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Research Article

FORMULATION, CHARACTERIZATION AND ANTI-CANCER ACTIVITY OF NOVEL BENZIMIDAZOLE DERIVATIVE NANOPARTICLES

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ABSTRACT

In an effort to establish novel benzimidazole derivative with improved anticancer activity, we report here the formulate novel benzimidazole derivative (4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine) loaded sustained release nanoparticles with the size of around 250 nm and to increase the encapsulation efficiency of the drug. The nanoparticles were prepared by simple ionic gelation method using various concentrations of chitosan and TPP Triphenyl phosphate (TPP) Solution. The prepared nanoparticles were evaluated for particle size, shape, charge, encapsulation efficiency, *in vitro* drug release and *in vitro* cytotoxicity. The optimized synthesised benzimidazole derivatives loaded nanoparticle showed size of 220±4 nm with PDI polydispersity index of 0.11 ±0.02, Zeta potential of +5.55 ±1mv, encapsulation efficiency of 69.2% and the drug release is 98.3% at 24 hrs. These results demonstrate that the possibility of delivering synthesised novel benzimidazole derivative 4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine to colorectum with enhanced encapsulation efficiency.

Keywords: Benzimidazole, Nanoparticles, Chitosan, CaCo-2, MCF-7.

1. INTRODUCTON

Benzimidazole derivatives are comprises a relatively huge, growing, key pharmacophore and privileged structure in modern drug discovery.[1] Nowadays is a moiety of choice which has numerous pharmacological properties. Substituted benzimidazole derivatives have found applications in diverse therapeutical areas.[2] Principally 2-substituted benzimidazole derivatives have been found to possess potential anticancer properties.[3]

Targeting of drugs specifically to colon is advantageous for the treatment of diseases associated with the colon such as Amoebiasis, Crohn's diseases,Ulcerative colitis and colorectal cancer. A drug delivery system is most often associated with particulate carriers such as emulsion, liposomes and nanoparticles which are designed to localize drugs at the target site. The efficacy of present cancer chemotherapy is mainly limited by the toxicity associated with the anticancer drugs to normal tissues. This limitations result from the fact that anticancer drugs presently used in chemotherapy lack efficient selectivity towards tumor cells. This necessitates the development of a novel nanoparticle delivery system to overcome these current obstacles in convention drug therapy. Nanoparticles due to their small size and target exact localization property offer several advantages compared to conventional dosage forms which includes reduced dose improved efficiency, reduced toxicity, patient compliance and convenience.[4]

Chitosan is a natural hydrophilic polysaccharide copolymer of glucosamine and N-acetyl glycosamine. It is considered as a safe excipient due to its biocompatibility, biodegradability and lack of toxicity, moreover it is cationic in nature and posses

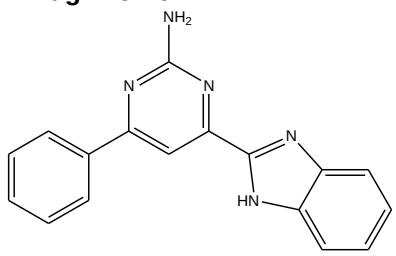
mucoadhesive property it will enhance the cellular uptake by ionic interaction[5,6].

The present study was aimed at the formulation and characterization of novel benzimidazole derivative (4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine) loaded chitosan nanoparticles additionally the nanoparticles have been evaluated for cytotoxicity in Caco2 and MCF- 7 cell lines, to overcome the above said obstacles for better therapy of colorectal cancer.

2. MATERIALS AND METHODS

The novel benzimidazole derivative (4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine) was a syntheised and characterized by RVS college of Pharmaceutical Sciences, Sulur, Tamilnadu, India. Chitosan was purchased from sigma Aldrich USA, Glacial acetic acid was obtained from Fischer scientific, Dialysis membrane with molecular weight cut off 12000-14000 Daltons was purchased from HIMEDIA laboratories, Mumbai.

2.1 Drug Profile[7]



4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine

Molecular Formula: $C_{17}H_{13}N_5$
Molecular Weight: 287.32
Mp: 146 – 149 ^{0}C
R_f : 0.75

The 2- substituted novel benzimidazole derivative were synthesised by using incorporated with heterocycles like pyrimidine and backbone of chalcones. The synthesised compound were screened for their *in vitro* anticancer activities against the CaCo-2 and MCF-7 cell lines. The 24 hours Caspase study revealed that compound 4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine showed 2 fold activities in caspase 3 and 9 pathway, single fold activity in caspase 8 pathways. These results revealed that the presence 2-substituted benzimidazole derivative could have the anticancer potential of the scaffold.

2.2 Preparation of novel benzimidazole derivative [4-(1H-benzo[d] imidazol-2-yl)-6-phenylpyrimidin-2-amine)] loaded chitosan nanoaprticles (BZI nano)[8]

The novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine] loaded chitosan nanoparticles were prepared using ionic gelation method. Determinate weight of chitosan was dissolved in glacial acetic acid 1% [v/v], 5mg of synthesized novel benzimidazole derivatives [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine] was added to the above solution and under constant magnetic stirring followed by addition of aqueous Triphenyl phosphate (TPP) solution in a drop wise manner, then the solution was kept on constant magnetic stirring for 30 mins and sonicator [vibrasonics]. The nanoparticle suspension was centrifuged at 13,000 rpm and 4^{0}C for 30 minutes using Eppendr of Ultracentrifuge to remove excessive amounts of TPP and unencapsulated novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine]. The pellets were dispersed in deionised water. Finally, nanoparticles were lyophilized for 24 hrs using freeze dryer [lyodel] for storage in powdered form. Optimization of benzimidazole derivative Nanoparticles of (CS-NP) on the basis of CS/ TPP ratio done.

2.3 Conditions for formation synthesised novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine] loaded chitosan nanoparticles (BZI nano)[9]

Chitosan Nanoparticles were prepared by simple scale up ionotropic gelation method similar to the method developed. Chitosan is a cationic polyelectrolyte the nanoparticles were formed by inducing the gelation by controlling its interaction with polyanion TPP which leads to reduce the aqueous solubility of CS this system based on inter and intramolecular linkages created between TPP and positive charge of charged amino groups of CS which are responsible for the successful formation of the nanoparticles. The particle size, PDI, drug encapsulation and zeta potential were analyzed.

2.3.1 Effect of Chitosan Concentration

The role of chitosan concentration (0.2, 0.4 and 0.6%) on formation of nanoparticles and its influence on particle size was evaluated.

2.3.2 Effect of Triphenyl phosphate (TPP) concentration

The role of TPP (0.2, 0.4 and 0.6%) concentration on particle size formation was studied. The sonication time in the formation of CS-NP played a crucial role in the formation of smaller size nanoparticles. Effect of sonification on particle size was studied.

2.4 Physicochemical characterization of nanoparticles[10]

2.4.1 Particle size and Zeta potential using photon correlation Spectroscopy

The average hydrodynamic diameter and polydispersity index (PDI) of the formulated nanoparticles were determined by dynamic light scattering (DLS) analysis using Zetasizer Nano ZS90 (Malvern Instruments limited, UK) 1ml of sample of nanoparticles dispersion was placed in disposable cuvettes for particle size measurements. Each experiment was conducted in triplicate. The electrophoretic mobility (zeta potential) measurements were made using the Malvern Zetasizer (Nano ZS90, Malvern Instruments) at 25ºC. Samples were diluted with double distilled water.

2.4.2 Transmission electron microscopy TEM images of synthesised novel benzimidazole derivative (BZI nano)

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through it. The surface morphology of the prepared NPs was determined for by using transmission electron microscopy (HRTEM). A drop of Nanosuspension was placed on a carbon film coated copper grid for TEM. Studies were performed at 80 kv using JOEL JEM 2100.The copper grip was fixed in to sample holder and placed in a vacuum chamber of the transmission electron microscope and observed under low vacuum and TEM images were recorded.

2.4.3 Encapsulation efficiency

Nanoparticles were separated from aqueous phase by ultracentrifugation (Eppendr of) at 13000 rpm and 4°C for 45 minutes. The supernatants were collected and evaluated for novel benzimidazole derivative residues by UV. The encapsulation efficiency (EE) was determined indirectly by measurement of the amount of free novel benzimidazole derivative in the supernatant after ultracentrifugation and was calculated according to the following equation:

$$EE = \frac{\text{Amount of total drug - Amount of free drug in supernatant}}{\text{Amount of total drug}} \times 100$$

2.4.4 *In vitro* Release[11]

A modified dialysis method was used to evaluate the *invitro* release of novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine] loaded chitosan NPs. Two milliliters of nanoparticles suspension (corresponding to 2 mg of novel benzimidazole derivatives) was placed in a dialysis bag (cellophane membrane, molecular weight cut off 10,000–12,000, Hi-Media, India) which was tied and placed into 20 ml of phosphate buffer (0.1M, pH 7.4) maintained at 37ºC with continuous magnetic stirring.At selected time intervals, aliquots were withdrawn from the release medium and replaced with the same amount of phosphate buffer. The sample was assayed spectrophotometrically for novel benzimidazole derivative at 235 nm.

2.5 *In vitro* cytotoxicity of nanoparticles[12]

2.5.1 Cell culture

MCF-7 (Human breast cancer cell line) and CaCo-2 (Human colon cancer cell line) were obtained from NCCS Pune.

2.5.2 Drug Preparation

The synthesized novel benzimidazole derivative (BZI 3 nano) were dissolved in DMSO to give a stock concentration of 50µg/µl. The stock solution was then serially diluted with culture medium. The test concentration were 0.1,0.3,1,3,10,30 µM. The concentration of DMSO never exceeded 1% in any of the 96 wells.

2.5.3 MTT assay

Cell lines were maintained in RPMI supplemented with 10% FBS, 2mM glutamine, amphotericin (3 µg/ml), gentamycin (400 µg/ml), streptomycin (250 µg/ml) and penicillin (250 units/ml) in a carbon dioxide incubator at 5% CO_2. Approximately 2 X 10^4 cells/well were seeded in 96 well plate using culture medium, the viability was tested using trypan blue dye with help of haemocytometer and 95% of viability was confirmed. After 24hrs, the fresh medium with the extracts were added at respective wells and kept incubation for 72 hrs. After incubation the following assays were performed. After 72hrs of the drug treatment the fresh medium was changed again for all groups and 10 µl of MTT (5 mg/ml stock

solution) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100µl of DMSO. The optical density was measured at 570nm. The percentage toxicity was calculated by using following formula. Graph pad prism software was used to calculated IC_{50} of the extracts.

% Toxicity =
1- treated cells/untreated cells x 100

3. RESULTS AND DISCUSSION

3.1 Preparation of novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine] loaded chitosan nanoaprticles (BZI nano)

Synthesised novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine (4a)] loaded chitosan nanoparticles were prepared using ionic gelation method. Optimization of benzimidazole derivative Nanoparticles of (CS-NP) on the basis of CS/ TPP ratio was shown in Table 01.

3.2 Conditions for formation synthesised novel benzimidazole derivative [4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine] loaded chitosan nanoparticles (BZI nano)[9]

3.2.1 Effect of Chitosan Concentration

When the amount of TPP was kept constant as 0.2% and an rise in Chitosan concentration from 0.2% to 0.6% showed a decrease in the particle size with favorable PDI value. When the amount of chitosan exceeded 0.6% of Chitosan a highly opalescent suspension is obtained and it also leads to aggregation. Recent studies reported that when the concentration of Chitosan is low (0.6%) it forms a low viscosity gelation medium resulting in a decrease in liquid phase dispersion, thus promoting formation of smaller particles.

3.2.2 Effect of Triphenyl phosphate (TPP) concentration

The role of TPP (0.2, 0.4 and 0.6%) concentration on particle size formation was studied. The increase inTPP concentration showed an increase in particle size. The TPP concentration with 0.2 and 0.6 chitosan forms particle 220 nm at the same time TPP concentration above 0.4% it results in highly opalescent suspension on storage it starts settling of particles.

3.2.3 Effect of Sonication on Particle Size

The sonication time in the formation of CS-NP played a crucial role in the formation of smaller size nanoparticles. The smallest nanoparticles (220± 4 nm) were obtained with the sonication time of two minutes. While employing ultra-sonication formation of acoustic cavitations is the main cause for decreasing particle size. Acoustic cavitations by creating a largeshear force on the chitosan molecules breaks the particles in to smaller ones. The increase in the sonication time from 30, 60 and 120 seconds showed the decreased particle size presented in (Figure 01). The sonication time beyond two minutes showed no further decrease in particle size.

3.3 Physicochemical characterization of nanoparticles

3.3.1 Particle Size And Zeta Potential

The nine formulations were prepared with various concentrations of chitosan and TPP. The particle size distribution of prepared CS nanoparticles was ranged from 220± 4 to 436±8 nm. With increasing the concentration of CS we observed decrease in particle size and increase in zeta value. At 0.2% concentration of TPP the cross linking with chitosan is high (0.6%) this result in more compact particle structure and the neutralization degree of charged amino acid is improved leading the good net charge of the particles. Due to the compact structure and net charge the particles prepared at this concentration have a smaller size.

The zeta potential of the prepared CS nanoparticles was ranged from +3 to +6 mV. When increase in the concentration of CS the zeta value increases due to the higher degree of protonation of amino group in the CS molecule with the strong positive charge which leads to the higher zeta potential.

The optimum concentration of CS/TPP was identified as 0.6% of CS with 0.2% TPP (BZI 3 nano) with size of (220± 4) nm and the zeta potential showed in (Figure No.2 and 3) novel benzimidazole nanoparticle loaded CS-NP (BZI 3 nano) was 5.55 ± 1 mV which indicates the good colloidal stability of the prepared CS NP.

3.3.2 Transmission electron microscopy TEM images of synthesised novel benzimidazole derivative (BZI nano)

The TEM images of the prepared novel benzimidazole nanoparticle loaded CS-NP (BZI 3 nano) indicate that nanoparticles were roughly spherical in shape with size of 200 nm shown in Figure 04.

3.3.3 Encapsulation efficiency

The encapsulation efficiency of 5 Fluorouracil loaded CS-NP were ranged from 54.7 to 69.2%. The increase in chitosan concentration from 0.2 to 0.6% increases in encapsulation was observed at constant TPP concentration of 0.2%. Out of these formulations BZI 3 nano was selected as the best formulation based on particle size, zeta potential and encapsulation efficiency. The optimized formulation was selected for further studies.

3.3.4 *In vitro* Release

The cumulative percentage release of optimized novel benzimidazole derivative nanoparticle loaded CS-NP (BZI 3 nano) was studied in phosphate buffer pH 7.4 and showed in Table No.2 and Figure 05. The percentage release was found to be 98.3 at 24 hrs. The release profile of 5 novel benzimidazole derivative nanoparticle loaded CS–NP exhibits a initial release burst release of 25% in one hour followed by the sustained release of 98% at 24 hrs. The observed burst effect was due to the dissociation of drug molecules that were loosely bound to the surface of the chitosan nanoparticles. The second part of the release was slow and sustained release of encapsulated novel benzimidazole derivative at an approximately constant rate from the nanoparticles.

3.4 *In vitro* cytotoxicity of nanoparticles

3.4.1 *In Vitro* Cyto Toxicity Study for synthesised benzimidazole derivatives Loaded Nanoparticles (BZI 3 nano)

The IC_{50} of synthesised benzimidazole derivatives Loaded Nanoparticles (BZI 3 nano) increased compare with 5-Fluorouracil (standard) and compound 4a against of MCF-7 and CaCo-2 cell compounds against of MCF-7 and CaCo-2 cell shown in Table 03.

4. CONCLUSION

In the present study we developed a nanoparticulate system which is composed of hydrophilic polymer chitosan possessing the following advantages like obtaining NP by mild agitations absence of organic solvents and high temperature and obtaining NP with positive charge which could enhance the cellular uptake chitosan produces low to high positive charge which could enhance the cellular uptake and has muco adhesive property. This study demonstrates the ionic gelation method can be used to load hydrophilic drugs and produce the size of less than 220 nm. The concentration of CS, TPP and sonication time strongly effect the particle size formation of the CS-NP. The CS-NP composed of 0.6% CS and 0.2% TPP was selected as the optimized formulation which produced smaller particle with better encapsulation. *In vitro* cytotoxicity study suggested the safety of the prepared novel 4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine loaded chitosan nanoparticles, which can be potential carrier to deliver hydrophilic drugs to target colorectum. Further *In vivo* will confirm the targeting efficiency of 4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine loaded chitosan nanoparticles to treat colorectal cancer.

Benzimidazole derivative nanoparticle explored to benefit modern pharmacotherapy and drug discovery. For reaping such benefit, improving future synthetic research in the cancer therapy is essential.

Table 1: Optimization of Nanoparticles of (CS-NP) on the basis of CS/ TPP ratio

S.No	Formulation code	CS %	TPP (%)	SIZE(nm)	PDI	Zeta Potential (mV)	EE (%)	Physical appearance And opacity
1	BZI 1 nano	0.2	0.2	357.3 ± 5.7	0.24 ± 0.05	4.89 ± 0.17	55.12 ± 3.58	Opalescent suspension
2	BZI 2 nano	0.4	0.2	297.5 ±6.3	0.27 ± 0.03	5.23 ± 0.24	61.47 ± 2.46	Opalescent suspension
3	**BZI 3 nano**	**0.6**	**0.2**	**220.2 ± 4.8**	**0.11 ± 0.02**	**5.55 ± 0.31**	**69.2 ± 2.41**	**Opalescent suspension**
4	BZI 4 nano	0.2	0.4	421.8 ± 3.7	0.32 ± 0.06	3.49 ± 2.41	64.51 ± 4.39	Highly Opalescent suspension
5	BZI 5 nano	0.4	0.4	376.2 ± 7.6	0.37 ±0.08	3.89 ± 1.04	56.62 ± 3.45	Highly Opalescent suspension
6	BZI 6 nano	0.6	0.4	343.4 ± 6.8	0.35 ± 0.10	4.05 ± 1.82	63.85 ± 2.74	Highly Opalescent suspension
7	BZI 7 nano	0.2	0.6	436.2 ± 8.7	0.37 ± 0.07	5.32 ± 2.64	60.23 ± 3.51	Highly Opalescent suspension
8	BZI 8 nano	0.4	0.6	359.2 ± 9.2	0.41 ± 0.05	6.23 ± 1.53	58.34 ± 4.65	Highly Opalescent suspension
9	**BZI 9 nano**	**0.6**	**0.6**	**362.8 ± 5.7**	**0.39 ± 0.06**	**6.25 ± 2.61**	**68.52 ± 3.29**	**Highly Opalescent suspension**

Table 2: Cumulative % Drug Release of synthesised benzimidazole derivatives Loaded Nanoparticles

S.No	Time (hr)	BZI 1 nano	BZI 2 nano	BZI 3 nano	BZI 4 nano	BZI 5 nano	BZI 6 nano	BZI 7 nano	BZI 8 nano	BZI 9 nano
1	**0**	0	0	0	0	0	0	0	0	0
2	**0.5**	27.8	25.4	24.8	28.5	20.8	30.7	29.3	17.6	22.4
3	**1**	47.4	48.1	45.2	40.3	43.6	42.4	48.2	36.2	38.6
4	**2**	59.3	55.2	49.7	51.7	50.4	47.5	53.6	41.2	43.7
5	**4**	63.5	63.6	54.1	58.2	57.3	52.7	59.2	51.5	50.6
6	**6**	69.8	72.8	62.6	63.6	62.9	57.3	66.4	58.7	63.5
7	**8**	75.7	79.5	74.2	72.1	69.2	68.4	71.8	64.2	69.2
8	**12**	79.4	84.8	79.2	76.8	75.1	73.6	77.3	74.2	76.8
9	**16**	84.3	89.3	83.1	81.3	79.4	78.7	83.1	79.3	80.3
10	**20**	89.2	92.6	89.7	89.3	84.1	86.6	89.4	87.3	88.2
11	**24**	98.5	97.3	98.3	99.6	95.7	99.4	96.6	94.5	99.4

Table 3: IC_{50} of the 5-Fluorouracil and synthesised benzimidazole derivatives Loaded Nanoparticle (BZI 3 nano)compounds against of MCF-7 and CaCo-2 cell

S.No	Compound code	IC_{50} ± SD (µM)	
		MCF-7	CaCo-2
1	**BZI 3 nano** (benzimidazole derivatives Loaded Nanoparticle)	6.26± 1.48	4.21 ± 1.25
2	4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine	8.22 ± 1.48	5.67 ± 1.25
5-Fluorouracil		7.26 ± 2.30	5.23 ± 2.36

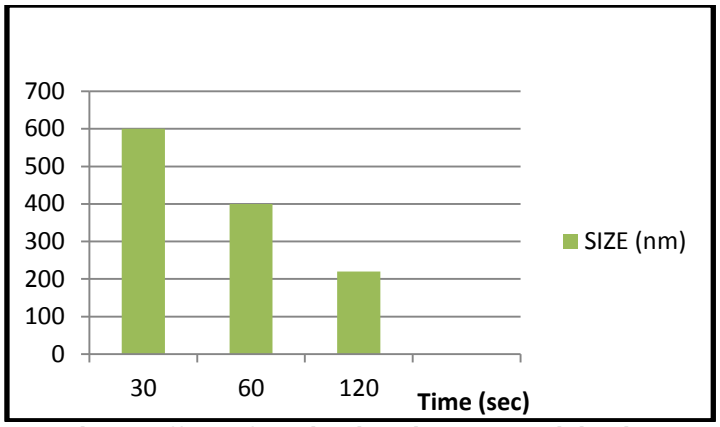


Fig. 1: Effect of sonication time on particle size

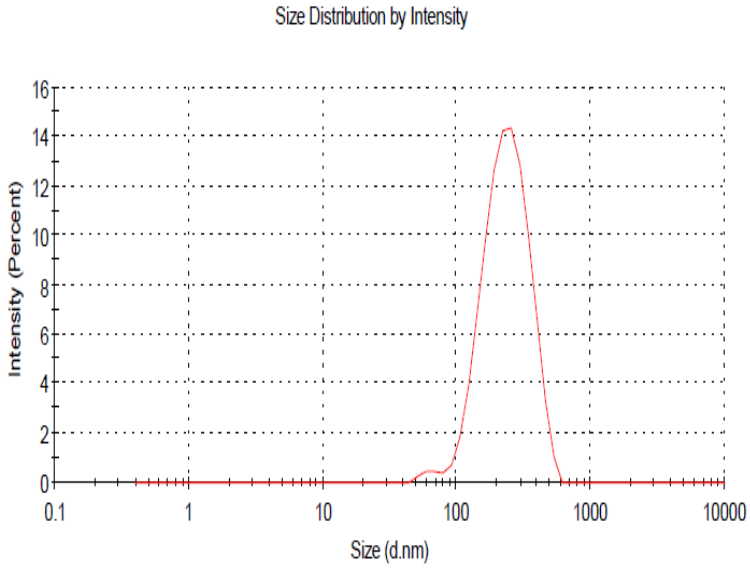


Fig. 2: Particle size of BZI 3

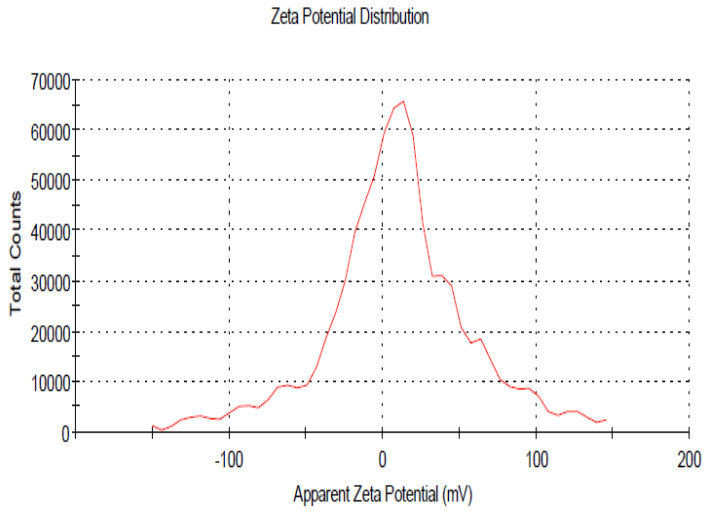


Fig. 3: Zeta potential of BZI 3

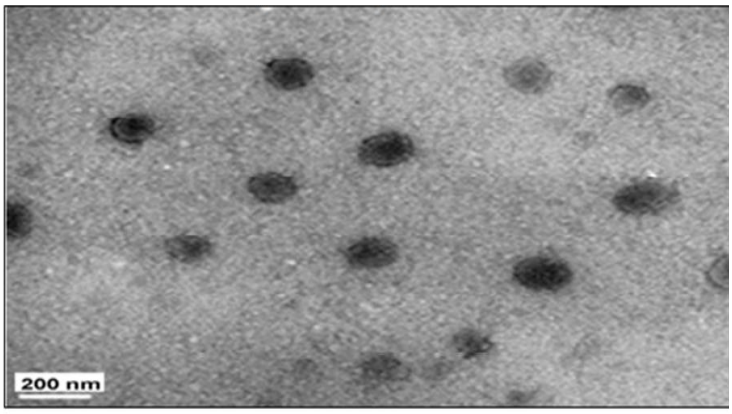


Fig. 4: TEM images of synthesized novel benzimidazole derivative

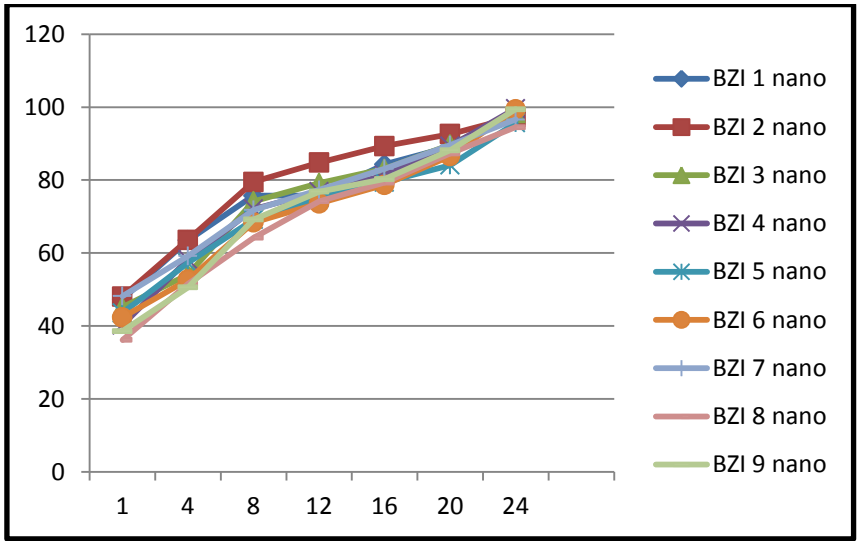


Fig. 5: Cumulative % release graph

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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