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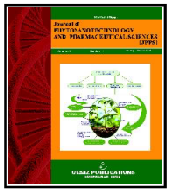

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Synthesis, characterization and anticancer activity of rutin loaded chitosan nanoparticles

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Abstract

Rutin is one of the well-known phytoconstituent with antioxidant property and also exhibiting anti-cancer potential. In this study, ionic gelation method is followed to prepare chitosan nanoparticles loaded with rutin (RCNPs). These nanoparticles are characterized by different techniques like fourier transform infrared spectroscopy (FTIR) for identifying functional groups, Zeta potential for assessing stability, X-ray diffraction spectroscopy (XRD) for determining structure, and scanning electron microscopy (SEM) for identifying surface morphology. FTIR spectra of RCNPs showed the presence of different functional groups of chitosan and also the peaks corresponding to functional group of rutin, indicating successful encapsulation of rutin in chitosan nanoparticles. Debye-Scherrer equation was applied for XRD data and RCNPs size was found to be 32.15 nm. SEM image revealed the porous and rough morphology of the surface of RCNPs. Zeta potential was found to be + 0.68 mV, indicating stability of nanoparticles. Entrapment efficiencies (EE %) was found to be 68.7%, Loading efficiencies (LE %) was found to be 50.8%, % yield of RCNPs was found to be 31.08%. Based on the above test values, RCNPs were further subjected to anti-proliferative activity by MTT assay on various cancer cell like HeLa (Cervical cancer cells), PC3 (Prostate cancer cells), MDAMB231 (Breast cancer cells), Panc-1 (Pancreatic cancer cells), SK0V3 (Ovarian cancer cells). The results showed that rutin and RCNPs have anti-proliferative effect on SK0V3 cell line only and effects of RCNPs were greater when compared to that of pure rutin. On this basis, we can suggest that rutin loaded chitosan nanoparticles have promising therapeutic role in cancer treatment.

1. Introduction

Globally, cancer is a second principal reason of fatality and includes 277 various types (Hassanpour and Dehghani, 2017). Cancer cells are characterized by abnormal growth and uncontrolled division (Balachandran and Govindarajan, 2005). Main treatment option is chemotherapy, surgery, radiotherapy, and hormone therapy (Shihabul *et al*., 2018). Alopecia, anemia, exhaustion, and nausea are the major side effects. Moreover, therapeutic application of above said options was also decreased due to high toxicity, high cost, and low efficacy (Shaûei *et al*., 2015). In this context, scientists all over the world are focusing on plant based therapies. Considering the significance of all phytoconstituents, in particular flavonoids play a vital role in management of life threatening diseases. A flavonoid 7-methyl gallic acid has a role in treatment of neurodegenerative related disorders (Pugazhendhi *et al*., 2018). Other flavonoids such as rutin, vinca alkaloids, etoposides, *etc*., are used in the treatment and management of cancer, as compounds derived from plants are more tolerant, non-toxic to normal human cells (Mariyappan *et al*., 2018). Majority of flavonoids are polar compounds and water soluble in nature which limits their absorption. Two are the important factors which decrease their bioavailability, first factor is molecular size which blocks their passive absorption and second factor is weak lipid solubility which restricts their entry through phospholipid layer. Hence, there is a challenge to increase bioavailability of these flavonoids, in order to increase their therapeutic efficacy (Khan *et al*., 2013; Li *et al*., 2015; Sepahvand *et al*., 2014).

Rutin is also known as quercetin-3-rutinoside (Mauludin *et al*., 2009) which is commonly found in tea, passion flower, apple, and buckwheat (Harborne, 1986). It is reported with many pharmacological activities, *viz*., cardioprotective (Annapurna *et al*., 2009), neuroprotective (Pu *et al*., 2007), anticonvulsant activity (Nieoczym *et al*., 2014), antidepressant effects (Machado *et al*., 2008), analgesic (Rylski *et al*., 1979), antinociceptive effects (Selvaraj *et al*., 2014), and anticancer effects (Alonso-Castro *et al*., 2013). It is reported to possess anticancer potential against HL-60 human leukemia cells (Lin *et al*., 2012), LAN-5 human neuroblastoma cells (Chen *et al*., 2013), colorectal cell (Araujo *et al*., 2011), OVCA 433 ovarian cancer cells (Scambia *et al*., 1990) and pancreatic cancer cells (Mouria *et al*., 2002).

Taking in to consideration of different pharmacological activities of rutin and its action profile against cancer cells many studies are conducted to increase its clinical use by increasing its bioavailability (Imad Uddin *et al*., 2020). Rutin loaded nanophytosomes was prepared with better antioxidant potential and bioavailability (Hooresfand *et al*., 2015). Targeted delivery to brain for treating

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cerebral ischemia was reported with rutin loaded chitosan nanoparticles (Ahmad *et al*., 2016). Chitosan nano formulations reported a key role in increasing bioavailability of many plant derived drugs. This is a polymer obtained by the de-acetylation (Ravi Kumar, 2001) of chitin which is a fibrous substance present in crab's exoskeleton, scales of fish, and cell walls of fungi (Shu and Zhu, 2002). Majority of biomedical uses of this polymer is due to its non-toxic, biodegradable, mucoadhesive, and biocompatible applications (Shanmuganathan *et al*., 2019). Moreover, it has the ability to attach to mucosal layer and fleetingly opens epithelial tight junctions (Qian *et al*., 2006). Based on above discussion, present work was designed to encapsulate rutin in chitosan to increase its therapeutic efficacy by improving its bioavailability.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and cell lines

Sodium tripolyphosphate (STTP) (Qualikems Fine Chemical Limited), ethanol, rutin (NR Chemicals). Chitosan with MW= 68 kDa and deacetylaton degree = 90% From Chemsworth Suppliers Pvt. Ltd., acetic acid, sodium hydroxide, DMSO (S.D. Fine Chemical Limited). HeLa, MDAMB231, PC3, SKOV3, Panc-1cell lines, Roswell Park Memorial Institute (RPMI), 3-(4,5-dimethyl-2-yl)-2,5-diphynyltetrazoliumbromide (MTT), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) are obtained from American type culture collection, United States. Sodium pyruvate, bovine insulin is procured from Sigma Aldrich.

2.1.2 Equipments and Instruments

Sensitive weighing balance (Citizen scale-CY220; India), pH Meter (Digisun Electronics-India), Sonicator (Citizen scale-CUD25; India), Magnetic stirrer (REMI-1MLH; India), Rapid cooling centrifuge (Remilabworld-CM12; India), Refrigerator (Whirlpool-DC20045; India), Hot air oven (Lab care-TC344; India), UV spectrophotometer (LabIndia-T60; India), Multimode plate reader (Perkin Elmer-HH35000500; USA). FTIR (BRUKER-ALPHA; USA), XRD (SCHIMADZU-XRD7000; Japan), Zeta potential (MALVERN-MAL1004428; United Kingdom), SEM (Hitachi-3000N; Japan).

2.2 Methods

2.2.1 Synthesis of RCNP's

1 mg/ml chitosan solution of pH 5.0 (adjusted with NaOH (0.1 M conc)) was prepared by using 1% CH_3COOH. 1 mg/ml STTP solution of pH 5.0 was prepared by using double distilled water (DDW). 17.5 ml of chitosan solution containing rutin (4 mg/ml) was kept in a volumetric flask on a magnetic stirrer with a speed of 200 rpm at room temp. To this solution, 9 ml of above made STTP solution was added drop-by-drop. Above solution is kept on overnight stirring for the formation of nanoparticles. After 24 h, solution is subjected for centrifugation at 8000 rpm at 4°C for 20 min. RCNPs are deposited at the base of centrifugation tube as a pellet which was washed thrice with 10% aqueous ethanol. Finally, pellet was stored at 4^0C after resuspending in 10% aqueous ethanol (Patil *et al*., 2010).

2.2.2 EE %, LE % and % yield of RCNP's

3 mg of synthesized RCNP's was dissolved in 10% DDW and sonicated for 10 min at amplitude of 40%. Above solution is centrifuged for 15 min at a speed of 3000 rpm and at a temp. of 4°C. Amount of rutin released from RCNPs in supernatant was estimated by measuring absorption at 354 nm by using UV visible spectroscope. Standard curve of pure rutin was obtained by taking absorbance of different concentrations of rutin, *viz*., 100, 200, 300, 400 and 500 µg/ml at 354 nm by using UV visible spectroscope (Anitha *et al*., 2011).

$$EE\% = \left(\frac{\text{Total amount of rutin with in the pellet}}{\text{Intial amount of rutin for loading studies}}\right) \times 100$$

$$LE\% = \left(\frac{\text{Total amount of rutin trapped with in the pellet}}{\text{Yield of RCNP's}}\right) \times 100$$

$$\%Yield = (W1/W2)\ 100$$

where W1= Dried wt. of RCNPs; W2= Wt. of Rutin + Wt. of STTP + Wt. of chitosan. (Sharma and Garg, 2010)

2.2.3 Fourier transform infrared spectroscopy of RCNP's

RCNP's are analyzed from 4000 to 500 cm^{-1} in FTIR spectroscopy. For FTIR mediated assessment, obtained RCNP's are diluted in DDW. To remove unbound moieties, these are centrifuged for 15 min at the speed of 5000 rpm. After discarding supernatant, pellet is suspended in DDW. To obtain pure product, above discussed procedure is repeated for 3 times. Finally to obtain a pure form of RCNP's, pellet is dried in hot air oven (60°C). This is subjected to analysis (Kiran *et al*., 2010).

2.2.4 X-Ray diffraction mediated assessment of RCNPs

Crystallographic structural pattern of RCNPs was determined by using XRD. Instrument is maintained at 31 mA and 42 kV voltage. 2-Theta scale was set from 2.0000° to 50.0014°, Step=0.0053°, step time = 13.93 with Anode Cu, wavelength- 1.5406 (Thomas *et al*., 2009). Size of RCNP's was measured by applying the Debye-Scherrer formula (Wang, 2000).

$$D = \frac{k\lambda}{\beta\cos\theta}$$

where, D = Average size of RCNP's , γ = x-ray wavelength (1.5406), θ = Bragg's angle in degrees, β = Full width at half the maximum (FWHM), k = constant (0.94).

2.2.5 Scanning electron microscopic analysis of RCNP's

SEM is used to analyze morphology, size and shape of RCNP's. For preparation of sample, copper grid coated with copper is used. Small quantity of RCNP's was added to this grid. Extra solution was removed by using blotting paper. Finally, grid was subjected to analysis (Imad Uddin *et al*., 2018).

2.2.6 Zeta potential measurement of RCNP's

Stability of RCNP's was measured by using a common light scattering method. Whole experiment was conducted at room temperature. Analysis was carried out by using zeta potential analyzer (Dodane and Vilivalam, 1998).

2.2.7 Anti-proliferative activity of RCNPs by MTT Assay

Dulbecco's modified Eagle's medium and 5% CO_2 was used to grow four different cell lines, *viz*., MDAMB231, SKOV3, Panc-1 and HeLa. 10% FBS is also added to DMEM. Experiment was conducted at 37°C. RPMI medium containing non-essential amino acids, 10% FBS, 10 mg/ml bovine insulin and 1 mM sodium pyruvate was used to grow PC3 cells.

100 µl aliquots of trypsinized cells were prepared. Prepared aliquots were added to a 96-well microtiter plates. These plates were incubated for 24 h in atmospheric conditions like 100% relative humidity, 5% CO_2, temp. of 37°C and 95% of air. After 24 h, rutin and RCNPs was added to each cell line at a conc. of 50 µM and was incubated for more 48 h. 10 µl of MTT (5%) was added to stop the reaction. This is incuba-ted for 60 min at 37°C. Air dried plates are eluted with DMSO (100 µl) and used to record absorbance at 560 nm on a multimode plate reader (Imad Uddin *et al.*, 2019).

3. Results

3.1 Characterization of RCNPs

Standard curve of rutin was obtained and R^2 value was found to be 0.999 (Figure 1). For screening nanocarrier property of chitosan EE% and LE % are estimated which were found to be 68.7% and 50.8%, respectively. FTIR analysis of chitosan nanoparticles are shown in Figure 2. –OH stretching vibration is indicated by peak at 3273.96 cm^{-1}, 1635.50 cm^{-1} indicates interaction between STTP and chitosan, 1539 cm^{-1} indicates –NH bending vibration of primary amine, C-O stretching is represented by peak at 1152.65 cm^{-1}, 1070.51 cm^{-1} and 1023.50 cm^{-1} indicates -CO vibrations. In case of RCNPs (Figure 3), 3341 cm^{-1} and 1650.50 cm^{-1} indicates –OH stretching vibration and C=O stretching vibrations, respectively. C-O-C vibrations, P=O vibrations and C=C vibrations are indicated by peaks at 1060 cm^{-1}, 1203 cm^{-1}, and 1454 cm^{-1}, respectively.

Physical nature of RCNPs was assessed by XRD technique and XRD-diffractogram is presented in Figure 4. 2θ peaks of RCNPs were found to be from 5.210 to 46.150 with intensity (cps) from 44.9 to 8.7. SEM image (Figure 5), revealed rough and porous surface of RCNPs. Stability of nano formulations is estimated by measuring potential on either surface or interface of particles. Irrespective of the charge particles with higher zeta potential are stable but the particles with lower value are unstable and are prone to flocculate. Zeta potential of RCNP (Figure 6) was found to be +0.68 mV which indicates low stability.

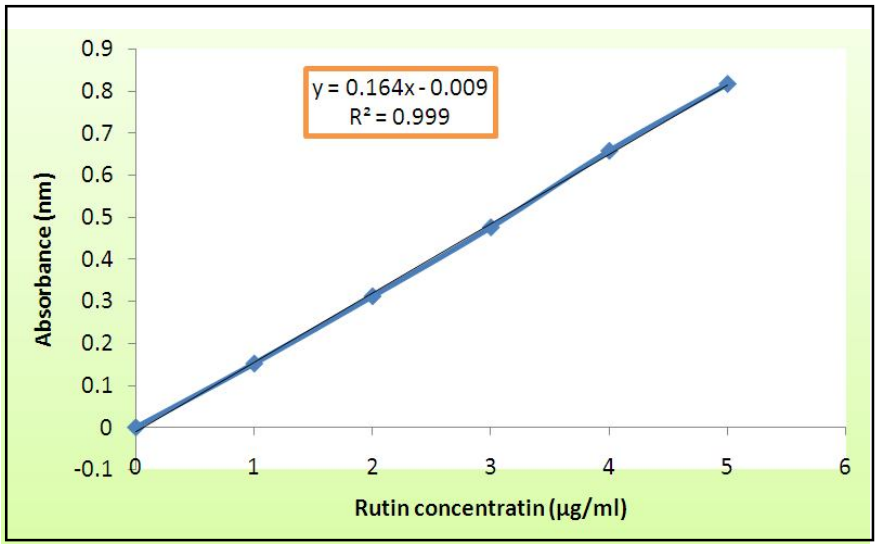

Figure 1: Standard curve of pure rutin.

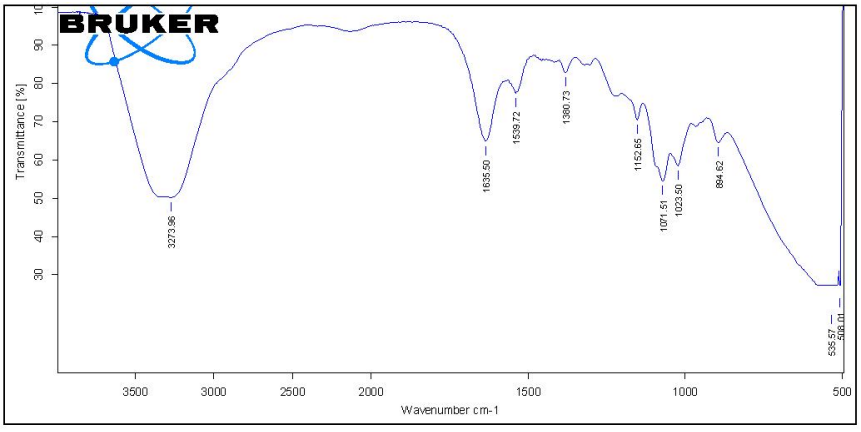

Figure 2: FTIR analysis of chitosan nanoparticle.

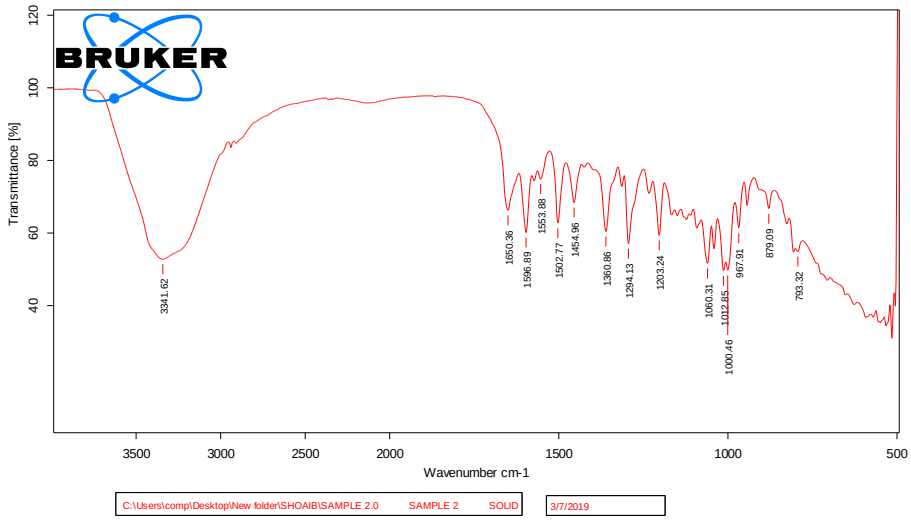

Figure 3: FTIR analysis of RCNP's.

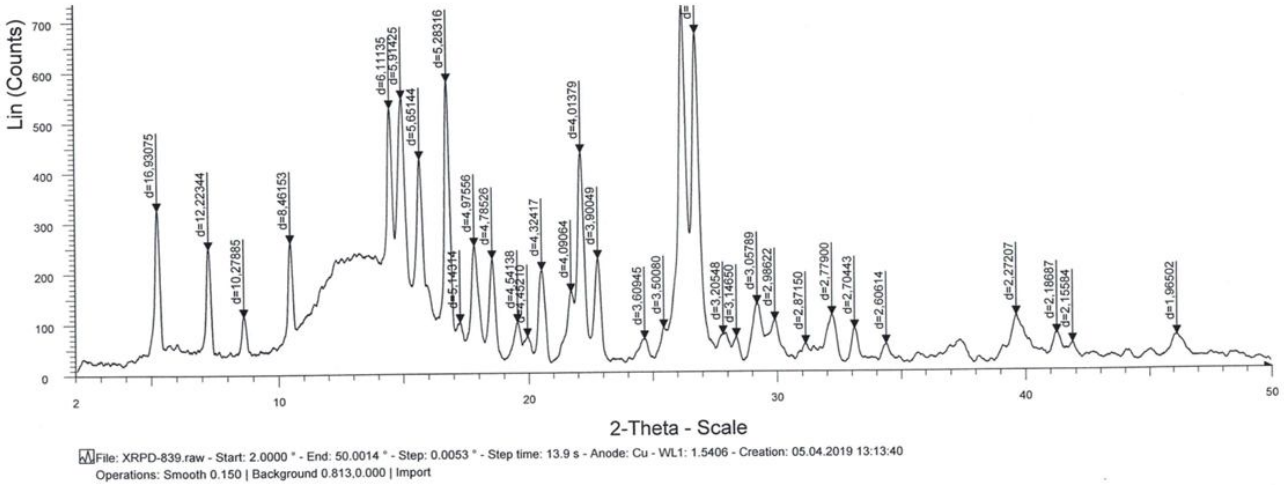

Figure 4: XRD pattern of RCNPs.

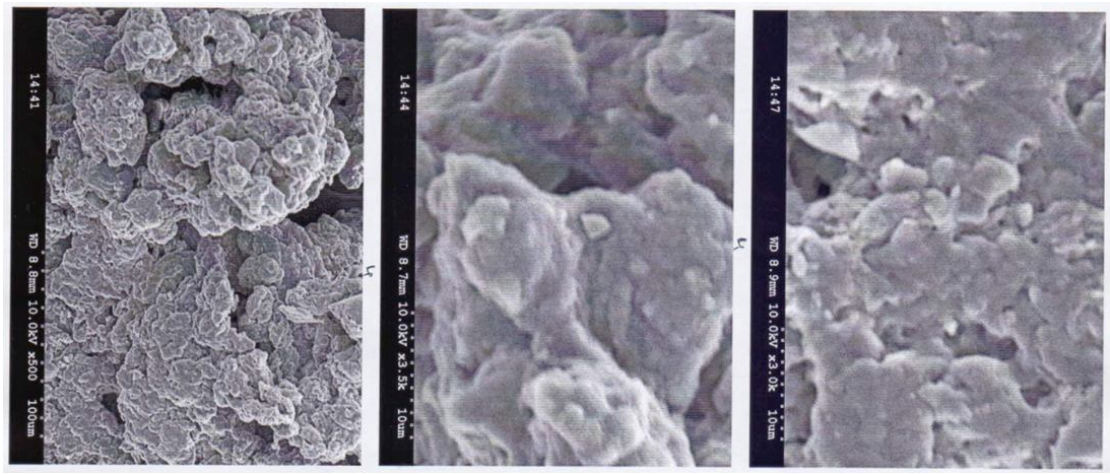

Figure 5: SEM image of RCNPs.

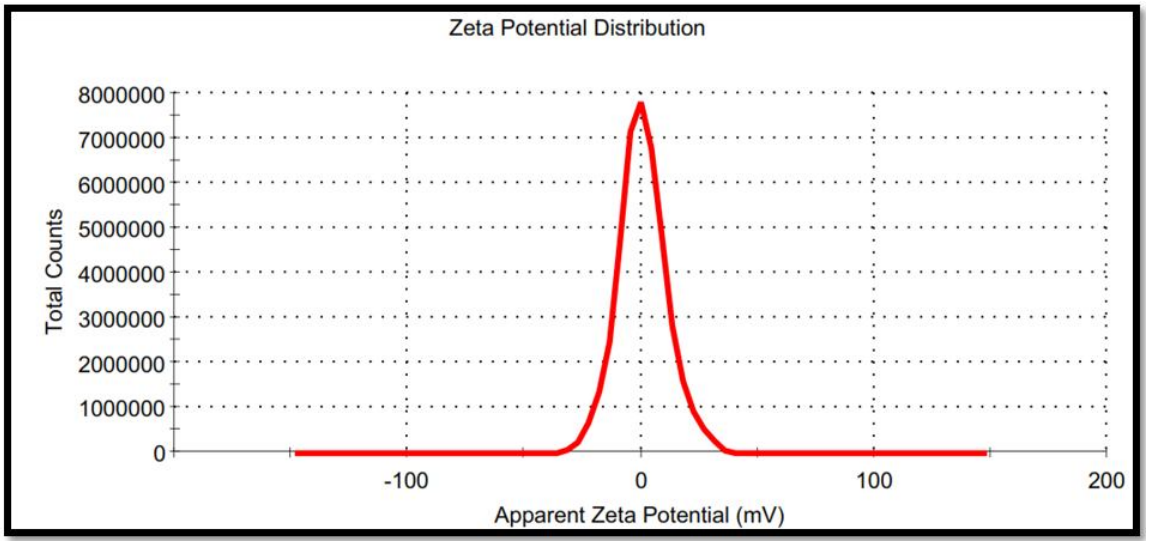

Figure 6: Zeta potential of RCNPs.

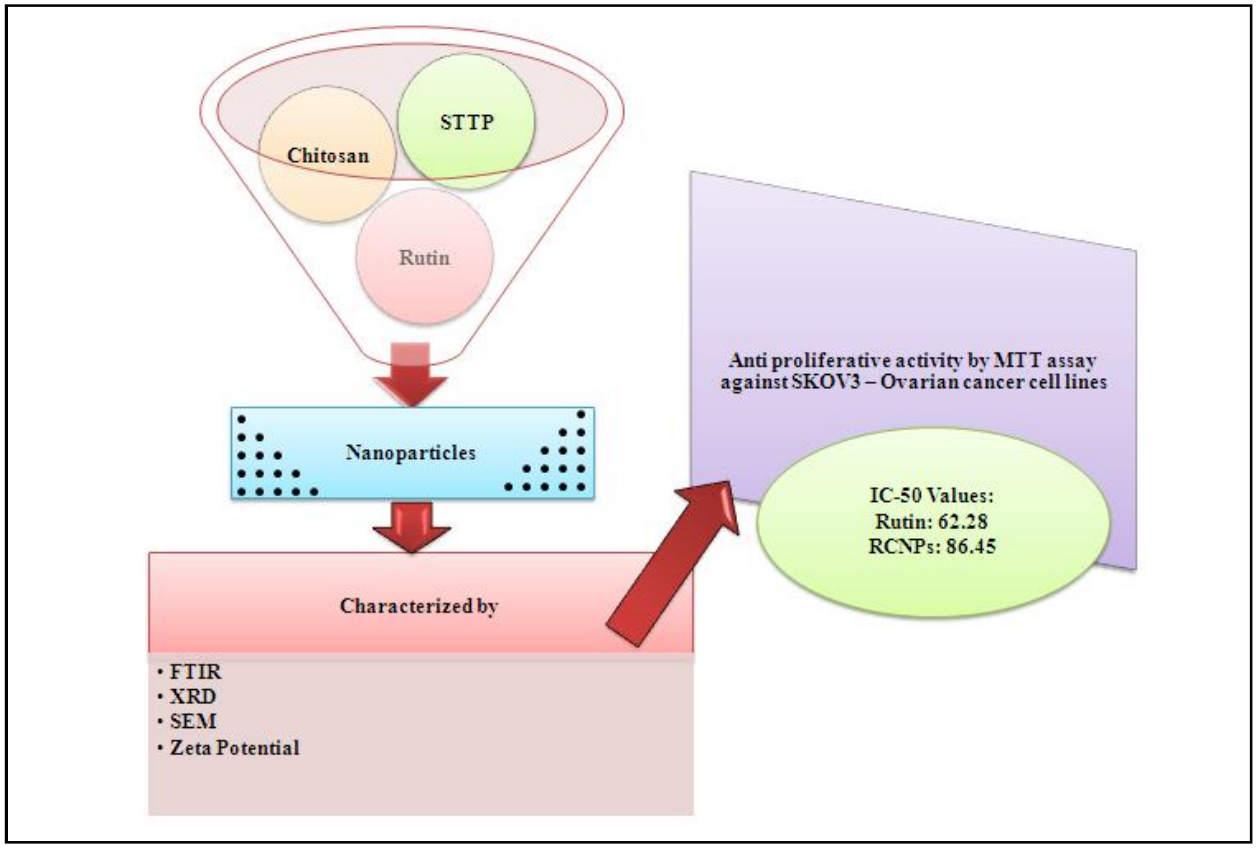

Figure 7: Graphical abstract explaining synthesis, characterization and antiproliferative activity of rutin and RCNPs.

Table 1: Antiproliferative activity of pure rutin and RCNPs

Compounds	HeLa	MDAMB231	Panc^{-1}	PC3	SKOV3
Pure rutin (50 µM)	--	--	--	--	62.28 ± 19.42
RCNP (50 µM)	--	--	--	--	86.45 ± 19.46

Antiproliferative activity of pure rutin and RCNPs by MTT assay. All values are expressed as Mean ± SEM, NA=Not active

3.2 Antiproliferative activity of RCNPs by MTT assay

The IC_{50} values against SK0V3 cell lines for rutin and RCNPs was found to be 62.28 ± 19.42 and 86.45 ± 19.46, respectively (Table 1). Thus, efficacy of rutin and RCNPs against ovarian cancer cell line is reported for the first time. However, in our study, both rutin and RCNPs have not showed any activity against HeLa, MDA – MB-231, Panc^{-1} and PC3. Graphical abstract explaining synthesis, characterization and antiproliferative activity of rutin and RCNPs is presented in Figure 7.

4. Discussion

RCNPs were prepared by adding rutin to the chitosan solution. To this, chitosan solution STPP was added. After overnight stirring, above mixture is subjected to centrifugation at 8000 rpm, then the nanoparticles are separated by removing the supernatant and then washed with ethanol and stored for further studies. EE% can be increased by optimizing the quantities of chitosan, drug and also by optimizing temperature, rotating speed, and pH. LE% is the actual amount of drug loaded in RCNPs. % yield of RCNPs was found to be 31.08 %. These results are similar to other studies, *viz*., Patil and Jobanputra (2015) got 36.72% of % yield and 32.24% of EE% when they prepared rutin loaded chitosan nanoparticles. % yield was less but EE% was far better in our study. Moreover, both efficiencies and % yield can be increased by optimizing the ratio between chitosan and STTP.

In FTIR peaks of chitosan nanoparticles 1635.50 cm^{-1} indicates interaction between STTP and chitosan, 1539cm^{-1} indicates –NH bending vibration of primary amine. In case of RCNPs (Figure 3), 3341 cm^{-1} and 1650.50 cm^{-1} indicates –OH stretching vibration and C=O stretching vibrations, respectively. 1454 cm^{-1}, 1203 cm^{-1}, and 1060 cm^{-1} indicates C=C vibrations, P=O vibrations and C-O-C vibrations of rutin, respectively. This confirms loading of rutin in chitosan nanoparticles. 1596 cm^{-1} indicates –NH bending vibration of primary amine of chitosan. Our results are in conformity with the study conducted by Patil and Jobanputra (2015) where they reported –OH stretching vibration is due to a broad peak at 3498 cm^{-1}, interaction between STTP and chitosan is due to a peak at 1637 cm^{-1}, confirmation of loading rutin is indicated by C-O-C vibrations of rutin which showed a peak at 1064 cm^{-1} (Ashwini *et al.*, 2015). Diffractogram showed many XRD peaks below 30^0 which indicates semi crystalline nature of chitosan. Size of RCNPs was found to be 22.31 nm to 42.32 nm with an average size of 32.15 nm by using Debye-Scherrer's equation. Similar results were obtained by Khan *et al.* (2016).

According to SEM study, porous morphology of RCNPs is reported. This facilitates in swelling and rapid release of drug. Surface morphology depicted in our study is in accordance with SEM results of Cahyono *et al.* (2017). Low cross linking densities of nanoparticles is credited due to porous, rough, loose and open surface morphology (Bhumkar and Pokharkar, 2006). Results of zeta potential measurement showed reduced stability of rutin loaded chitosan nanoparticles. This is due to various factors such as types of biopolymers and their quantity, ionic strength, and pH of the solution. Value near to zero may be due to –vely charged groups of rutin (Ashwini *et al.*, 2015). RCNPs are effective against SKOV3 cells. Rutin is a citrus flavonoid found in many plants. Long term and high intake of flavonoids will decrease the incidence of ovarian cancer (Evans, 2011). Results indicated that RCNPs are more effective than rutin. This increased activity of rutin loaded chitosan nanoparticles is due to targeted delivery of nanoparticles. Moreover, both pure rutin and RCNPs are not active against other cell lines, *viz*., HeLa, MDAMB231, Panc^{-1} and PC3.

5. Conclusion

Current investigation portrays easy method for incorporation of insoluble phytoconstituent rutin in chitosan nanoparticles. Synthesized RCNPs are stable and water soluble. Moreover, successful encapsulation of rutin in nanoparticles improves bioavailability of rutin and their by increasing its therapeutic effect. Obtained results more prominently authenticate the effectiveness of rutin loaded chitosan nanoparticles; this may be attributed to increased antiproliferative action, intracellular accumulation and cellular uptake. Thus, chitosan loaded rutin nanoparticles provide a proficient tool for encapsulation and delivery of rutin by increasing its stability and water solubility for making it an efficient treatment option for cancer. Further studies should be carried out to screen anticancer potential by using higher conc. of RCNPs against other cell lines (MDAMB231, Panc-1, HeLa and PC3). Study also directs the conduct of *in vivo* studies of RCNPs against ovarian cancer.

Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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