

# Synthesis of Chitosan Based Nanocomposite with 3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene) indolin-2-one (RAJI) for Efficient Therapy Against Triple Negative Breast Cancer

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## ABSTRACT

Cancer is defined by the unregulated and excessive growth of abnormal cells, eventually leading to the destruction of body tissues. Among various cancer types, breast cancer is notably prevalent in females generally originating in the ducts or lobules forming lumps or tumours and subsequently metastasizing to other regions of the body. The treatment of breast cancer is contingent upon its stage and typically involves surgery, chemotherapy, radiation, hormone therapy, etc. Drugs currently available on the market frequently induce substantial adverse effects, which has prompted the development of innovative therapeutic agents. In this study, we propose the use of a novel compound, 3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene) indolin-2-one (RAJI), with chitosan to create a nanocomposite for the purpose of developing an effective breast cancer treatment. RAJI-loaded chitosan nanocomposite was synthesized using a high-pressure homogenizer and subsequently characterized to assess their physicochemical properties and drug loading profile. The *In-vitro* toxicity analyses of RAJI on MDA-MB-231 cells - a Triple Negative Breast Cancer (TNBC) cell line, demonstrated its potent anti-cancer properties. Consequently, we speculate that the efficaciousness of RAJI-loaded chitosan nanocomposite in the treatment of TNBC will be superior to that of other commercially available medicines.

**Keywords:** Breast cancer, Triple Negative Breast Cancer, Drug, RAJI, Chitosan, Nanocomposite.

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## INTRODUCTION

The uncontrolled growth of aberrant cells is termed cancer that can invade surrounding tissues and metastasize to distant body sites. Its underlying mechanism includes the loss of control over cell proliferation, differentiation, and apoptosis, often driven by genetic mutations and epigenetic modifications. Cancer ranks as the second most common cause of mortality worldwide, following cardiovascular diseases<sup>1,2</sup>. Recent reports state that lung cancer is the most common in men, while breast cancer is predominant in women. In accordance with the GLOBOCAN 2020 report, an approximation of nearly 19.3 million new cancer cases and 10 million cancer deaths were recorded worldwide in 2020, with breast cancer (BC) becoming the most diagnosed cancer globally<sup>3</sup>.

BC is a malignant tumour originating from the breast cells, commonly from the ducts or lobules. It is the leading reason behind cancer-related deaths among women worldwide, with increasing incidence due to factors such as age, genetic predisposition, lifestyle, and hormonal influences. In 2020, nearly 2.3 million new BC cases were reported, accounting for approximately 24.5% of all cancer cases among women globally<sup>4,5</sup>. In India, it is the most prevalent cancer among women, with over 178,000 new cases annually and a

significant mortality rate due to late-stage diagnosis and limited access to effective treatment<sup>3</sup>.

Triple-negative breast cancer (TNBC) is a predominant subtype of BC distinguished by the lack of Estrogen receptors (ER), Progesterone receptors (PR), and HER2 protein overexpression. This lack of receptors makes TNBC distinct from other breast cancer types, leading to unique challenges in treatment and prognosis<sup>6</sup>. TNBC is frequently associated with a younger age, higher tumour grade, and a more aggressive clinical progression. Constituting around 10-20% of all breast cancers, this kind is more common in women who have BRCA1 mutations<sup>7</sup>.

The treatment of breast cancer involves several modalities, each of which has unique merits and limitations. Surgical intervention continues to be a fundamental approach for treating early-stage breast cancer, often providing the possibility of a cure. Nevertheless, it is fundamentally intrusive, posing risks of substantial adverse reactions<sup>4</sup>. Post-operative radiation treatment is efficacious in specifically targeting any remaining cancerous cells, hence reducing the likelihood of cancer relapsing. However, patients may encounter negative consequences such as skin irritation, exhaustion, and chronic cardiovascular issues<sup>8</sup>. Chemotherapy, a kind of treatment that affects the whole body, is very effective in reducing the size of tumours and

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controlling the spread of cancer to other parts of the body. Although chemotherapy is successful, it is also linked to significant deleterious effects like nausea, hair loss, and a greater vulnerability to infections<sup>9</sup>. Hormone treatment is specifically designed for breast cancers that have hormone receptors, thereby decreasing the likelihood of cancer relapse. Nevertheless, this therapy may cause adverse reactions including osteoporosis, and an elevated risk of thromboembolism<sup>1</sup>.

The heterogeneity of TNBC necessitates personalized treatment strategies to effectively manage this complex disease. Hence, a novel drug - 3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene) indolin-2-one (RAJI) was synthesized as a 'one pot protocol' encompassing the consecutive addition of Piperidine and concentrated Hydrochloric acid to a reaction mixture containing Isatin and 3,4-dimethoxy Acetophenones. Piperidine has been recognized as a potential compound in the development of various new pharmaceutical agents. Isatin is a heterocyclic molecule that possesses an extensive history of application as a building block for a wide range of pharmaceutical compounds. Furthermore, various other research has referenced the antimalarial effects of 3,4-dimethoxy acetophenones. Despite their well-documented functions, the potential cancer applications of these chemicals have received little attention. With this knowledge gap in mind, RAJI was synthesized from the above-mentioned elements.

Previous studies indicate that RAJI exhibited no significant toxicity in zebrafish and mice at low doses while demonstrating potent cytotoxicity against TNBC cells<sup>10</sup>. Additionally, RAJI significantly reduced tumour volume in MDA-MB-231 xenografted BALB/c nude mice and modulated key genes in the AKT/PI3K pathway, underscoring its potential as a promising therapeutic agent for breast cancer<sup>11</sup>.

Bio-nano polymers have garnered substantial attention owing to their biocompatibility, biodegradability, and potential applications in various biomedical fields<sup>10,11</sup>. Chitosan-based nanocomposites are notable for their distinctive characteristics and wide range of uses. Chitosan, a cationic polymer obtained from chitin, has exceptional biocompatibility, biodegradability, and lack of toxicity, making it a perfect choice for biomedical intents<sup>12</sup>. Chitosan-based nanocomposites are synthesized using many techniques, such as electrostatic interaction, covalent crosslinking, adsorption, and hydrogen bonding. These

techniques enable the incorporation of chitosan with other nanomaterials to augment its mechanical robustness, stability, and functional characteristics. Chitosan nanoparticles may be merged with magnetic nanoparticles and quantum dots to produce versatile nanocomposites that have many uses in drug delivery and bioimaging<sup>13</sup>. Therefore, we expect that the incorporation of RAJI might enhance the efficacy of treatment for triple-negative breast cancer by improving the therapeutic index and reducing systemic toxicity via targeted drug distribution.

## MATERIALS AND METHODS

### *Synthesis of Chitosan Nanocomposite*

Chitosan solution was prepared when 1% (w/v) chitosan was dissolved in 2% (v/v) acetic acid solution under constant magnetic stirring at 1000 rpm, until it turned transparent.

### *Preparation of RAJI-Loaded Chitosan Nanocomposite*

To 25ml of the chitosan - acetic acid solution, 50 $\mu$ g of RAJI (drug) was added and subjected to constant magnetic stirring at 1000 rpm.

### *Lyophilization of RAJI-Loaded Chitosan Nanocomposite*

The RAJI-chitosan mixture was poured into a petri plate and was kept in freezing conditions of -20°C for four hours. To retain the porous structure in the chitosan scaffold, the frozen materials were placed in the freeze drier for 12 hours to totally sublimate the water in the material<sup>14</sup>.

### *Physicochemical Characterization of the Synthesized Nanocomposite*

#### *Ultraviolet-Visible Spectroscopy (UV-vis)*

The validation of RAJI-loaded chitosan nanocomposite formation was accomplished by recording the UV-visible spectra using Shimadzu UV-visible 1800 spectrophotometer which is a common method for characterising nanoparticles synthesis<sup>15</sup>.

#### *Fourier Transform Infrared Spectroscopy (FTIR)*

Fourier Transform Infrared Spectroscopy (FTIR) was employed to analyse the distinctive functional groups present in the RAJI loaded chitosan nanocomposite. Thermo Nicolet Nexus, Smart Orbit spectrometer (Shelton, USA) was used to record the spectra for the RAJI loaded chitosan nanoparticles, RAJI, and chitosan alone using 1% in 200 mg spectroscopic-grade potassium bromide (KBr) under 10 tonnes of pressure<sup>16</sup>. Spectral scanning was taken in the wavelength region between 4000 and 400  $\text{cm}^{-1}$  at a resolution 158 of 4  $\text{cm}^{-1}$  with scan speed of 2 mm/sec.

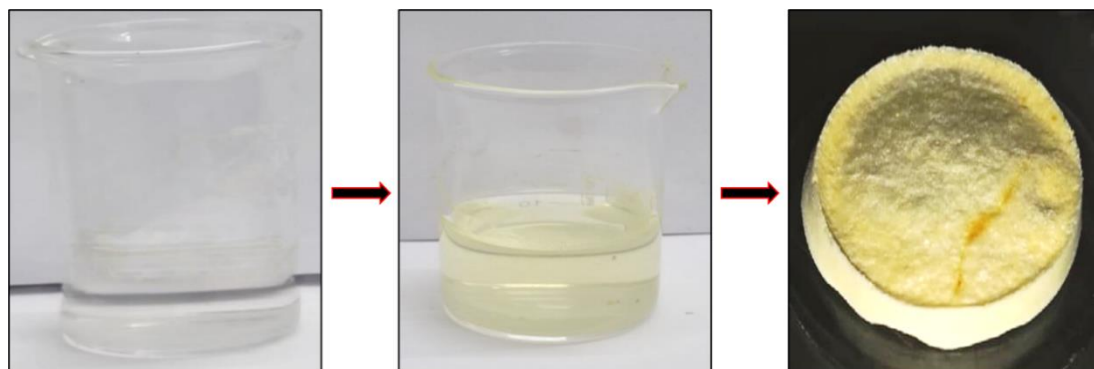


Figure 1: Synthesis of chitosan Nanocomposite, RAJI loaded chitosan Nanocomposite and RAJI incorporated chitosan nanocomposite scaffold post lyophilization

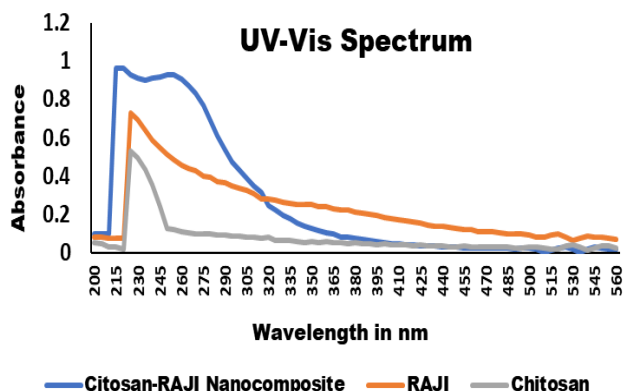


Figure 2: UV-visible spectrum of RAJI, chitosan and the synthesized RAJI-chitosan nanocomposite

#### X-Ray Diffraction (XRD)

The RAJI-loaded chitosan nanocomposites' powder X-ray diffraction (XRD) patterns were acquired on a Shimadzu XRD-6000 device (Shimadzu Corporation, Kyoto, Japan). The tests were carried out using CuK $\alpha$  radiation ( $\lambda = 1.5418 \text{ \AA}$ ), produced at an operating voltage of 30 kV and a current of 30 mA, spanning a  $2\theta$  range of  $10\text{--}80^\circ$  <sup>16</sup>.

#### Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is an essential device for analysing the physical characteristics of nanoparticles. It offers precise and high-quality pictures that reveal important details such as size, shape, and surface texture. The sample was degassed at  $100^\circ\text{C}$  overnight in an evacuated, heated chamber. Double-sided conductive tape was used to attach the dried sample to the specimen stub before they were scanned using 2700 (Hitachi Ltd, Tokyo, Japan) SEM <sup>16</sup>.

#### Cell Culture and Maintenance

The TNBC cell line - MDA MB 231, was obtained from National Centre for Cell Sciences (NCCS) in Pune, India. It

was cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, along with 10% Foetal Bovine Serum (FBS) and 1% antibiotic-antimycotic solution. The cells were maintained in a 5% CO<sub>2</sub> environment at a temperature of  $37^\circ\text{C}$ .

#### Assessment of Cell Viability using MTT Assay

To assess the cytotoxic effect of RAJI on MDA-MB-231 cells, a 96-well plate was seeded with  $1 \times 10^4$  cells and then incubated for 24 hours. Following this, the cells were subjected to different concentrations of RAJI and RAJI loaded chitosan nanocomposite (10 to 50  $\mu\text{g/ml}$  for RAJI and 100 to 500  $\mu\text{g/ml}$  for RAJI loaded chitosan) for a duration of 24 hours. The control group consisted of cells that were not subjected to any treatment. The viability of cells was assessed using the MTT reagent (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) from Sigma-Aldrich, following the manufacturer's guidelines, and the IC<sub>50</sub> values were calculated <sup>17</sup>.

#### Evaluation of Drug Loading Efficiency of RAJI-Loaded Chitosan Nanocomposite

To determine the drug loading efficiency (DLE) of RAJI loaded chitosan nanocomposite, a small section of the nanocomposite was dissolved in DMSO and the concentration of the RAJI was quantified at 481 nm <sup>16</sup> using the formula:

$$\text{DLE (\%)} = (\text{Mass of RAJI in NPs}) / (\text{Total mass of NPs}) \times 100\%$$

## RESULTS

#### Synthesis of Chitosan Nanocomposite

Chitosan solution was formulated by dissolving chitosan at a concentration of 1% (w/v) in a 2% (v/v) acetic acid solution. The procedure was carried out with continuous magnetic agitation at a speed of 1000 rpm until the solution turned clear, indicating the full dissolving of the chitosan as shown in figure 1. Acetic acid enhances the solubility of

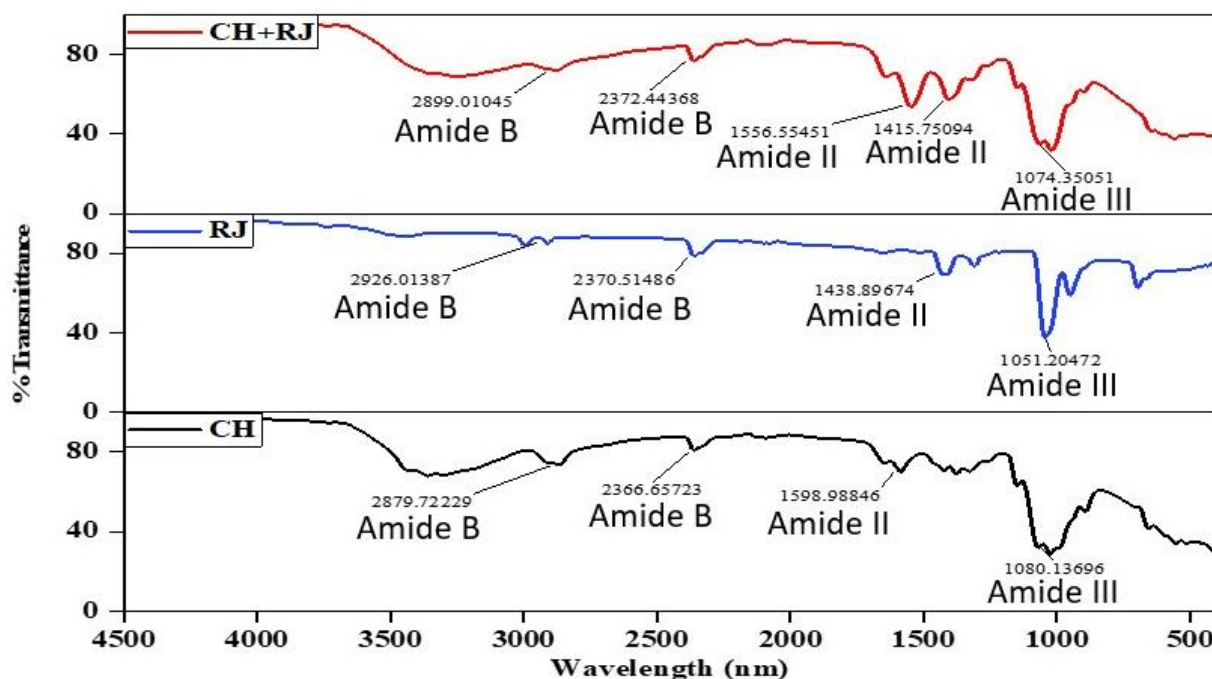


Figure 3: Fourier transform infrared (FTIR) spectrum of RAJI, chitosan and the synthesized RAJI-chitosan nanocomposite

chitosan by protonating its amino groups, resulting in increased solubility in water. The increased stirring velocity guarantees consistent blending and effective dispersion of chitosan with acetic acid.

*Preparation of RAJI-Loaded Chitosan Nanocomposite*

50 µg of RAJI - the drug of choice, was added to a 25 ml solution of the above-mentioned chitosan-acetic acid solution. Subsequently, the combination was subjected to continuous magnetic stirring at a speed of 1000 revolutions per minute. This guarantees comprehensive and consistent dispersion of RAJI throughout the chitosan solution, facilitating efficient interaction between the drug and chitosan resulting in a mild yellow colour as shown in figure 1.

*Lyophilization of RAJI-Loaded Chitosan Nanocomposite*

The RAJI-chitosan mixture when subjected to freeze drying post exposure at -20°C, facilitated in the intactness of the pore architecture of the resulting scaffold. The first freezing process transformed the mixture into a solid state, resulting in the formation of a stable framework with the intended porosity pattern. As a result, the freeze-drying technique allowed for the entire conversion of water from the frozen matrix into vapor. This approach effectively retained the pore architecture by preventing any collapse or distortion of the scaffold structure as depicted in figure 1. Preserving the porous architecture is crucial for the scaffold's performance since it affects important features including mechanical strength, drug release kinetics, and biocompatibility. This

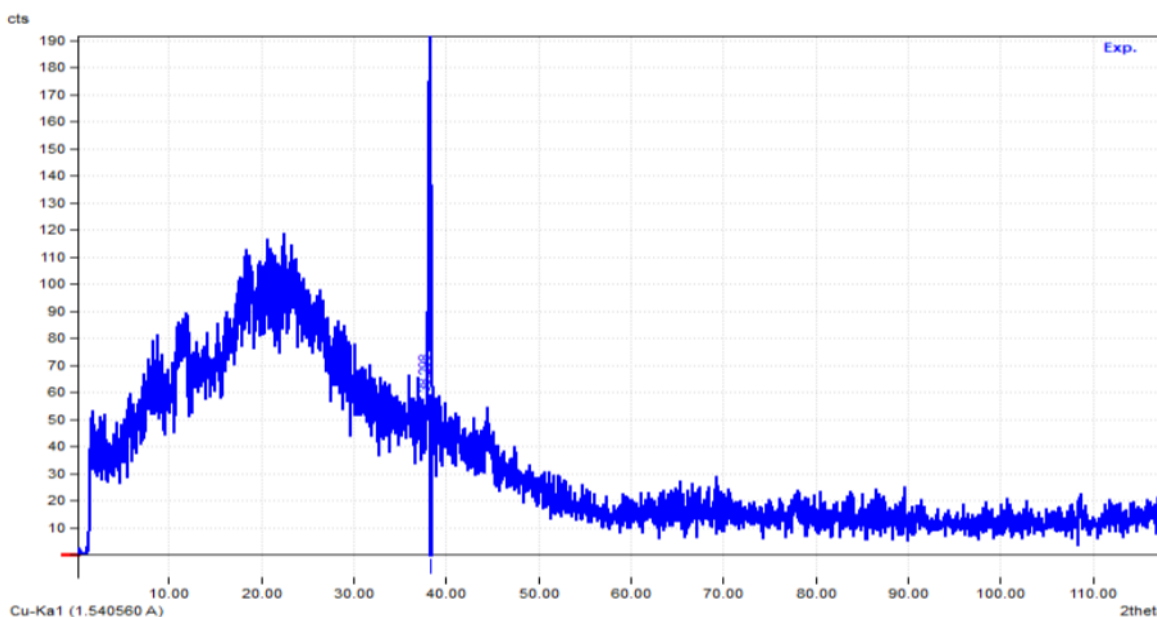


Figure 4: Scanning Electron Microscope (SEM) images of RAJI, chitosan and the synthesized RAJI-chitosan nanocomposite

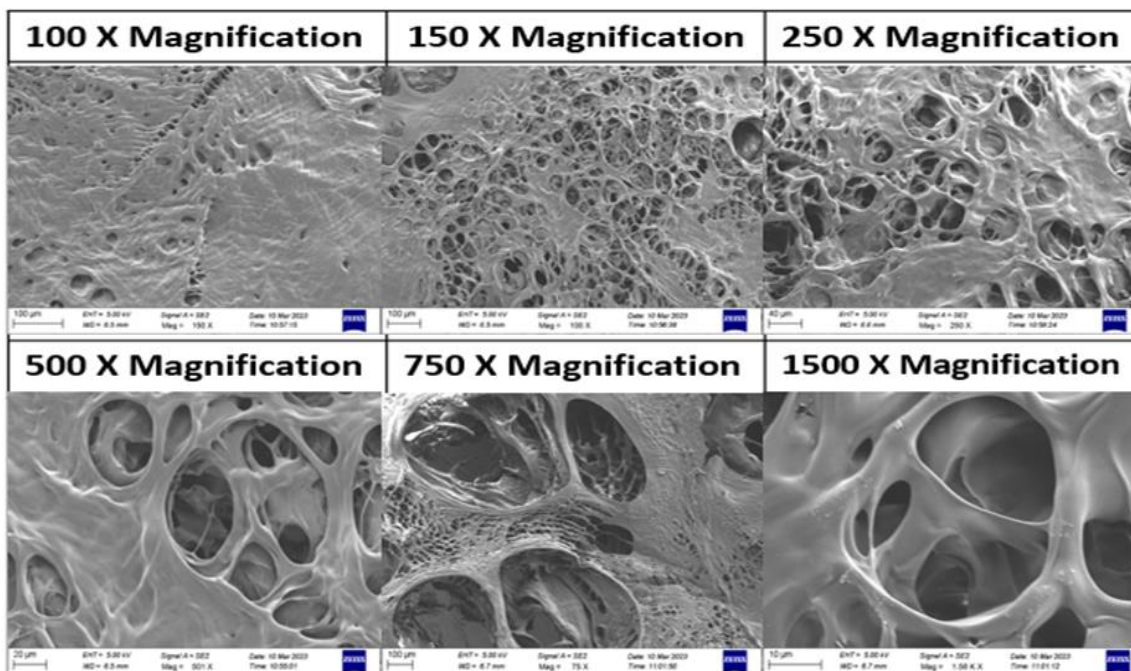


Figure 5: Scanning Electron Microscope (SEM) images of the synthesized RAJI-chitosan nanocomposite

preservation allows the scaffold to be utilized in various of biomedical applications.

#### Physicochemical Characterization of the Synthesized Nanocomposite

##### Ultraviolet-Visible Spectroscopy (UV-vis)

The lyophilized Chitosan-RAJI nanocomposite was subjected to UV-Vis spectroscopic analysis. The absorption peaks obtained conclusively confirmed the existence of both chitosan, RAJI and the RAJI-loaded chitosan nanocomposite in their stable forms as depicted in figure 2. The presence of specific absorption peaks of chitosan and RAJI in the spectrum analysis suggests that the drug has been well integrated and stabilized within the chitosan matrix. The investigation not only confirmed the synthesis of nanocomposite but also demonstrated its chemical stability and the retention of functional groups after lyophilization.

##### Fourier Transform Infrared Spectroscopy (FTIR)

The chemical interactions and functional features of chitosan-based nanocomposites are dependent on the presence of certain amide groups, namely Amide B, Amide II, and Amide III. The presence of these amide groups may be detected by distinct peaks in spectroscopic investigations, especially through Fourier Transform Infrared (FTIR) spectroscopy.

The FTIR spectra of RAJI, chitosan, and RAJI-loaded chitosan nanoparticles show a wide band at  $2899\text{ cm}^{-1}$ ,

which indicates the presence of the amide group in the chitosan spectrum, which is clearly displayed in figure 3. This discovery validates that the nanoparticles loaded with chitosan have maintained their functional integrity, hence keeping their distinctive amide group.

##### X-Ray Diffraction (XRD)

X-ray diffraction patterns provide evidence that the nanoparticles formed either a molecular or an amorphous dispersion inside the chitosan matrix. The X-ray diffraction (XRD) study of nanocomposites that comprised RAJI was able to confirm that RAJI was completely encapsulated within the chitosan matrix as shown in figure 4.

##### Scanning Electron Microscopy (SEM)

The scanning electron microscopy (SEM) pictures, taken at different levels of magnification, demonstrate the notable variation in both particle sizes and shapes present in the synthesized nanocomposite as represented in figure 5. The microscopic images at various magnifications demonstrate that the nanoparticles are distributed unevenly, showcasing the variances in shape, emphasizing the effective creation of the nanocomposite with a wide variety of structural features.

##### Assessment of Cell Viability using MTT Assay

The cytotoxic potential of RAJI and RAJI incorporated chitosan nanocomposite was studied in MDA-MB-231 (TNBC) cell line for 24 & 48 hours, in varying concentrations of RAJI and RAJI incorporated chitosan nanocomposite - 10 to 50  $\mu\text{g/ml}$  for RAJI and 100 to 500  $\mu\text{g/ml}$  for RAJI loaded chitosan nanocomposite. Untreated cells served as control and cells treated with Doxorubicin served as the standard for reference. The results suggest that the  $\text{IC}_{50}$  value of RAJI treatment in the cells was 20  $\mu\text{g/ml}$ , however, the  $\text{IC}_{50}$  value of RAJI incorporated chitosan nanocomposite was 400  $\mu\text{g/ml}$  as represented in figure 6.

##### Evaluation of Drug Loading Efficiency of RAJI-Loaded Chitosan Nanocomposite

The drug loading efficiency was calculated and correlated with the  $\text{IC}_{50}$  value determined using the cytotoxic MTT assay on MDA-MB-231 cells for both RAJI and the synthesized RAJI incorporated chitosan nanocomposite. The results suggest that the drug loading efficiency of RAJI in the nanocomposite was comparatively low as shown in figure 7. Furthermore, it was noted that the nanocomposite released a much less quantity of RAJI compared to the administration of RAJI alone in treating the MDA-MB-231 cells, resulting in reduced induction of cell death. This indicates that the nanocomposite has a regulated release profile and the ability to deliver drugs in a sustained manner.

## DISCUSSION

BC continues to be one of the most prevalent and difficult malignancies to treat, necessitating the development of innovative approach to enhance therapeutic outcomes. The biocompatibility, biodegradability, and versatile chemical properties of chitosan, a natural polymer derived from chitin, have garnered prominent attention in the development of nanocomposites for cancer therapy. Current research advancements have highlighted the potentiality of

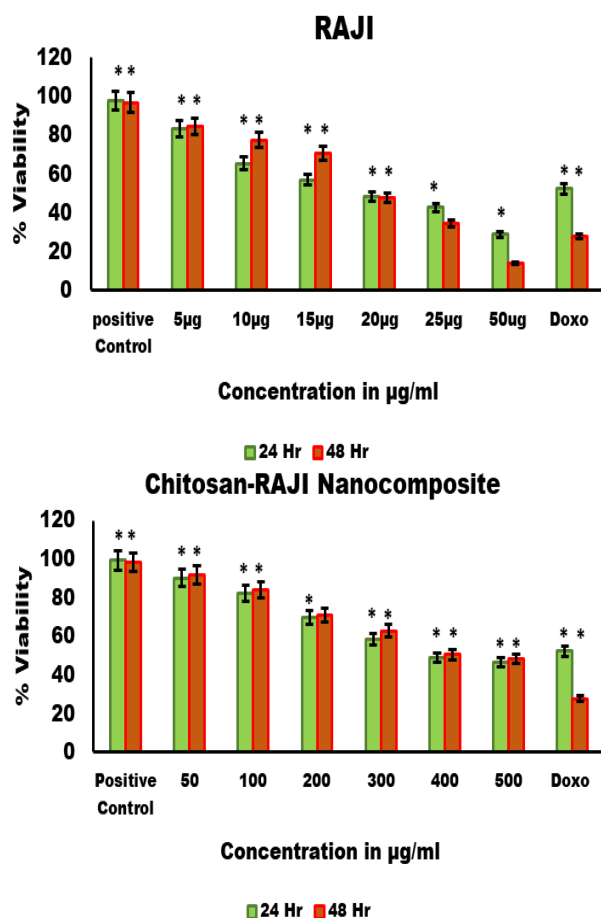


Figure 6: Cytotoxic potential of RAJI and RAJI incorporated chitosan nanocomposite in TNBC cell line MDA-MB-231

chitosan-based nanocomposites as a promising design in treating BC<sup>13</sup>.

Engineering chitosan nanocomposites to specifically target cancer cells can reduce systemic toxicity and improve therapeutic efficacy. Chitosan nanoparticles can selectively bind to receptors that are overexpressed on breast cancer cells by incorporating targeting ligands, such as antibodies or peptides, onto their surface. This targeted approach not only enhances the accumulation of the drug moiety at the tumour site but also reduces the adverse effects on healthy tissues<sup>18</sup>.

Additionally, the retention duration of the nanocomposites at the site of administration is increased by the muco-adhesive properties of chitosan, which improves drug absorption and efficacy. This is especially beneficial in the treatment of breast cancer, as localized delivery can be instrumental in preventing metastasis and reducing tumour growth<sup>19</sup>.

The employment of UV-visible spectroscopy was used in order to study the incorporation of RAJI into the synthesized nanocomposite. An absorbance peak in the vicinity of the 260 - 275 nm area may be associated with the usual  $\pi \rightarrow \pi^*$  transition of the pterin ring that is present in the synthesized nanocomposite, also indicating the appropriate incorporation of RAJI into the synthesized nanocomposite<sup>20</sup>.

The Amide B band is seen as a critical parameter for distinguishing N-H stretching vibrations. The stretching seen in chitosan is a resultant of the presence of unreacted free amino groups (NH<sub>2</sub>), which provide prospects for further chemical modification or interaction between other molecules. In our study, peaks specific for the presence of Amide B were observed around 2800 – 2900 cm<sup>-1</sup> which could be rationalized to the C-H stretching of the synthesized chitosan nanocomposite<sup>20</sup>. Amide II

predominantly originates from the bending vibrations of N-H bonds combined with the stretching vibrations of C-N bonds. The band is often seen in a wavelength range of 1400–1600 cm<sup>-1</sup> in the FTIR spectra<sup>21</sup>. The presence of the Amide II band in proteins indicates their secondary structure, and in the case of chitosan, it also reflects the degree of acetylation. In addition, the Fourier transform infrared (FTIR) spectra displayed distinct bands at 1400 and 1500 cm<sup>-1</sup>, which were affiliated with the stretching vibration of the carboxyl group (C=O) and the bending vibration of the CONH (N-H), therefore verifying the existence of Amide - II group. The Amide III region corresponds to the vibrations of C-N stretching and N-H bending. This band generally manifests between the range of 1000 cm<sup>-1</sup>. The Amide III band plays a pivotal role in comprehending the conformation and secondary structure of the chitosan polymer thereby describing the molecular interactions in chitosan, namely the hydrogen bonding between the amide groups and the polymer backbone<sup>20</sup>. In a similar manner, the absorption peaks occurring at 1000 cm<sup>-1</sup> observed in our study might be linked to the C-N vibration, which highlights the existence of Amide - III groups in the synthesized nanocomposite<sup>22</sup>.

Chitosan often shows a maximum value at an angle of  $2\theta = 20.20^\circ$ . However, our investigation revealed a marginal displacement towards an increased diffraction angle. The intensity proclaimed by the diffraction peaks of RAJI-chitosan nanocomposite proclaimed that they are also amorphous in nature emphasizing good integration of RAJI with chitosan. This highlights the successful integration of RAJI with chitosan. Therefore, it can be inferred that the interaction between RAJI and -NH<sub>3</sub><sup>+</sup> of chitosan molecules has led to the creation of a RAJI-chitosan nanocomposite, eventually resulting in the production of an amorphous RAJI-chitosan nanocomposite<sup>23</sup>.

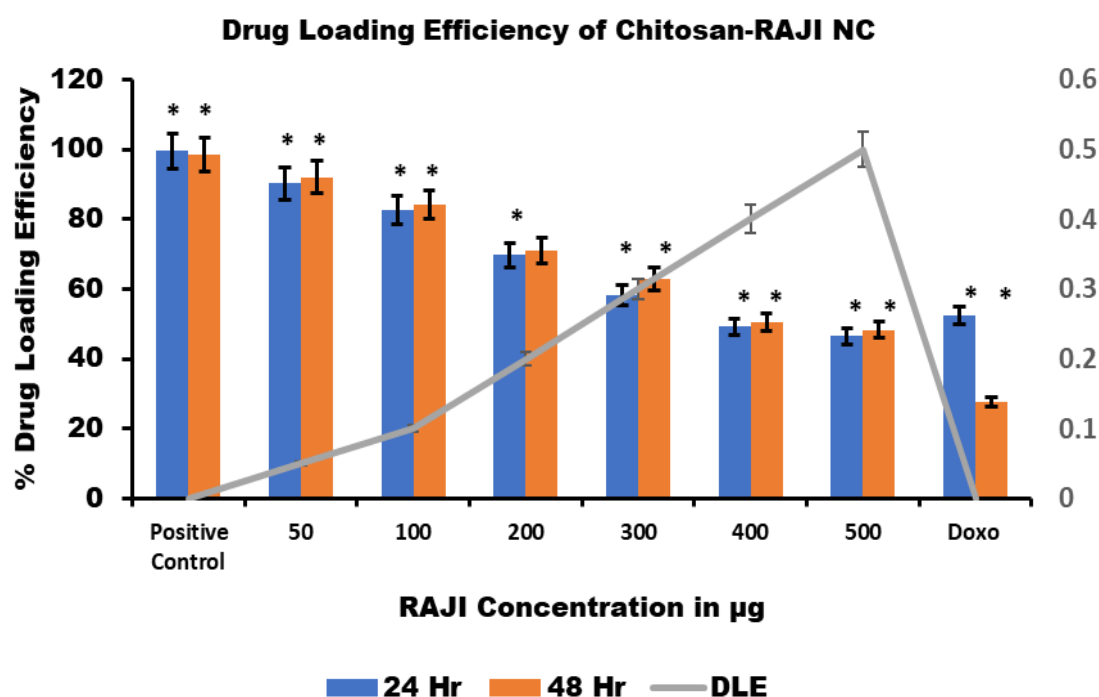


Figure 7: The drug loading efficiency of RAJI and RAJI incorporated chitosan nanocomposite in TNBC cell line MDA-MB-231

The SEM imaging of the synthesized RAJI-chitosan nanocomposite revealed the existence of particles of a variety of forms with good porosity. In addition, the image's lower magnification showed that the chitosan comprised particles that were uneven and sheet-like in shape highlighting greater porosity. Additionally, the surface of the chitosan particles was smooth, and was also lacking voids which can be attributed for better cell-cell interactions and efficient nutrient transportation which drug administration<sup>23</sup>.

RAJI and RAJI-incorporated chitosan nanocomposite were assessed for cytotoxic potential in TNBC cell line MDA-MB-231 for 24 and 48 hours at various concentrations (10-50 µg/ml for RAJI, 100-500 µg/ml for RAJI-loaded nanocomposite). The results indicate that both RAJI and RAJI-incorporated chitosan nanocomposite have strong cytotoxic effects on MDA-MB-231 cells. The nanocomposite formulation enables precise and prolonged delivery of RAJI, which has the potentiality to enhance the effectiveness of treatment and depreciate the adverse effects linked to large systemic dosages of free RAJI. Integrating RAJI in chitosan nanocomposites is a potential approach to provide targeted drug delivery and enhance the drug's bioavailability.

Hence, in conclusion, chitosan-based nanocomposites have the ability to encapsulate and release medications in a regulated manner, which makes them very promising materials for drug delivery. Nanocomposites may be effectively triggered to release drug moieties by certain factors such as pH, temperature, or magnetic fields. A chitosan-based system with Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles controls doxorubicin release. This technology delivers targeted drugs and allows bimodal cellular imaging<sup>24</sup>.

## CONCLUSION

The observations from this study indicate that the incorporation of RAJI with chitosan was successful, leading to the formation of a stable and competent nanocomposite. The stability of the produced nanocomposite was confirmed by evaluating its physicochemical characteristics using UV-Visible spectroscopy, FTIR, XRD, and SEM like techniques. Assessments of cytotoxicity on MDA-MB-231 (TNBC) cells demonstrated that RAJI, had significant toxicity even at low dosages when administered alone. However, when RAJI was incorporated with chitosan, it exhibited a controlled and prolonged release pattern. These findings suggest that using chitosan encapsulation is a successful approach to provide a gradual release of drugs, which might possibly minimize any negative effects on the surrounding healthy cells. It is also anticipated that incorporating RAJI might improve their effectiveness in treating triple-negative breast cancer as it might improve the therapeutic index and reduce systemic toxicity by targeting drug distribution. Also, RAJI's capacity to alter TNBC-related molecular pathways and chitosan's muco-adhesive qualities may increase RAJI's bioavailability and cellular absorption. This novel technique might thus combine targeted medication with real-time bioimaging to manage aggressive TNBC and

improve patient outcomes. Thus, the RAJI-chitosan nanocomposite is proposed to be a potential advancement in cancer treatment.

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