A.F., and M.A.M. prepared and revised the manuscript. All authors read and approved the manuscript.

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Tables

Table 1: List of primers of Ikap α , BCL2, SURVIVN, TGF, and β - Actin genes that used for quantitative real time PCR

Primers	Sequence
Bcl2 forward	5'-TATAAGCTGTCGCAGAGGGGCTA-3'
Bcl2 reverse	5'-GTACTCAGTCATCCACAGGGCGAT-3'
IKap- α forward	5'CATGAAGAGAAGACACTGACCATGGAAA3
IKap- α reverse	5'TGGATAGAGGCTAAGTGTAGACACG3'
SURVIVN forward	5'-TGCCCCGACGTTGCC-3'
SURVIVN reverse	5'-CAGTTCTTGAATGTAGAGATGCGGT-3'
TGF forward	5'CAAGGGCTACCATGCCAACT3'
TGF reverse	5'AGGGCCAGGACCTTGCTG3'
β - Actin forward	5'- GTGGGGCGCCCCAGGCACCA-3'
β - Actin reverse	5'- CTCCTTAATGTCACGCACGATTTTC -3'

Figures

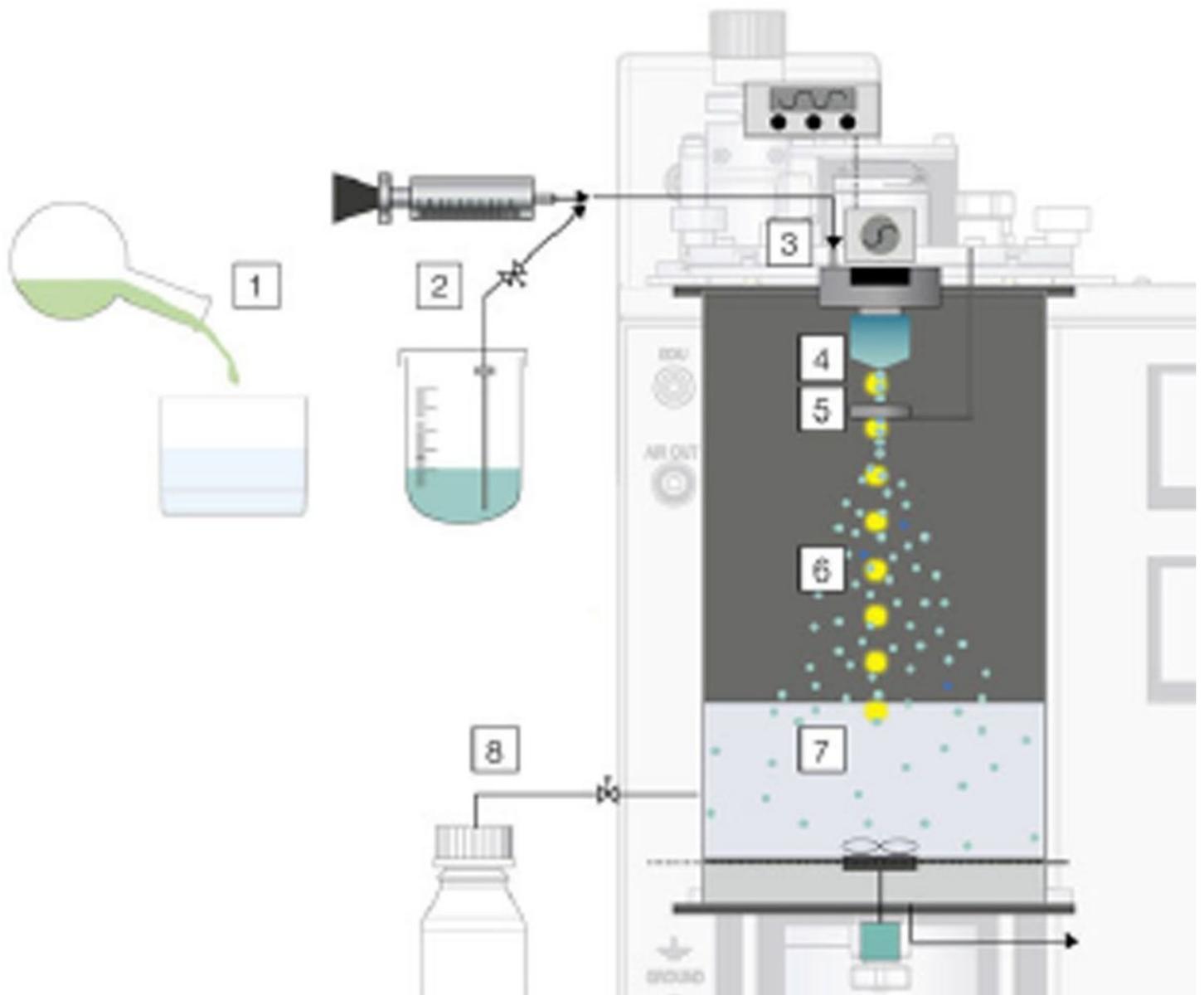


Figure 1

Schematic diagram represents the formation of Alg/ κ -carrageenan*polysaccharide microcapsules using encapsulator (Mahdavinia et al. 2004)

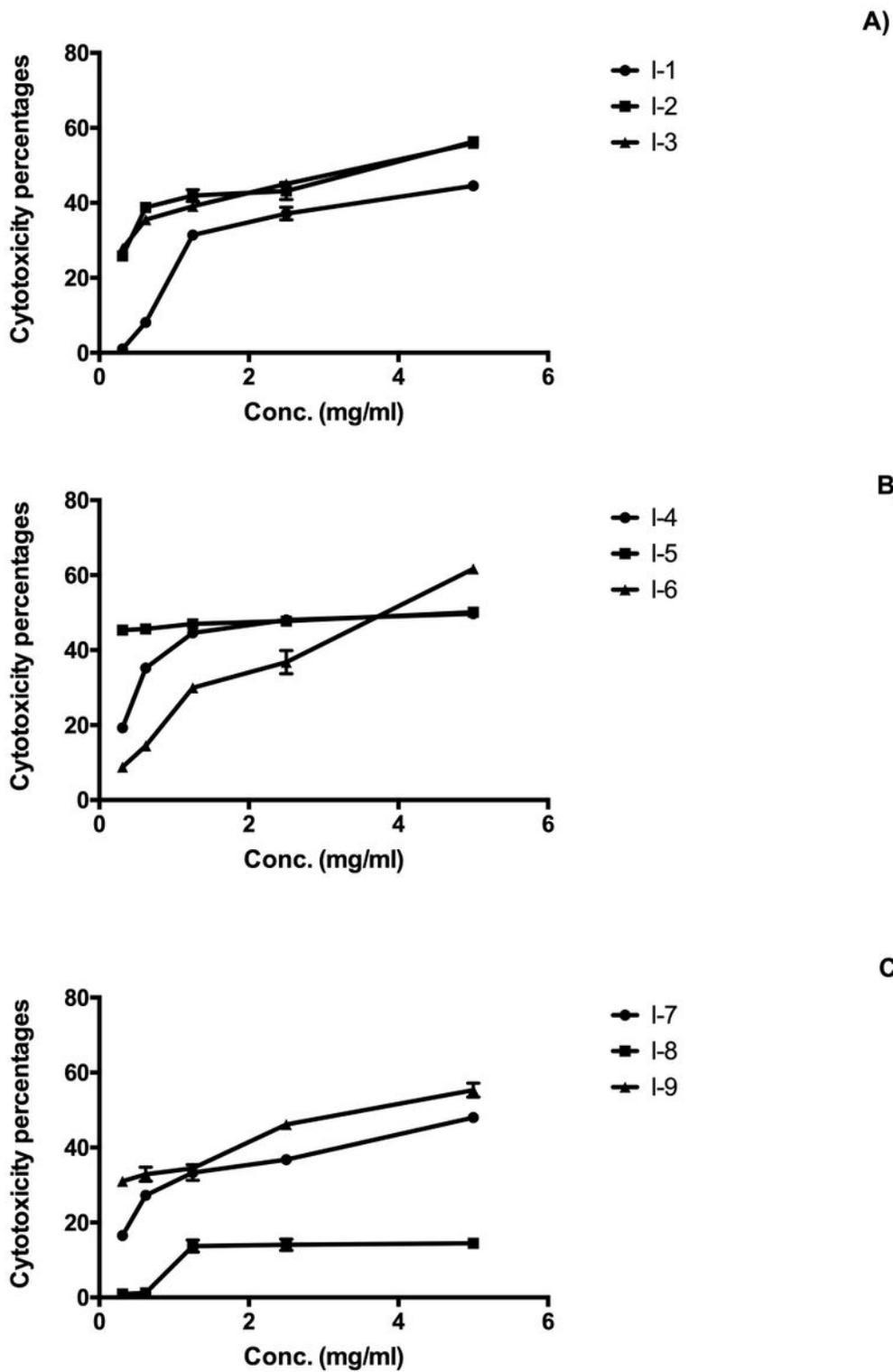


Figure 2

Safety assay of the extracted polysaccharides on PBMC cells Different concentrations of mushroom polysaccharides (1-5 mg/ml) were tested on PBMCs to detect the safety patterns. MTS assay was used to select the used mushroom strain based on its safety results A) Isolates from 1 to 3; B) Isolates from 4 to 6; C) Isolates from 7 to 9.

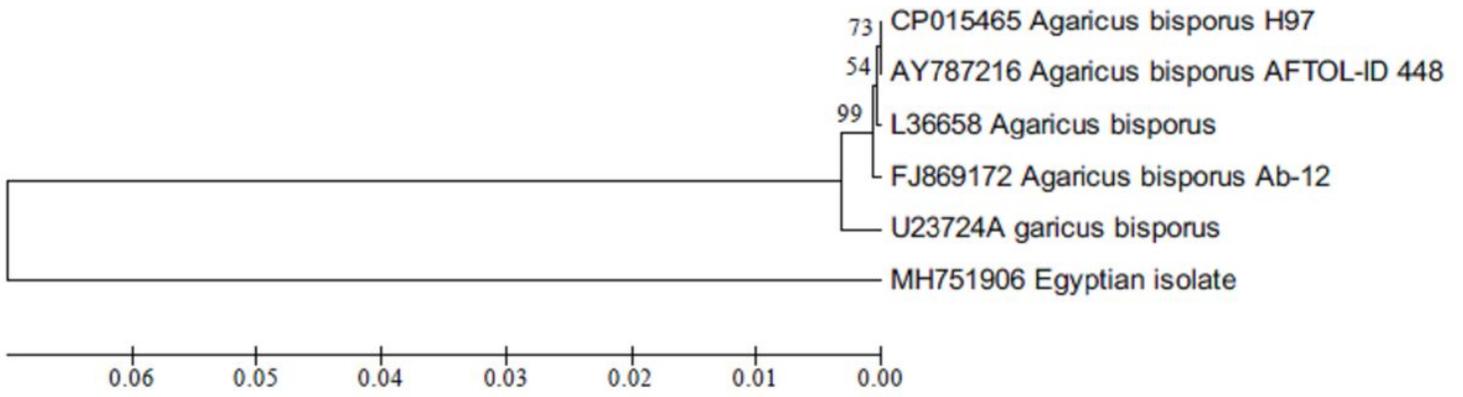


Figure 3

Phylogenetic tree of *Agaricus bisporus*. 18S rRNA gene sequencing of the selected mushroom strain and the obtained sequence was analyzed using NCBI-BLAST. Using the mycelial culture genomic DNA, fragments of the rDNA region was amplified using universal primers and subjected to nucleotide sequencing.

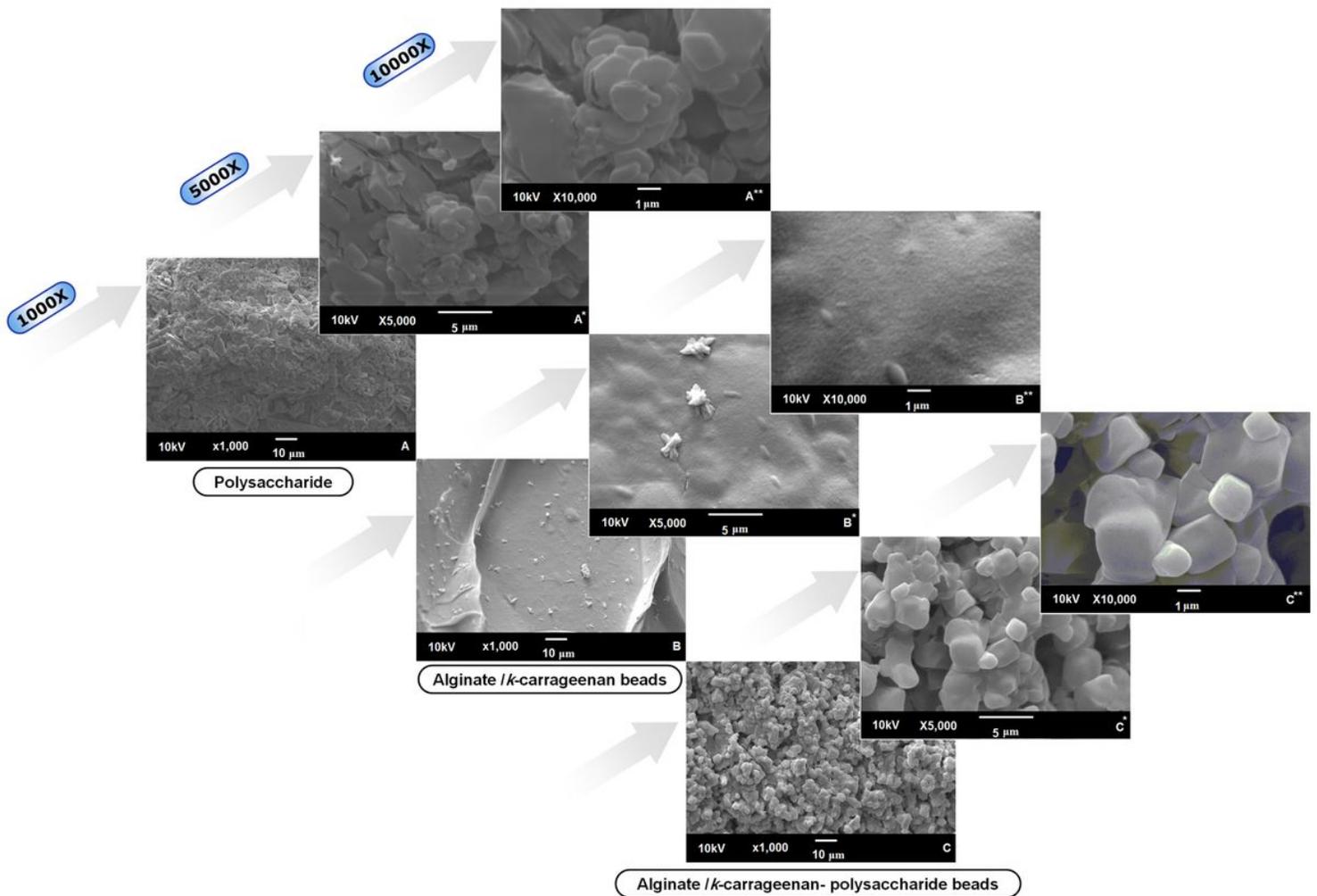


Figure 4

SEM micrographs of the surface of (A, A*, A**) Polysaccharide, (B, B*, B**) Alg/κ-carrageenan microcapsules, and (C, C*, C**) Alg/κ-carrageenan*polysaccharide microcapsules at 1000x, 5000x and 10000x magnification, respectively.

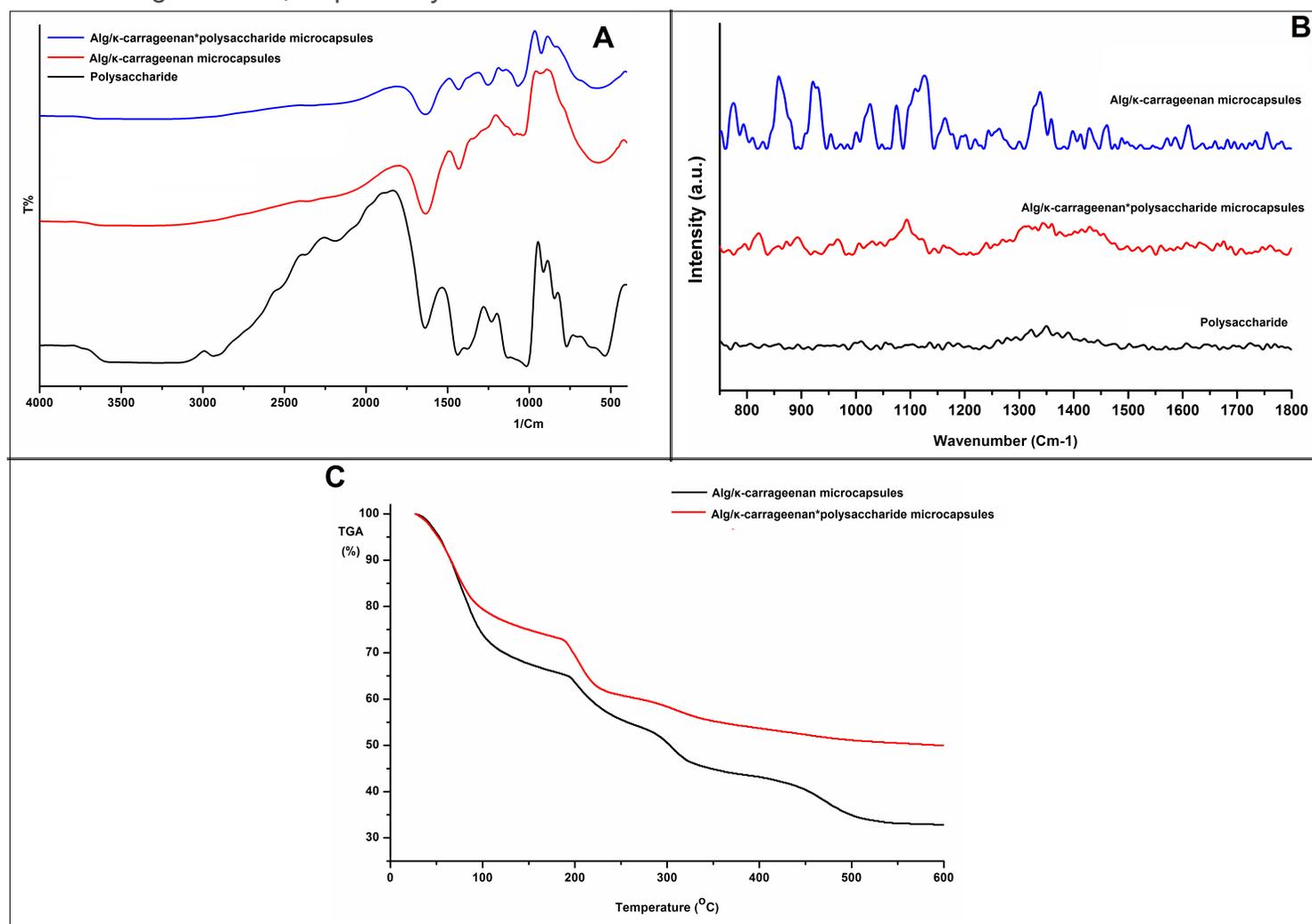


Figure 5

(El-Aassar MR) FTIR spectrum of polysaccharide, Alg/κ-carrageenan microcapsules, and Alg/κ-carrageenan*polysaccharide microcapsules; (El-Aassar MR) Raman Spectroscopy of polysaccharide, Alg/κ-carrageenan microcapsules, and Alg/κ-carrageenan*polysaccharide microcapsules; (El-Aassar MR) TGA of A polysaccharide, Alg/κ-carrageenan microcapsules, and Alg/κ-carrageenan*polysaccharide microcapsules.

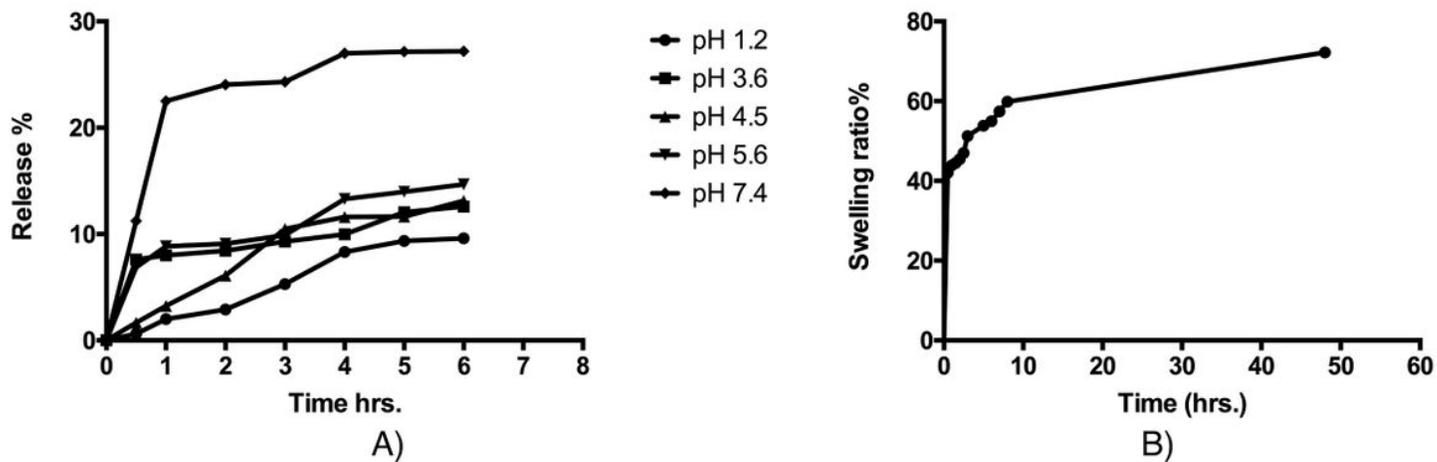


Figure 6

Flow cytometric analysis of CD markers of polysaccharides activated NK cells. The NK cells (ANK cells) were collected after 36 hrs. of activation with 1 ml of polysaccharides microencapsulated beads and its activation markers were quantified by Flowcytometry using antibodies against CD56, CD16 and CD11b.

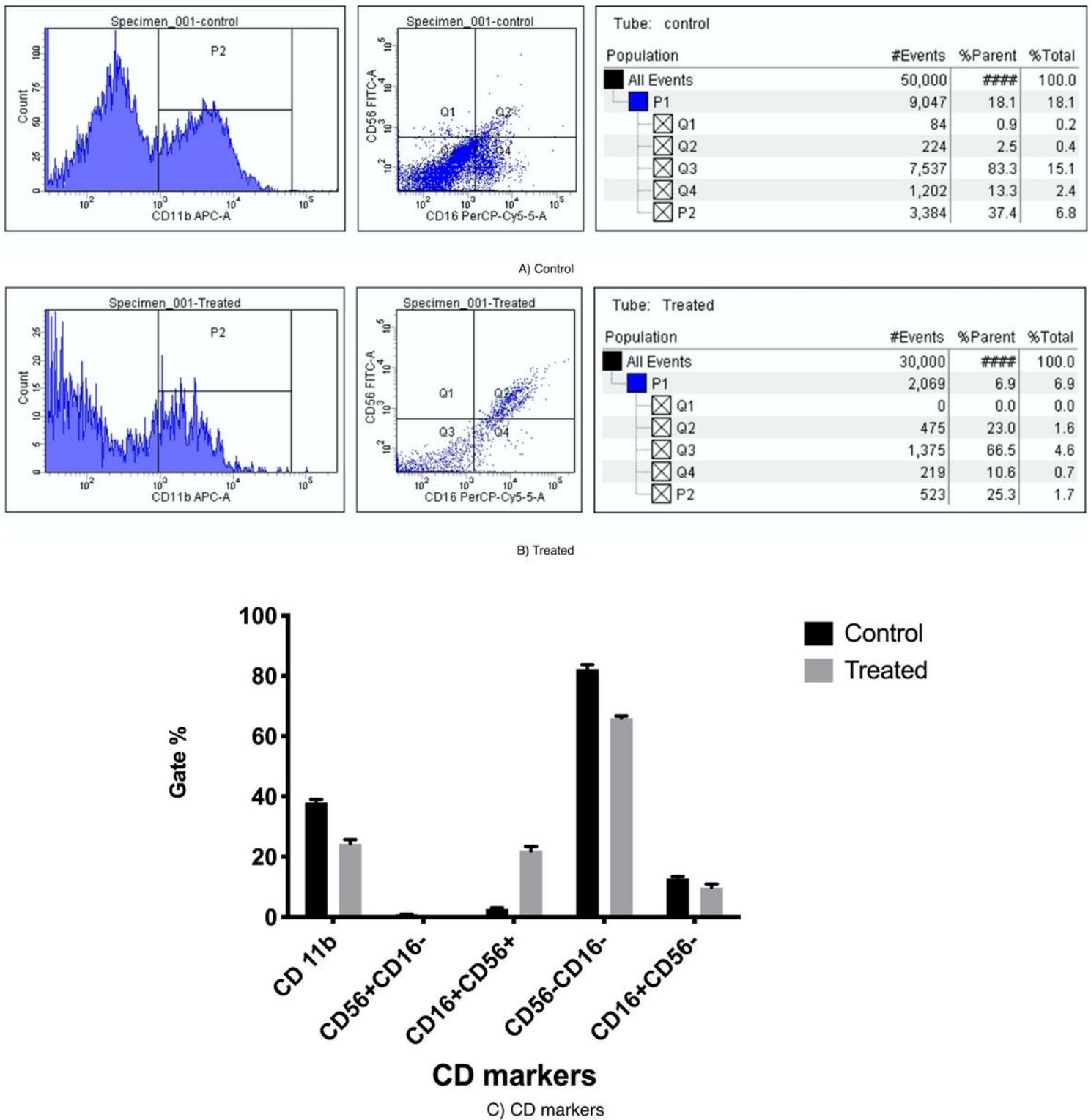


Figure 7

Anticancer activity of ANK cells against CaCO-2 cells A) Cytotoxicity assays of ANK cells against CaCO-2 cells; B) Morphological changes on CaCO-2 cells upon treatment with polysaccharides-activated NK cells. CaCo-2 cells were treated with ANK cells for 72 hrs. indicated the presence of apoptotic cells with fewer viable and attached cells that characterized by shrinking and blebbing of cellular membrane.

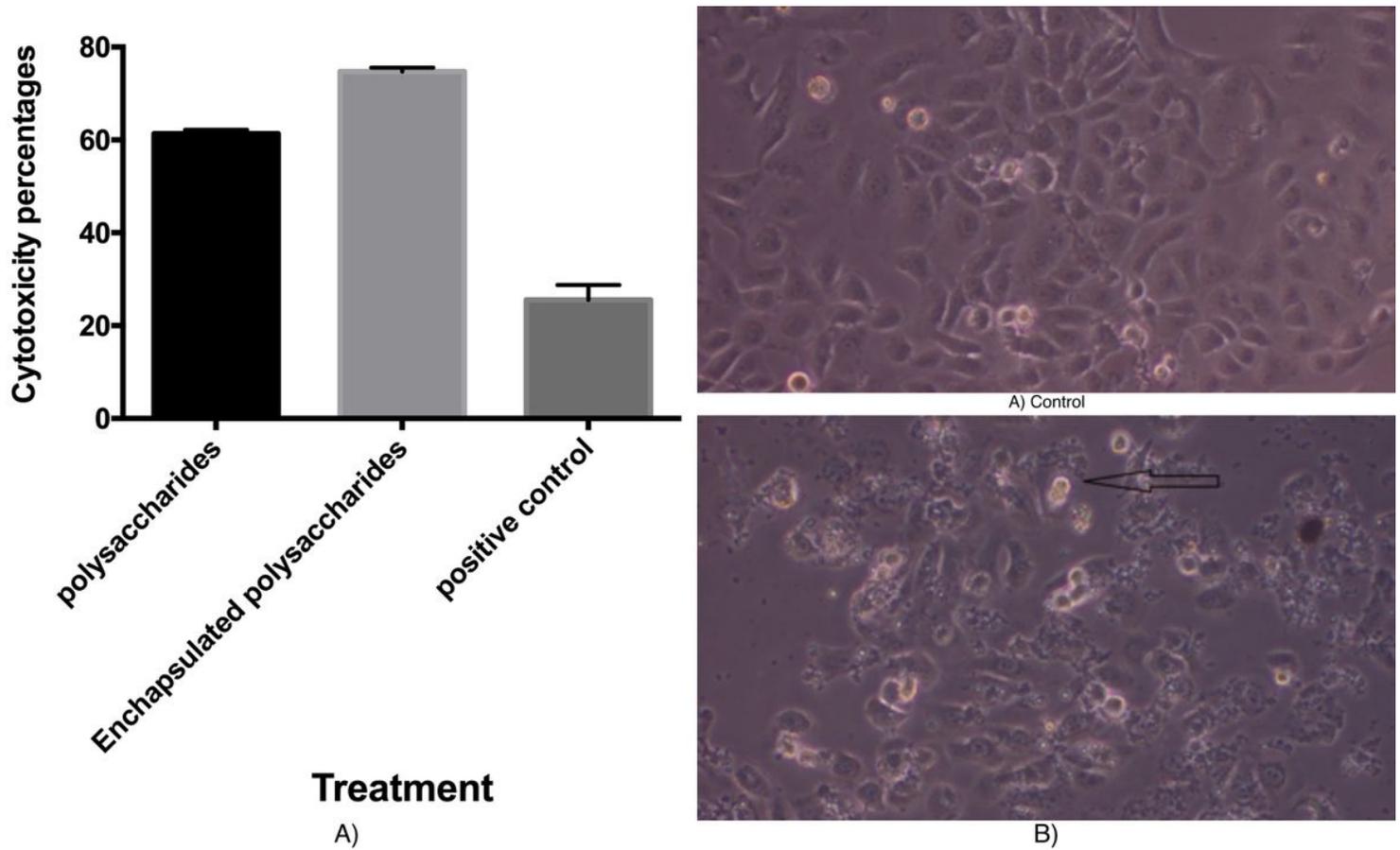


Figure 8

The mode of ANK cells against CaCO-2 cells: A) cell cycle analysis of CaCO-2 cells after treatment with ANK cells; B) RTqPCR gene expression analysis of CaCO-2 cells treated with ANK cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials1.pdf](#)
- [Supplementarymaterials2.pdf](#)
- [Supplementarymaterials3.pdf](#)