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Quercetin loaded and alginate sealed β-Glucan particles based drug delivery system against Prostate Cancer

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Abstract

Prostate cancer remains a challenge in healthcare, being second most common male cancer demanding innovative therapeutic approaches and treatment techniques. This study investigated*in vitro*methodsfor the investigation of the potential anticancer effects of quercetin loaded and alginate-sealed β-Glucan particles derived from yeast against the DU145 cell line. *In vitro* evaluation using MTT assays demonstrated the cytotoxic effects of quercetin loaded and alginate-sealed particles against DU145 prostate cancer cells. Apoptosis induction, ROS generation, and lysosomal pH alterations underscore the potential of quercetin loaded and alginate sealed β-Glucan particles as promising therapeutic agent for prostate cancer. Future studies focusing on preclinical validation, pharmacokinetic profiling, and clinical trials to assess translational potential and optimize therapeutic strategies can help to use Quercetin loaded β-Glucan as an therapeutic option for the treatment of prostate cancer.

Keywords:β-Glucan, prostate cancer, Quercetin, network pharmacology, Apoptosis

1. Introduction

Prostate cancer is the tumor that occurs in prostate gland due to uncontrolled growth of the cells, represents a huge challenge in the landscape of oncology. When cells of the prostate cancer start to metastasize to different organs, the survival rate of the patient start to decline significantly. The process of metastasis and the tendency of prostate cancer cells to target specific organs are influenced by various cellular subtypes and the unique microenvironments of those organs, along with the interactions between them (Klusa et al., 2021). The androgen hormone, testosterone, and its metabolite dihydrotestosterone (DHT) play pivotal roles in prostate cancer development and progression. Binding of androgen to the androgen receptor leads to the activation of cell proliferation signaling leading to the development of prostate cancer. Androgen receptor signaling pathways lead to theproliferation and survival of prostate cells in themalignant stage, making androgen deprivation therapy (ADT) through the removal of gonads a cornerstone in the management of advanced disease (Hou et al., 2021). Gonadotropin-releasing hormone (GnRH) is found to be involved in the inhibition of steroidogenesis leading to the suppression of androgen receptor resulting in good management of prostate cancer. Symptoms of the prostate cancer are nonspecific or may remain asymptomatic until later stages. Screening strategies, including serum prostate-specific antigen (PSA) and digital rectal examination (DRE) measurement are the techniques that are used for the early-stage detection of prostate cancer (Morote et al., 2022). Currently available treatment protocols for prostate cancer, including surgical interventions, radiation therapy, hormone therapy, chemotherapy, and immunotherapy, offer valuable options for patients still, each treatment comes with its set of limitations and potential side effects that can significantly impact well-being and quality of life of patient that leads to the continued research in prostate cancer treatment. Surgical interventions, such as radical prostatectomy, are effective in removing localized tumors, however, they can lead to complications such as urinary incontinence, erectile dysfunction, and bowel dysfunction.

Polysaccharides are digestible and indispensable component present in a variety of foods that provide essential calories and glucose necessary for regular bodily functions along with various health benefits. One extensively studied and well-documented bioactive polysaccharide is β-

Glucan having monomer units linked through glycosidic bonds at β 1→3, 1→4, and/or 1→6 positions, forming branched or unbranched structures (Kaur et al., 2019). β-Glucans are carbohydrate polymers and they are the main constituents of the cell wall of algae, lichens, yeasts, fungi, bacteria, as well as in many plants and have a molecular weight in the range between 50 kDa to 4100 kDa contributing to their diverse biological activities(Nakajima et al., 2024).β-Glucans exhibit a rigid structure characterized by β-1→3 and 1→6) glycosidic linkage in the case of yeast and mushrooms, whereas β-1→3 and 1→4) glycosidic linkages in cereals. The molecular weight of β-Glucans is influenced by various factors, including the type of detectors utilized, reaction conditions, solvents employed, and the specific compounds present in the sample. These parameters collectively determine the size and distribution of β-Glucan molecules, impacting their functional properties and physiological effects (Kupetz et al., 2015).β-Glucans exhibit diverse biological activities influenced by several key parameters. These include molecular weight, structural characteristics, conformational arrangement such as single helix, triple helix, or random coil, charge on the polymer, and density of branching. Changes or modifications in the conformation of β-Glucans can profoundly impact their immune-modulatory properties and overall functionality. Despite being insoluble in water, β-Glucans find extensive applications in the food and pharmaceutical industries. They serve as valuable raw materials for various beverages and medicinal products, contributing to the therapeutics for numerous diseases includingcholesterol management,diabetes, and cancer (Sletmoen et al.,2008).

Quercetin is a flavonoid in fruits and veggies, having aketocarbonyl group and four active hydroxyl groups with scientific name 3, 3', 4', 5, 7-pentahydroxyflavone. Phenolic hydroxyl group and double bond presence makes it strong antioxidant (Yang et al., 2020). It boasts anti-inflammatory, antioxidant, and anticancer properties along with offering vasodilatory, anti-hypertensive, anti-hypercholesterolemic, anti-obesity, and anti-atherosclerotic effects. Its standout quality is triggering tumor cell death (apoptosis), hindering the progression of various human cancers like prostate cancer while sparing normal cells. Specifically with prostate cancer, quercetin indirectly hampers key genes like the prostate-specific antigen (PSA), and androgen receptor (AR) potentially impeding cancer development(Fard et al., 2021). Although, quercetin is reported to have various biological activities, its less solubility is one of its drawbacks in medicine so many researchers tried to load it in the nano/micro particles for its targeted delivery and found positive more effective and improved biological activity.Quercetin have potential for

the treatment of cancer but it has some limitations such as low aqueous solubility, gets clear very rapidly from body, high metabolic rate and very poor absorption that make it restricted for the use in cancer therapy. To overcome these limitations, use of a delivery agent/carrier may enhance its absorption, solubility and target specificity (Nam et al., 2016).In a variety of cancers including prostate cancer, Quercetin is found to inhibit a variety of enzymes those are responsible for the activation of carcinogens and cell proliferation signaling pathways. In this study, we have loaded Quercetin in the hollow β-Glucan particles and sealed it with alginate for the slow and sustained release of the Quercetin for the possible treatment of prostate cancer. The novelty of the study lies in the innovative approach of encapsulating quercetin within β-Glucan carrier and then sealing it with sodium alginate for targeted therapy against prostate cancer. This method addresses several limitations associated with the use of quercetin in cancer therapy, including its low aqueous solubility, rapid clearance, high metabolic rate, and poor absorption.

2. Materials and Methods

2.1. Reagents and chemicals

Quercetin (HiMedia), MTT, DAPI, PI, H2DCFDA, LysoTracker Red DND 99, MitoTracker Red CMX-ROS, Acridine orange, and Ethidium bromidewere purchased from Invitrogen (Thermo Fisher Scientific).DMEM (HiMedia), fetal bovine serum (FBS), antibiotic, and antimycotic solution, were purchased from Gibco^{TM}.

2.2. Maintenance of the Cell culture

DU145and RAW264.7 cell line for the prostate cancer was obtained from the National Centre for Cell Science (NCCS)Pune, India. Cells were maintained at 37°C and 5% CO2 in a CO2 incubator. Complete DMEMmedia was supplemented with 10% FBS and 1% antibiotic and antimycotic solution.

2.3. Particle preparation and characterization

β-Glucan particles were prepared using acid base extraction method from the yeast and characterization was performed using FTIR analysis. We have already published the preparation and characterization methodologies (Upadhyay et al., 2022). Loading of quercetin into β-Glucan and alginate sealing was performed according to the previously published protocol (Upadhyay et al., 2017).

2.4. Assessment of the cell viability of DU145 cells with MTT assay

We have performed the assessment of the cell viability using previously described protocol(Vodnik et al., 2021). 10,000 cells DU145 placed in each well of a 96-well plate and left to grow for 24 hours in a humid environment with 5% CO2 at 37°C. Following this, the cells were exposed to particles prepared in concentrations of IC50 and below and above IC50 for another 24 hours. After treatment, the old media was removed, and MTT dye (10 µl) was added to each well, followed by anincubation of4 h at the temperature 37°C. Next, 100 µl DMSO was added to solubilize the formazan crystals formed by viable cells, and after 10 minutes, absorbance of the dissolved crystals was computedat 490 nm. Cell viability was then calculated using a provided formula

$$\%\text{Viability} = \left(\frac{\text{Absorbance of treatment}}{\text{Absorbance of control}}\right) \text{X}100$$

Concentrations of IC_{50} and below and above IC_{50} were selected for the further analysis.

2.5. Morphological observations

Morphological changes of the treated cells were observed as per the previously defined protocol (Khan et al., 2020). In brief, 50,000 cells/well were addedin a 24 well plate and left overnight for the attachment. After 24 h, cells were given treatmentwith the selected concentrationsof the particles and Quercetin and further left for incubationfor 24 h at37^{o}C. After incubation, cells were washed with the PBS and morphological changes were observed and captured with the help of EVOS FLoid imaging station.

2.6. Assessment of Reactive oxygen species (ROS) generation

The assessment of reactive oxygen species (ROS) production subsequent to particle treatment was conducted according to the protocol described by (Zhu et al., 2022). Initially, a cell density of 50,000 cells per well was seeded into a 24-well plate and permitted to incubate for 24 hours to ensure attachment. Following this, the cells were treated withselected concentrations of the β-Glucan and Quercetin, and then maintained in culture for an additional 24 hours at 37°C with 5% CO2. Subsequent to the incubation period, the cells were rinsed through phosphate-buffered

saline (PBS) and stained with 20µM DCFDA dye, followed by incubation at 37°C for 20 minutes. Finally, images were captured using an EVOS FLoid imaging station.

2.7. Determination of nuclear morphology and DNA fragmentation with DAPI staining

DAPI staining was carried out based on a previously described protocol with slight adjustments, as outlined in the work by (Sana et al., 2020). In brief, 50,000 cells/ well were seeded in a 24-well plate and allowed to incubate for 24 h. Following this, the cells were exposedtoselected concentrations of the particles and further incubated for 24 h, at temperature 37°C. Once the incubation period was completed, the cells were rinsedwith PBS and the cells were stained with 1µg/mL of DAPI for 15 minutes. Morphological changes were then observed and documented.

2.8. Apoptosis detection with Propidium iodide (PI) staining

Cells undergoing apoptosisweredetermined using PI staining with the help of already established protocol with minormodification (Yang et al., 2022). In Brief, 50,000 cells per well were seeded in a 24 well plate and incubatedovernight. After that, cells were treated with selected concentration of Quercetin and prepared particles and further incubated for 24 h. After incubation, cells were stained with 1µg/ml of the PI (prepared from 1mg/ml of PI) and further incubated for 10 min at 37^{o}C following which images were obtained with the EVOS FLoid imaging station.

2.9. Acidic organelles activity analysis through LysoTracker Red DND-99

Acidic organelles were detected by usingLysoTracker Red (100nM) as per the formerly described method(Yang et al., 2022). In brief, 50,000 cells/well seeded in 24 well plate and incubated overnight. After attachment of the cells, treatment with the various concentrations of particles was performed and further incubated for 24 h. Postincubation, cells were washed with PBS and stained with LysoTracker for 30 min and imaged under FLoid imaging microscope.

2.10. Mitochondrial membrane potential (MMP)assessment

MMP was assessed as per the previously reported protocol (Ning et al., 2020). Briefly, 50,000 cells/well seeded in 24 well plate and incubated for 24 h,afterwards, cells were treated with the concentrations of particles and further incubated for 24 hrs. After incubation, cells were stained

for 30 min using the dyeMitoTracker red CMX ROS (300nM) and imaged under EVOS FLoid imaging microscope.

2.11. Acridine orange and Ethidium bromide (AO/EtBr) dual staining

AO/EtBr dual staining was performed conferring to the earlierdescribed protocol with slight modification (Fernandes et al., 2013). 50,000 cells/well seeded in 24 well plate and after gaining confluency, treated with selectedconcentrations of particles and left for 24 h incubation in CO_2 incubator. After that,cells were stained with 5 µl of AO and 5 µl EtBr (5mg/ml each) and cell death was observed with FLoid Imaging microscope.

2.12. Statistical analysis

In vitro experiments were accomplished in triplicates and One-way ANOVA was used for the statistical analysis using GraphPad Prism 8.0 and ImageJ. A probability value of $p < 0.05$ was deliberated statistically significant where *** means highly significant, ** means less significant and * means least significant.

3. Results

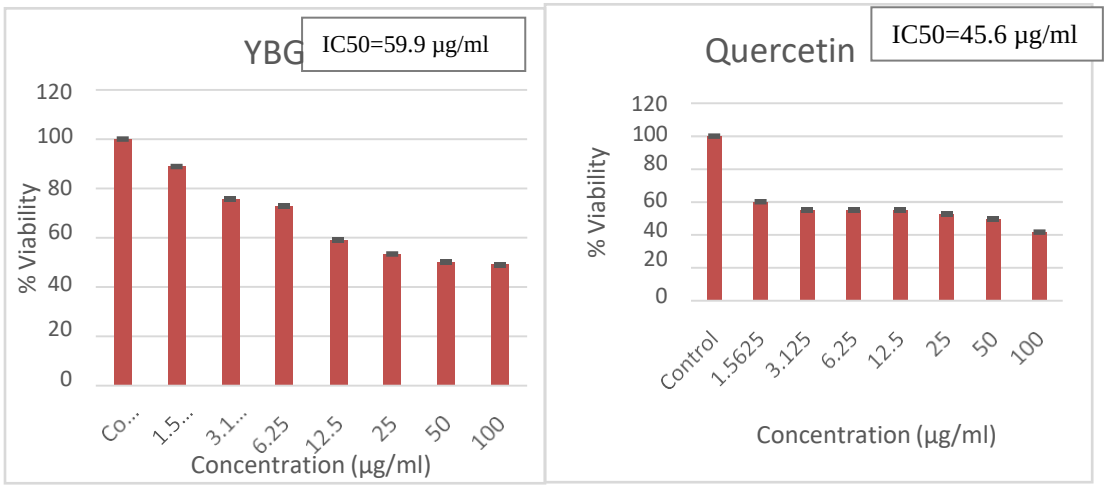
3.1. Particle preparation

Prepared quercetin loaded and alginate sealed particles were used for the determination of *in vitro* anti-cancer activity. We named hollow β-Glucan particle, Quercetin loaded β-Glucan particles, and Quercetin loaded alginate sealed particles as YBG, YBG+Q and YBG+Q+Alg respectively.

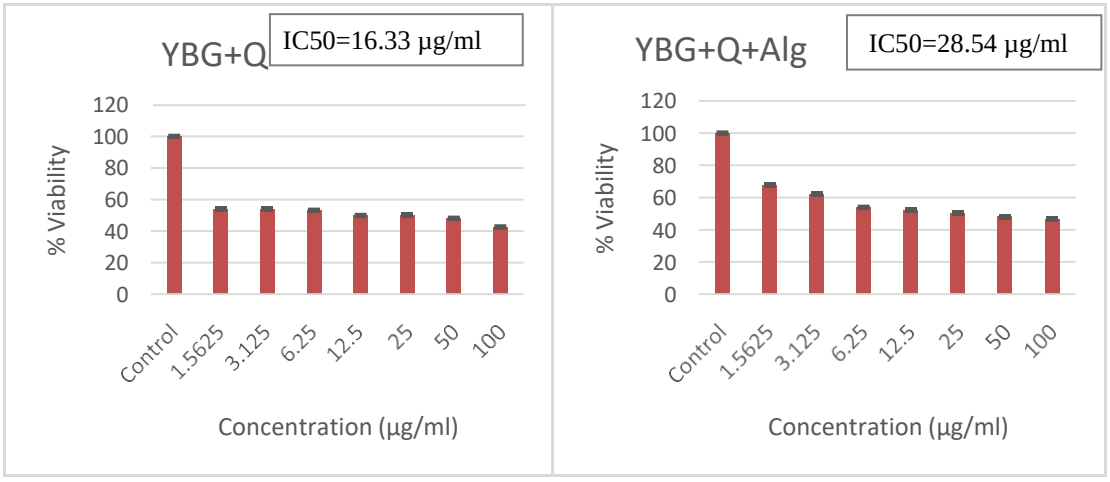
3.2. Assessment of the cell viability with MTT assay on DU145 and RAW264.7 cells

Cell viability of the DU145 cells upon exposure to the particles derived from yeast were found to decrease as shown in the (Fig. 1) and (Fig. 2). In case of yeast derived particles, Quercetin loaded and alginate sealed particles have lowest IC50. To evaluate the consequence of the particles on the healthy cells we treated RAW264.7 cells with the serial dilution from 500µg/ml to 1.56µg/ml. We found that particles are not having cytotoxic effects at initial concentrations as

shown in (Fig. 6) and at higher concentration of 500 and 250µg/ml, particles were having very little cytotoxic effects on the RAW264.7 cells.

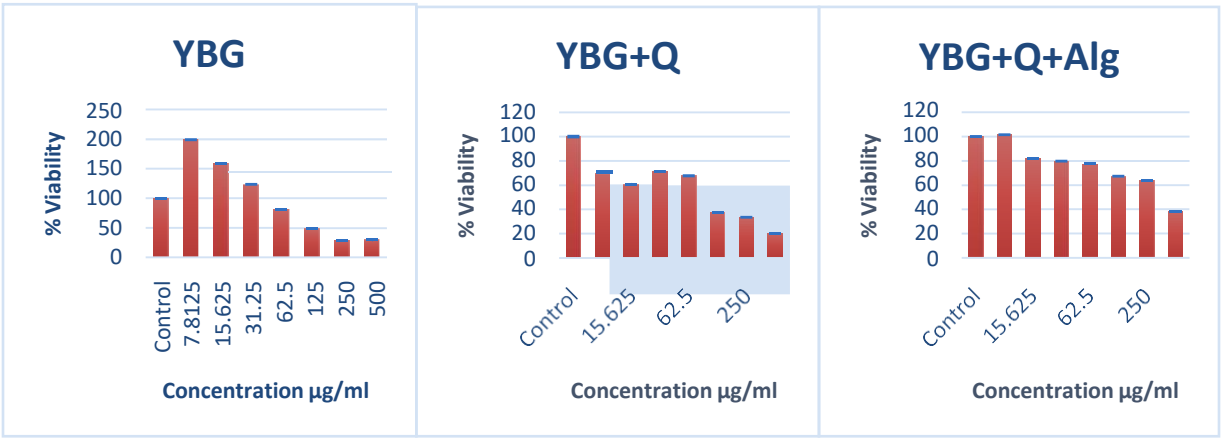


(a) **(b)**

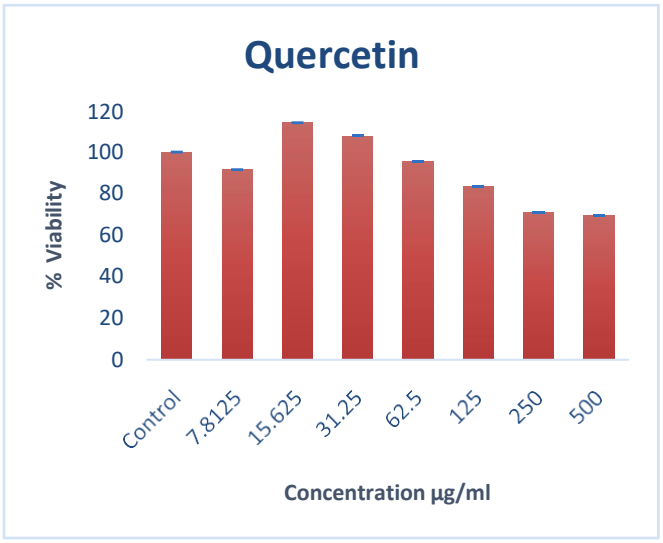


(a) **(b)**

Fig. 1. MTT of the particles derived from yeast β-Glucan. There was dose dependent reduction in cell viability after treatment (a) % Viability of DU145 cells after treatment with YBG (b) Cell viability of DU145 after treatment with Quercetin (c) % Viability of DU145 cells after treatment with YBG+Q (d) % Viability of DU145 cells after treatment with YBG+Q+Alg.



(a) **(b)** **(c)**



(d)

Fig. 2. Evaluation of the cell viability with MTT on RAW264.7 cells. (a, b, c) are the cell viability of the cells treated with yeast derived particle while (d) is the cell viability of the cells treated with quercetin.

3.3. Morphological observations

With the help of FLoid imaging microscope, we observed clear morphological changes in the shape of the DU145 cells. At the highest concentration, cells were highly disrupted and they lost their actual shape as shown in (Fig. 3).

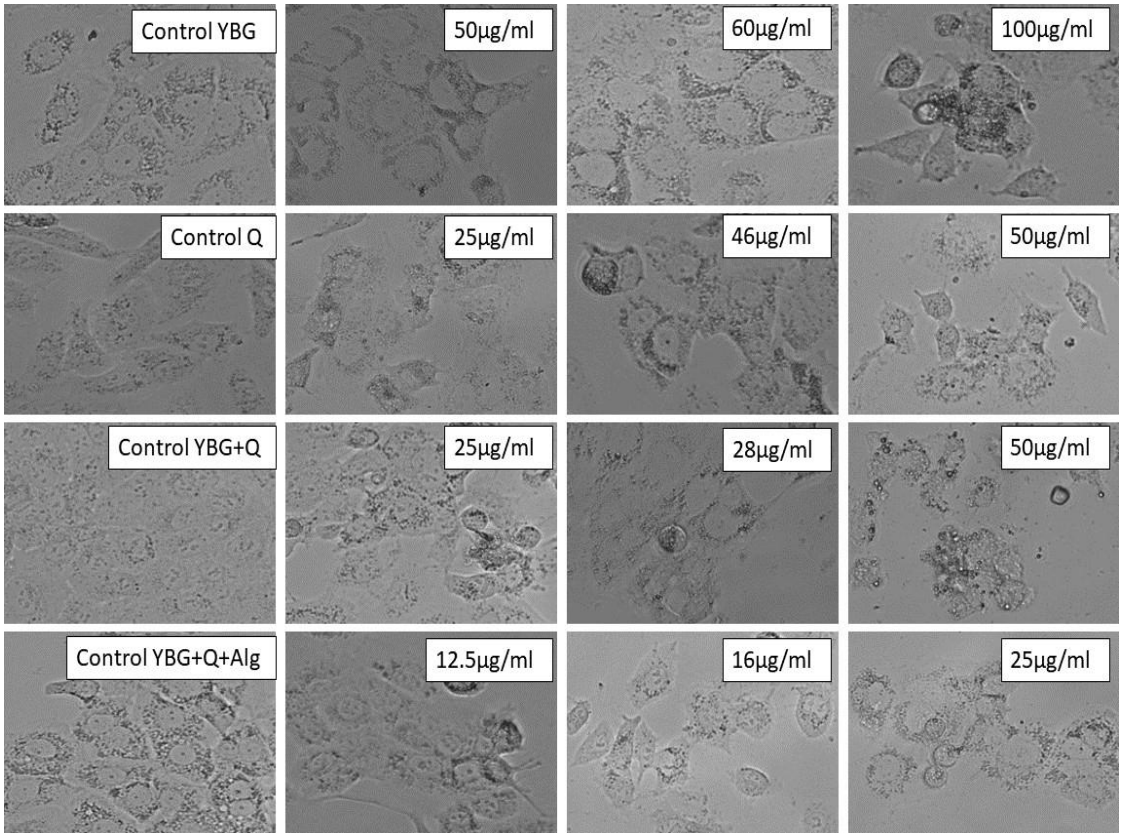
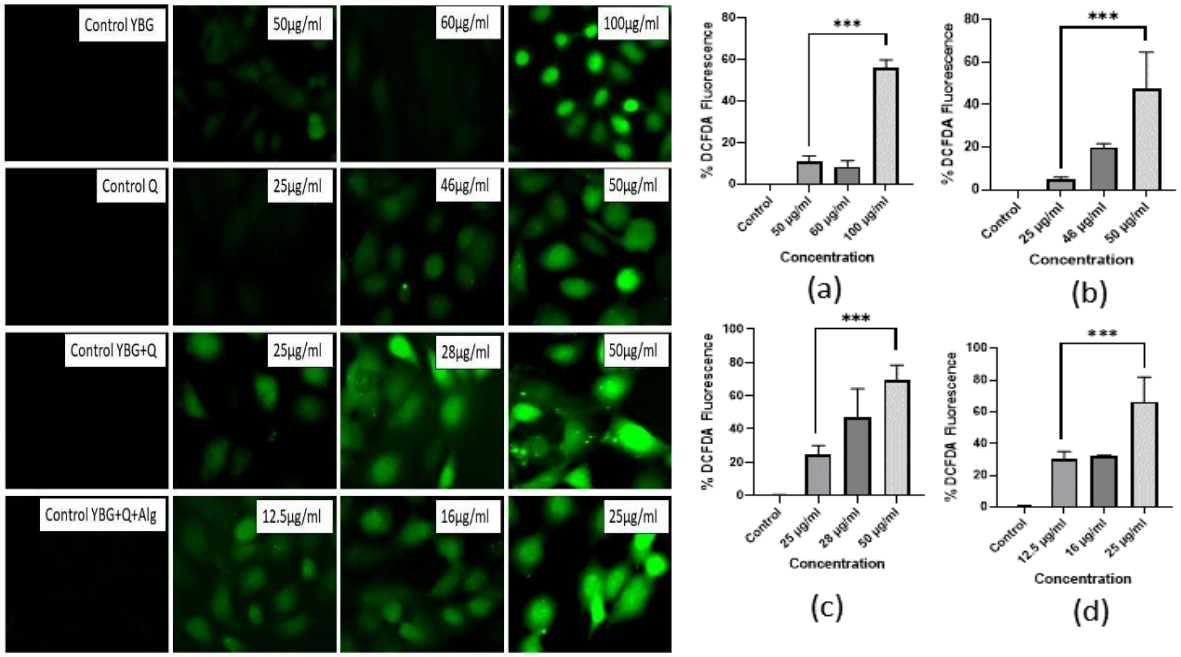


Fig. 3.DU145 cells were showing visible morphological changes after treatment for 24 h with yeast derived particles.

3.4. Assessment of Reactive oxygen species (ROS) generation

ROS are a natural derivative of oxygen metabolism in body and have a variety of important role in cell signaling. However, excessive ROS generation can lead to oxidative damages leading to the apoptosis of the cancer cells. In thisstudy, we found that β-Glucan and the quercetin loaded β-Glucan particles were having potential to generate efficient amount of ROS in DU145 cells asshown in (Fig. 4).



(i) **(ii)**

Fig. 4.(i) DU145 cells showing efficient amount of ROS generation after treatment with yeast derived particles. (ii) is the statistical analysis of the YBG, Quercetin, YBG+Q+Alg, and YBG+Q+Alg. All the values areshowing high significance with p value less than 0.0005 ($p<0.0005$)

3.5. Determination of nuclear morphology and DNA fragmentation with DAPI staining

The influence of particles on the DU145 cells was assessed with DAPI staining that is specific for the binding with nucleus. Nuclear condensation and abnormalities are quite observable and can be seen in (Fig. 5). We have found that nuclear deformities were increasing with the increase in the treatment concentration of the particles.

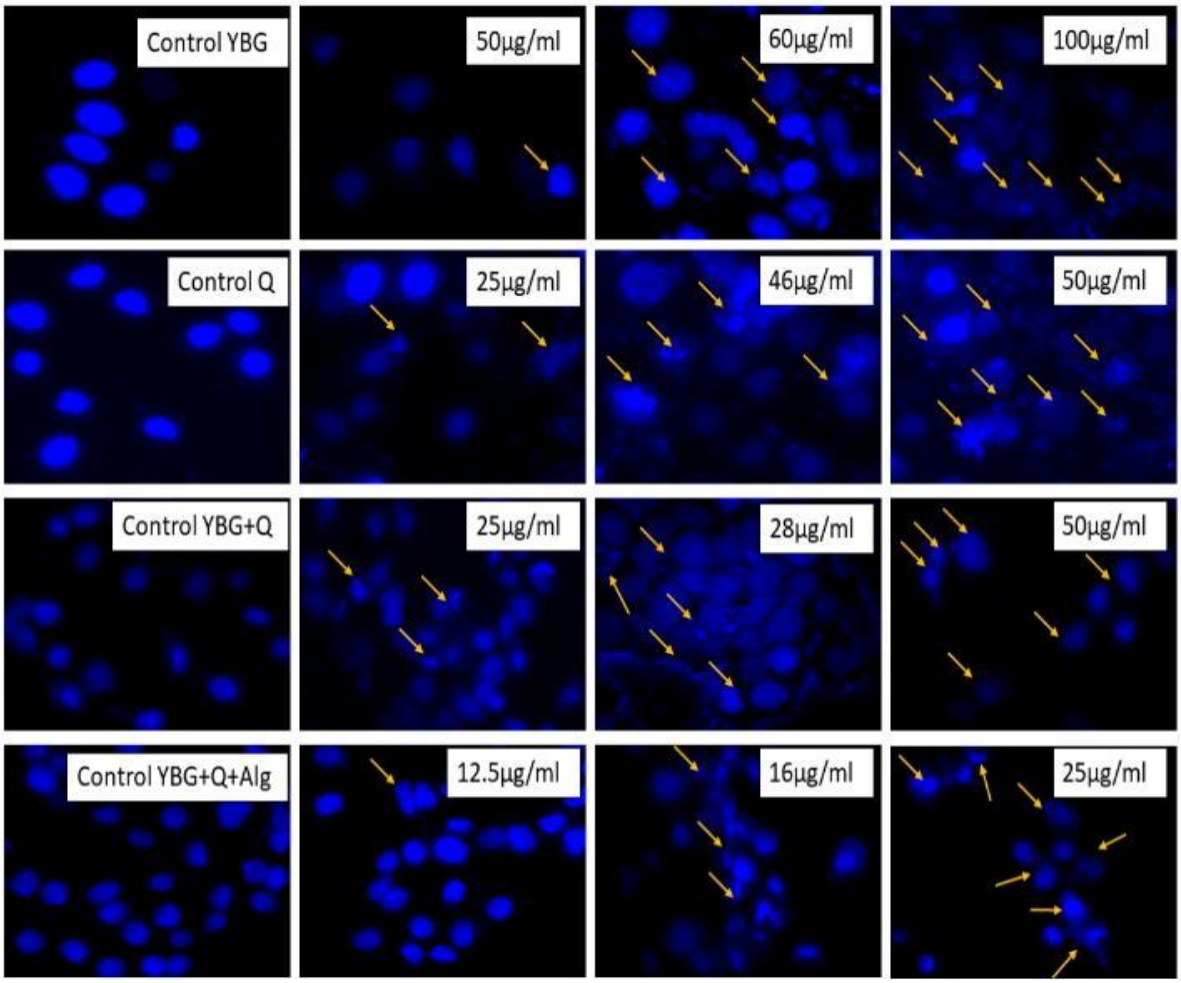


Fig. 5. Morphological changes in nucleus observed with the DAPI staining after treatment with yeastderived particles.

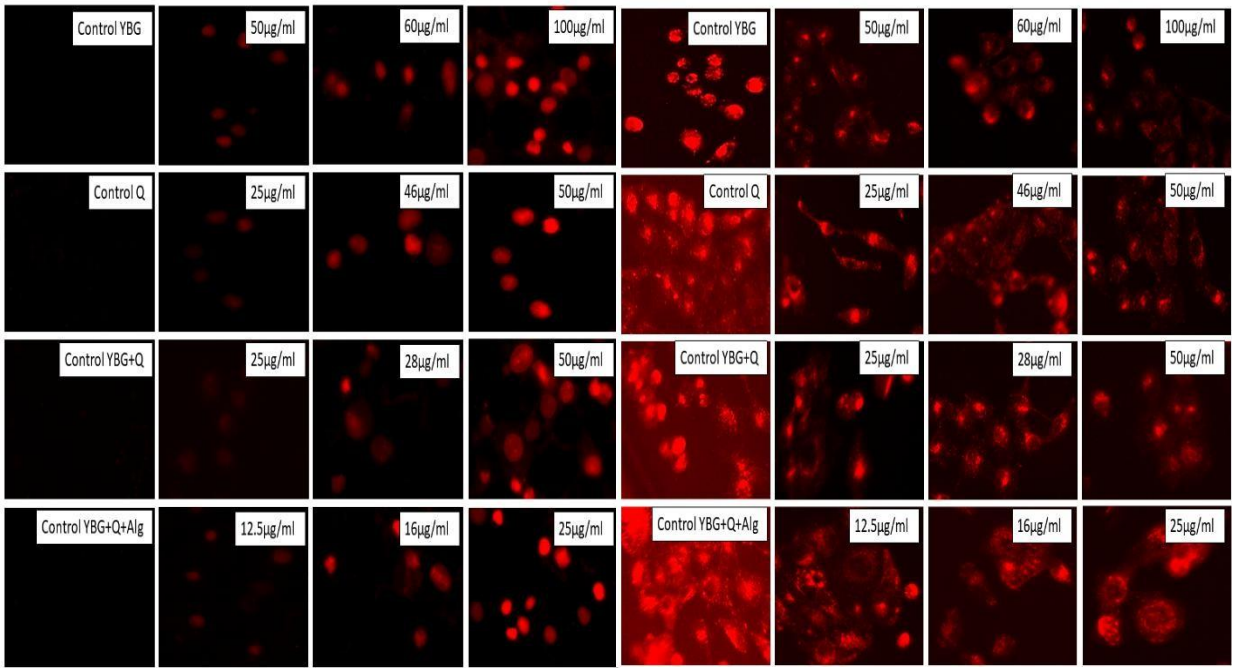
3.6. Apoptosis detection with Propidium iodide (PI) staining

PI is a very specific dye that only binds with the dying or apoptotic cells resulting in red color fluorescence. Cells in the late apoptotic or early necrotic stage stain red. In our study we have found the significant increase in the apoptotic cellswith the increase in concentration as shown in Fig. 6 (i). In non-treated (control) cells, there was no significant percentage of apoptosis was observed.

3.7. LysoTracker Red DND-99 staining to monitor pH change in lysosome

Lysosome functioning depends on the lysosomal pH and decrease in the fluorescence intensity of the red color indicated the elevation in the pH of lysosomes. We have found a dose dependent

decrease in the florescence intensity indicating the pH alteration that can result in the apoptosis of the cells as shown in Fig. 6 (ii).

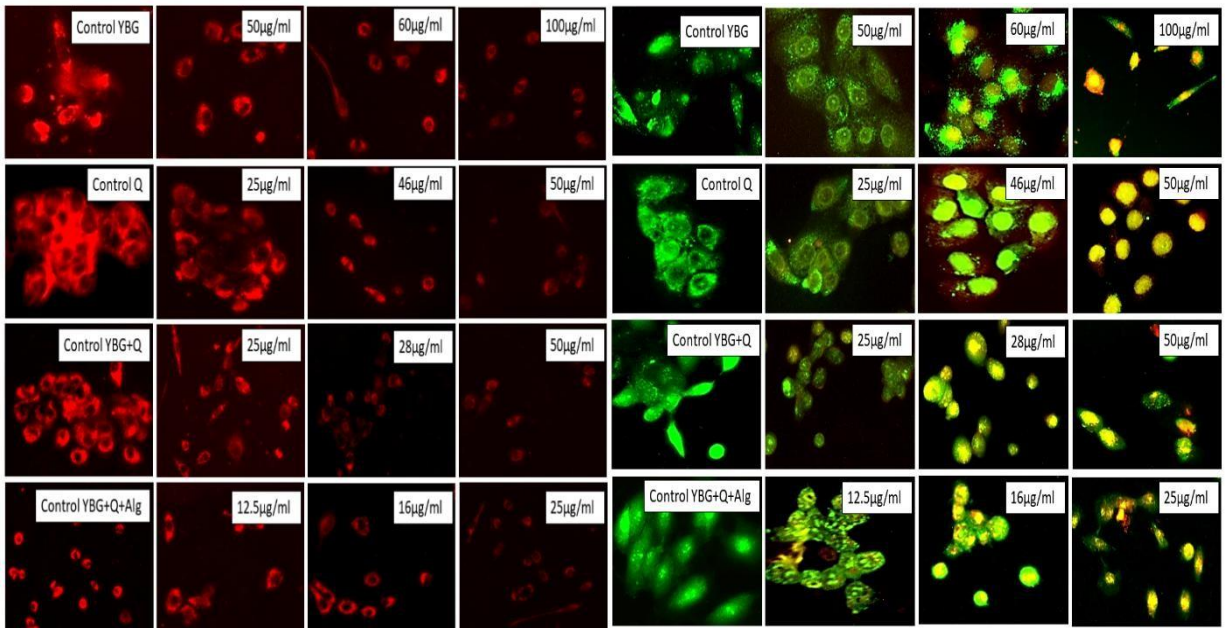


(i) (ii)

Fig. 6.(i) Apoptosis determination in the DU145 cells as observed with the FLoid imaging microscope after treatment with yeast derived particles(ii) LysoTrackerred DND-99 staining in the DU145 cells observed with the fluorescence microscope

3.8. Mitochondrial membrane potential determination

MitoTracker Red CMX-ROS is a probe-based cationicdye that enables to assess the change in MMP. In our study, DU145 cells showed significant decline in MMP after treatment with yeast derived particles as shown in Fig. 7 (i).



(i) (ii)

Fig. 7.(i) MitoTracker Red staining in the DU145 cells as observed with the fluorescence microscope for the determination of mitochondrial activity. (ii)AO/EtBr dual staining of the DU145 cells showing cells in light yellowish color cells are in early and orange color of cells in the late stage of the apoptosis

3.9. Acridine orange and Ethidium bromide (AO/EtBr) staining for the detection of cells in early and late stage of apoptosis

AO/EtBr dual staining is considered very efficient method for the detection of early and late apoptotic cells. AO dye is permeable to all the cells and gives florescence in green color while EtBr is only permeable to the cells undergoing apoptosis and gives red fluorescence. In the dual staining, cells in early apoptotic stage show yellowish-orange color while cells in late apoptotic stage show orange-red color. We have found cells appeared as yellow-orange-red color showing cells in early and late apoptosis as shown in Fig. 7 (ii).

4. Discussion

In this study, we have presented experimental investigation into potential therapeutic agents for prostate cancer. We made formulation by loading of quercetin into β-Glucan derived from yeast and alginate sealing was done for the slow and sustained release of quercetin from the hollow β-Glucan particles. Alginate sealed formulation was reported to slow down the release of the compound in many studies (Soto et al., 2010). We conducted MTT assays on DU145 cells to evaluate the cytotoxic effects of particles derived from yeast, demonstrating a dose dependent decrease in cell viability. Importantly, yeast-derived particles, particularly quercetin-loaded and alginate-sealed particles, exhibited promising cytotoxicity against prostate cancer cells. We found that the alginate sealed particles i.e. YBG+Q+ALG are having lowest IC_{50} values so they can be considered as good anticancer agent against prostate cancer. We also conducted MTT on the healthy cell line RAW264.7 to assess the effect of these particles and found that particles are not affecting the viability of the cells at initial concentration. On the higher concentrations such as 500µg/ml, cell viability was decreasing up to 20%. A study conducted the MTT of β-Glucan on RAW264.7 cell line and found that control group and β-Glucan treated group were not having any significant difference in the cell survival and this study aligns with our study in which we found no significant cell death after the treatment with β-Glucan and its formulations (Choi et al., 2016).

Through fluorescence microscopy and staining techniques, we tried tofind out mechanistic insights into the mode of action of the particles. The observed increase in ROS generation, alterations in lysosomal pH, decline in MMP, and induction of apoptosis highlight the significant anti-cancer potential of these compounds. Quercetin is investigated in many studies and is found to attenuate cell survival, inhibit cell proliferation pathways and induce cell death due to the generation of ROS (Ward et al., 2018) and slow and sustained release of the Quercetin from the hollow β-Glucan particles due to alginate sealing will enhance its potential towards efficient anticancer activity. In this study, ROS generation increased in a dose dependent manner that was confirming the apoptosis of the cancer cells due to the oxidative stress. Nuclear fragmentation and condensation in DU145 after treatment with β-Glucan particles was aligning with a study in which Farnesol was found to induce apoptosis in DU145 cells as assessed through fluorescent microscope using DAPI staining (Park et al., 2014).

AO/EtBr dual staining was found to show cells in the early and late apoptotic stage. AO stains live cells while EtBr stains the cells undergoing apoptosis or having compromised membrane. In a study, PAX2 siRNA knocked down DU145 cell showed condensed yellow nuclei due to the co-localization of AO and EtBr dye (Bose et al., 2009). Overall, our findings suggested that particle derived from yeast are having efficient anticancer activity and further *in vivo* studies clinical investigations can give impactful findings to use these formulations for the treatment of prostate cancer.

5. Conclusion

The *in vitro* study underscores the potential of quercetin loaded and alginate sealed β-Glucan particles as promising therapeutic candidates for prostate cancer treatment. The study elucidated the cytotoxic effects of quercetin-loaded particles, derived from yeast sources, against DU145 cells. ROS generation and other fluorescent assays support the anti-cancer properties of these particles. Future directions could involve preclinical studies to assess efficacy in animal models, pharmacokinetic profiling to determine bioavailability, and clinical trials to evaluate safety and efficacy in human patients. These efforts hold promise for advancing precision oncology and improving patient outcomes in prostate cancer treatment.

Conflict of Interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors declare no known competing interests.

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