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Benzyl Isothiocyanate Loaded Gelatin Nanoparticles Display Unique *in Vitro* Antioxidant Prospects

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Abstract:- This present investigation focuses on the fact that nanoformulation of phytochemicals could enhance the therapeutic capacity in different physiological systems by enhancing hydrophilicity and bioavailability. In this study gelatin nano-formulation of benzyl isothiocyanate (BITC) was prepared and characterized by dynamic light scattering and UV-Visible spectrometry. Then antioxidant activity of BITC and BITC-gelatin NPs was determined in different concentrations through measuring 2,2- diphenyl-1-picrylhydrazyl (DPPH), superoxide, hydroxyl radical, nitric oxide scavenging and lipid peroxidation inhibition activities. DLS and UV-Vis study revealed the production of uniform nanosized particles and effective encapsulation of BITC respectively. The results of antioxidant assays suggested that BITC-gelatin NPs more effectively scavenged free radicals and inhibited lipid peroxidation compared to free BITC. The findings proposed that gelatin formulated BITC nanoparticles could be effective against oxidative stress related disorders.

Keywords:- *Benzyl Isothiocyanate, Gelatin Nanoparticles, Oxidative Stress, Antioxidant Activity, Lipid Peroxidation Inhibition.*

I. INTRODUCTION

Oxidative stress caused by an imbalance of cellular reactive oxygen species (ROS) and the self-antioxidant capacity of the body.[1][2] Endogenous production of ROS is often related to enzymatic reactions involved in respiratory chain, prostaglandin synthesis, phagocytosis, cytochrome P450 system.[3][4][5] Exogenous free radical production can occur as a by-product from exposure to environmental pollutants, heavy metals, certain drugs, cigarette smoke, alcohol, and radiations.[6][7][8] The pivotal role of ROS plays a key regulatory role in apoptosis, mutagenesis and proper body functioning. In one hand, ROS helps host defence system to fight pathogens and regulates intracellular signalling cascades in fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue.[9][10][11] On the other hand, excess free radicals and oxidants can negatively affect several cellular components such as membranes, lipids, proteins, lipoproteins, and DNA.[12] Oxidative stress related lesions of DNA lead to formation of 8-oxo-2′-deoxyguanosine (8-OHdG) which is responsible for both the loss of epigenetic function and mutagenesis. [13] Oxidative stress is believed to be the cause of many pathological changes, including inflammation, cardiovascular diseases, hypercholesterolemia, atherosclerosis, hypertension, diabetes, heart failure and carcinogenesis.[14][15].Several research studies have already pointed out the role of phytochemicals in neutralizing oxidative stress.[16][17] Increasing evidence suggests that dietary isothiocyanates go beyond simple antioxidant roles, influencing several cellular pathways linked to increased resistance to oxidative damage. [18][19]

Isothiocyanates (ITCs) are predominantly obtained from Cruciferous vegetables of Brassicaceae family like broccoli, brussels sprouts, cabbage, cauliflower etc.[20][21][22] ITCs contain –N=C=S as functional groups which arise through the enzymatic hydrolysis of glucosinolates (GLS) by myrosinase enzyme.[23][24] Benzyl isothiocyanate (BITC) is a pharmacologically isothiocyanate found mainly in *Alliaria petiolata* and papaya seeds.[25] BITC (C_8H_7NS M.W- 149.21) or (Isothiocyanatomethyl) benzene is a pale yellowish liquid with strong penetrating aroma and density of 1.121-1.127. BITC exhibits potent anti-oxidant, anti-inflammatory, anti-fungal and anticancer effects as evidenced in different preclinical studies.[19][26][27] But its pharmacological effects are often hindered by its strong lipophilic nature.[28][29] Moreover, it is degraded rapidly in liver by glutamyl transpeptidase and excreted as mercapturic acid in urine narrowing its therapeutic window.[30][31] These limitations could be minimized by encapsulating in nanoparticles thereby increasing solubility, bioavailability, retention-permeation and extender drug release.[32][33] Biopolymers like gelatin is one of the most versatile, naturally occurring substance with a long history of safe use in pharmaceutical, cosmetics, and food products.[34][35] It is non-toxic, non-carcinogenic, biodegradable, non-irritable and classified as a Generally Recognized As Safe (GRAS) material from the United States Food and Drug Administration (FDA).[36] In addition, gelatin contain amino acid sequence Arg-Gly-Asp (arginine, glycine, aspartic acid), which modulates cell adhesion.[37] Thus, encapsulation of BITC with gelatin could be beneficial for its overall therapeutic efficacy. The present study

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demonstrates the synthesis of BITC loaded gelatin nanoparticles and simultaneously investigates anti-oxidant efficacy of BITC and BITC-encapsulated gelatin nanoparticles.

II. MATERIALS AND METHODS

➢ *Chemical and Reagents*

Benzyl isothiocyanate (BITC) was purchased from Sigma Aldrich, USA. Analytical grade gelatin powder (Bloom type A), glutaraldehyde, Ascorbic acid, hydrogen peroxide, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methanol, phenazine methosulfate (PMS), hydrogen peroxide (H2O2), nitro blue tetrazolium (NBT) and other chemicals were purchased from SRL chemicals, India.

➢ *Synthesis of BITC loaded gelatin nanoparticles:*

BITC loaded gelatin nanoparticles were synthesized by modified two step desolvation method. At first, 300 mg of bloom type A gelatin powder was dissolved thoroughly by stirring in 10 ml of double distilled water in hot water bath. Then 10 ml of acetone was added to the solution to precipitate high molecular weight gelatin. High MW gelatin was separated by centrifugation and was redispersed in 10 ml double distilled water through sonication. The pH of the solution was adjusted in 8.5 using 1(N) NaOH.[38]

Then 100 mg of BITC in acetone was added drop by drop to the gelatin solution under constant stirring and kept for 2 hours. After that, 25 µl of 8% glutaraldehyde was added as cross-linking agent and further stirred for 18 hours for solvent evaporation. Then, BITC loaded gelatin nanoparticles were separated by centrifugation at 10000 rpm for 10 minutes. The pellet was washed twice with acetone-water (30:70) and then the pelletwere lyophilized to obtain BITC loaded gelatin nanoparticles (BITC-gel NPs). [39]

➢ *Characterization of BITC Loaded Gelatin Nanoparticles*

- *Dynamic Light Scattering (DLS)*

Mean hydrodynamic particle diameter and polydispersity index of the synthesized BITC-gel NPs were determined by dynamic light scattering in Zetasizer Nano ZS instrument (Malvern Instruments, U.K.). Freshly prepared nanoparticles were diluted in Milli-Q water and was filtered with 0.22 µm syringe filter. The measurement was taken thrice at 25°C with a detector angle of 90°[40]

- *Ultraviolet-Visible Spectroscopic (UV-Vis) Analysis*

The production and stabilization of the synthesized BITC-gel NPs was monitored by UV-vis spectrophotometric analysis. Absorption spectra of the nanoparticles were determined at the spectral range of 200-800 nm using Hitachi Spectrophotometer U-3900/3900H (Japan). Analysis of the absorbed light was performed through the resultant spectrum. The recorded spectra were then replotted in Origin 8.5 software [41].

➢ *In-Vitro Anti-Oxidant Activity*

- *DPPH Radical Scavenging Activity*

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a popular, quick, easy, and affordable approach for the measurement of antioxidant properties of a compound or an extract. DPPH itself act as a stable uncharged free radical because delocalization of the pair electron on the whole substance prevents it from dimerization. In brief, different concentrations (50,100,200,500,100 ug/ml) of BITC, BITC-gel NPs and standard ascorbic acid solution were mixed with 0.2 ml of 100 µM methanolic solution of DPPH. The mixture was then incubated in room temperature for 30 min under dark condition. After incubation, the absorbance of the reaction mixture was measured at 517 nm using a spectrometer (Shimadzu UV-1800, Japan**)**. The DPPH radical scavenging activity nano was computed using the formula [42][43].

$$Percentage(\%)\ of\ scavenging = \frac{(OD\ of\ control - OD\ of\ sample)\times 100}{OD\ of\ control} \quad \ldots\ldots[1]$$

- *Superoxide Radical Scavenging Activity*

The principle of this assay is the conversion of yellow coloured Nitro blue Tetrazolium (NBT) into purple coloured NBT di-formazan via superoxide radical. At first, 3 ml of different concentrations of BITC, BITC-gel NP and ascorbic acid was mixed with 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 0.1 ml of 60 µM phenazine methosulphate (PMS) solution and incubated for 5 minutes. All the reagents and solutions were prepared in phosphate buffer saline. After incubation optical density was measured at 560 nm using spectrophotometer to determine the extent of radical scavenging [44][45].

- *Hydroxyl Radical Scavenging Activity*

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. This assay used iron-EDTA model of hydroxyl radical generating system. The radical degrades deoxyribose to produce by-product which reacts with TBA-TCA to produce pink chromogen. The assay was performed by mixing 0.1 ml EDTA, 0.5 ml of phosphate buffer0.01 ml of FeCl3, 0.1 ml of H_2O_2,0.3ml of deoxyribose, 1.0 ml of various concentrations (50, 100, 250, 500 µg/ml) of BITC, BITC-gel NP and ascorbic acid. After that the mixture was incubated at room temperature for 1 h. Then 1.0 ml of TCA (10%) and TBA (0.8%) was added and mixture was placed in boiling water bath for 20-30 min. The absorbance of produced pink chromogen was measured at 412 nm and the percentage of inhibition of deoxyribose degradation as well as hydroxyl radical formation was calculated by equation 1 [46].

- *Nitric Oxide Scavenging Activity*

Aqueous solution of sodium nitroprusside act as a source of nitric oxide which reacts with oxygen to produce

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nitrates and nitrites. The concentration of produced nitrites was determined by Griess reagent. In brief, 1ml of sodium nitroprusside (10 µM aqueous solution) was mixed with 1 ml of each concentration of BITC, BITC-gel NP and ascorbic acid and incubated for 2.5 h at 25°C. 1 ml of Griess reagent (1% sulphanilamide, 2 % orthophosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) then was added in this incubated mixture. Optical density of the solution was determined at 546 nm and the percentage inhibition was calculated [47].

- *Lipid Peroxidation Inhibition Assay*

At first, liver tissue homogenate was prepared in ice-cold tris buffer and 1 ml of the homogenate was mixed with various concentrations (50, 100, 250, 500 172 µg/ml) of BITC, BITC-gel NP and ascorbic acid, in presence of 30mM KCl, 0.16 mM FeSO4, 0.06 mM ascorbic acid and incubated at 37°C for 1 h. After that the reaction was terminated by adding 1.0 ml of TCA (10% w/v) and 1.5 ml of TBA (1% w/v) the mixture was kept in boiling water bath for 30 min. Then, 5 ml of n-butane: pyridine (15:1v/v) was added to it and centrifuged at 4000 rpm for 10 min. Organic layer containing pink chromogen was separated and optical density was measured at 532 nm (Ohkawa et al., 1979). The percentage inhibition was calculated in similar way [48].

➢ *Statistical Analysis*

All experiments were performed thrice and the results were expressed as mean± SD. Significant difference and multigroup comparisons of the data was analysed by using one and two-way analysis of variance (ANOVA) using GraphPad Prism and Origins 8.5

III. RESULT AND DISCUSSION

A. Characterization

➢ *Dynamic Light Scattering*

The hydrodynamic diameter and size distribution pattern of synthesized nanoparticles were determined by dynamic light scattering. Results reported that the average diameter and poly-dispersity index of the synthesized nanoparticles was 197.5nm and 0.084 respectively. That indicates small and mono-dispersed nature of BITC-gelatin nanoparticles.

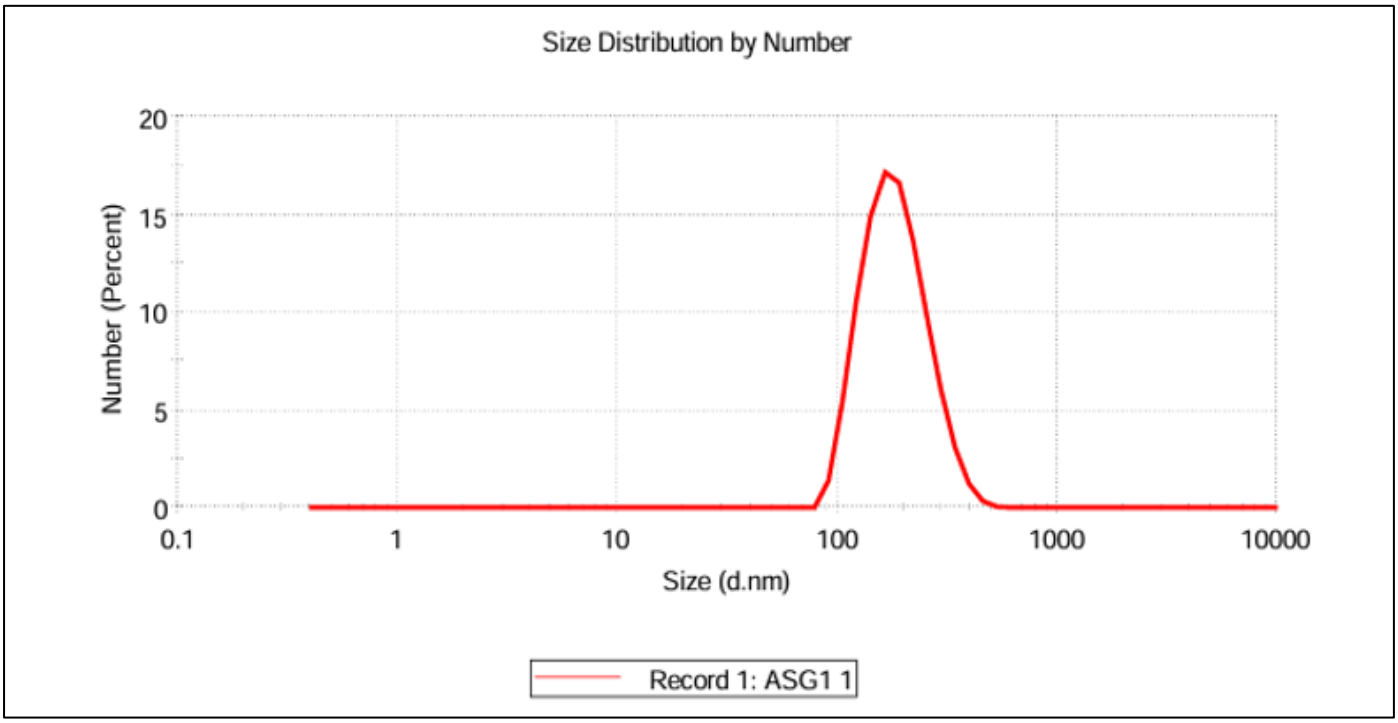

Fig 1 Size distribution of synthesized BITC-gelatine nanoparticles analysed by dynamic light scattering reported uniformly distributed nanoparticles with average diameter <200nm.

➢ *UV-Vis analysis*

UV-vis spectral analysis of BITC and BITC-gel NP shows absorption spectra between 210-290 nm wavelength. Characteristic peak of BITC-gelatine NP confirms the presence of BITC in synthesized nanoparticles.

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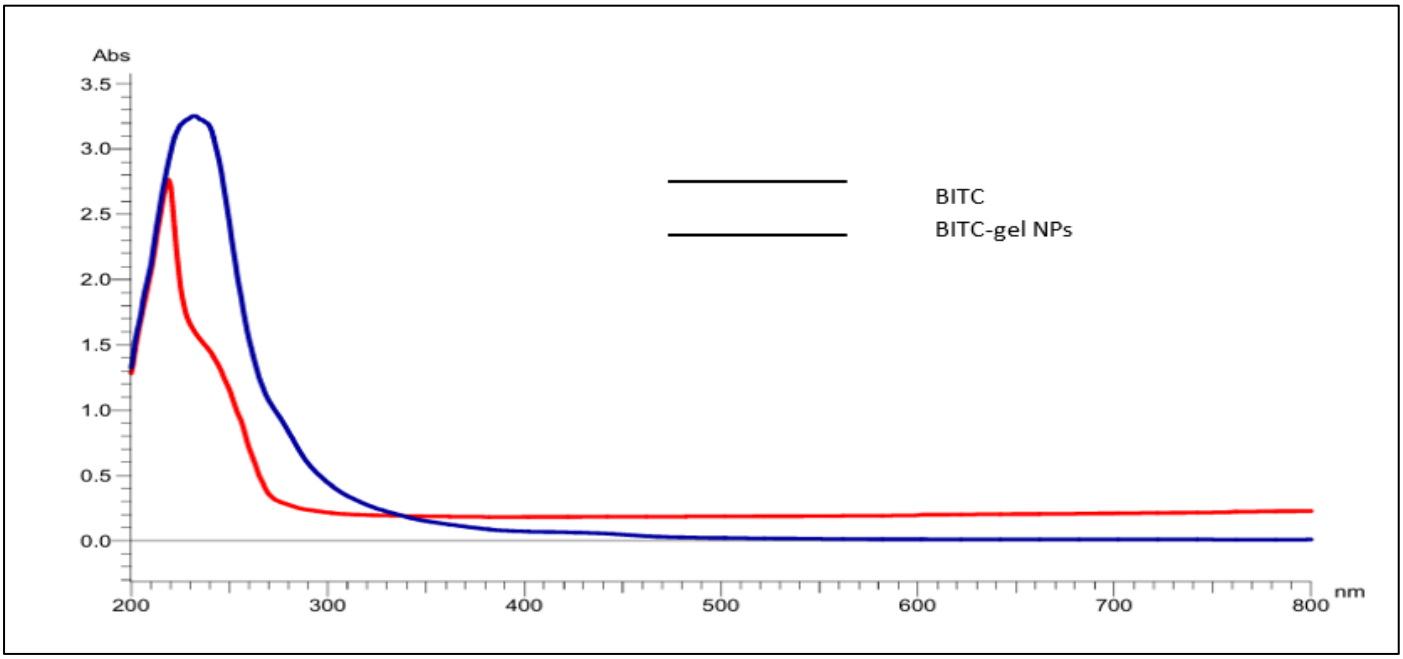

Fig 2 UV-Vis spectra of both BITC and BITC-gelation nanoparticles at the spectral range of 200-800 nm shows absorption band at 210-290nm

B. In-Vitro Anti-Oxidant Activity

➢ *DPPH Radical Scavenging Activity*

The result suggests both BITC and BITC-gel NP was able to scavenge DPPH in a dose-dependent manner. IC 50 values for BITC, BITC-gel-NP and ascorbic acid were 49.38, 37.66 and 13.21 µg/ml respectively [Figure: 3]. Delocalization of electrons on the whole substance gives the characteristic violet colour of DPPH solution [49]. When it reacts with an antioxidant it donates the electrons and is reduced to di-phenyl picryl hydrazine and loses its violet colour and becomes colourless [50]. Here the study confirms that BITC -gel NP was able to reduce DPPH more potently than free BITC.

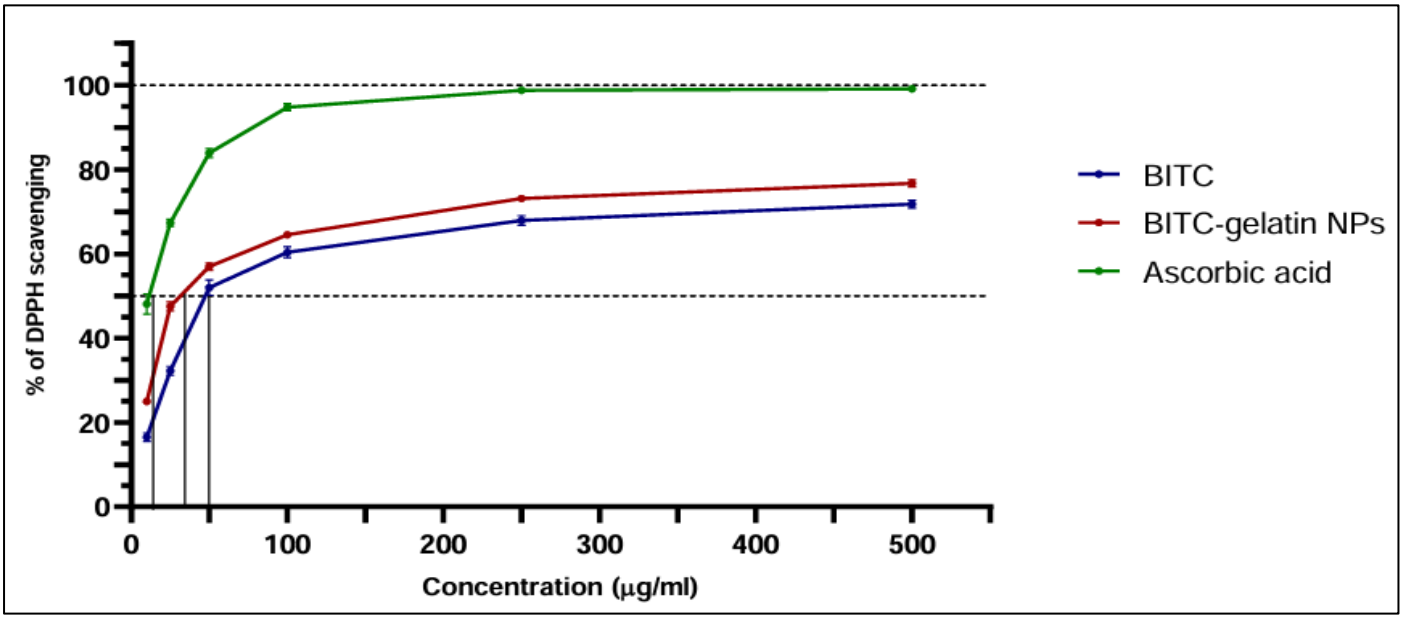

Fig 3 DPPH Scavenging Activity of BITC, BITC-gel NPs and Ascorbic acid. Results are Described as Mean ±SEM.

➢ *Superoxide Radical Scavenging Activity*

This study demonstrates that BITC-gel NPs was effectively able to scavenge superoxide anions than free BITC. Respective IC 50 value of BITC, BITC-gel NPs and ascorbic acid was 48.03, 44.33 and 32.57 µg/ml. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide- phenazine methosulfate (NADH/ PMS) reaction generates superoxide radicals, which reduce NBT to a purple coloured formazan [51]. BITC and its nano-formulation was able to scavenge superoxide anions, and as a result less formazan was produced.

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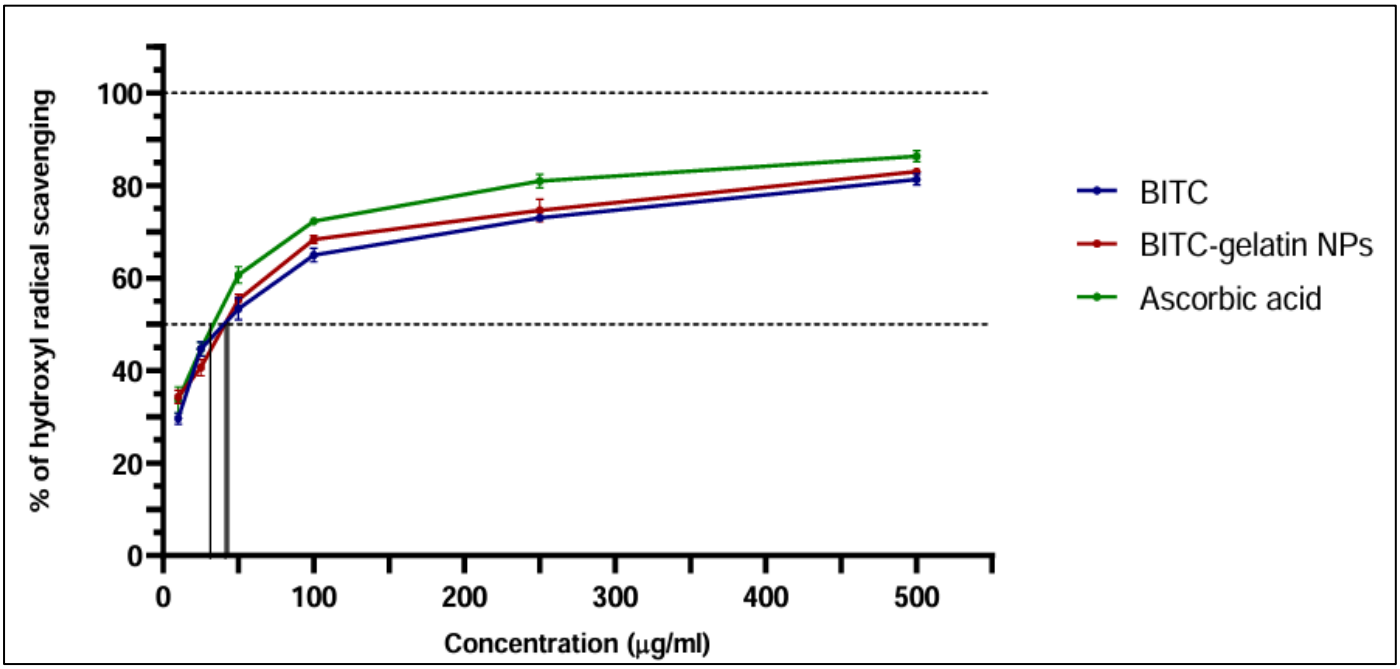

Fig 4 Hydroxyl radical scavenging activity of BITC, BITC-gel NPs and ascorbic acid. Results are described as Mean ±SEM. BITC and BITC-gel NPs effectively scavenged hydroxyl radical.

➢ *Hydroxyl Radical Scavenging Activity*

Iron-EDTA system generates hydroxyl radical which degrades deoxyribose and produce pink chromogen by reacting with Thiobarbutaric acid [52]. In presence of BITC and BITC loaded gelatin nanoparticles hydroxyl radicals were scavenged effectively resulting less chromogen formation. BITC, BITC-gel NPs and ascorbic acid was able to scavenge up to approximate 69%, 73% and 78% of total hydroxy radical at concentration of 500 µg/ml.

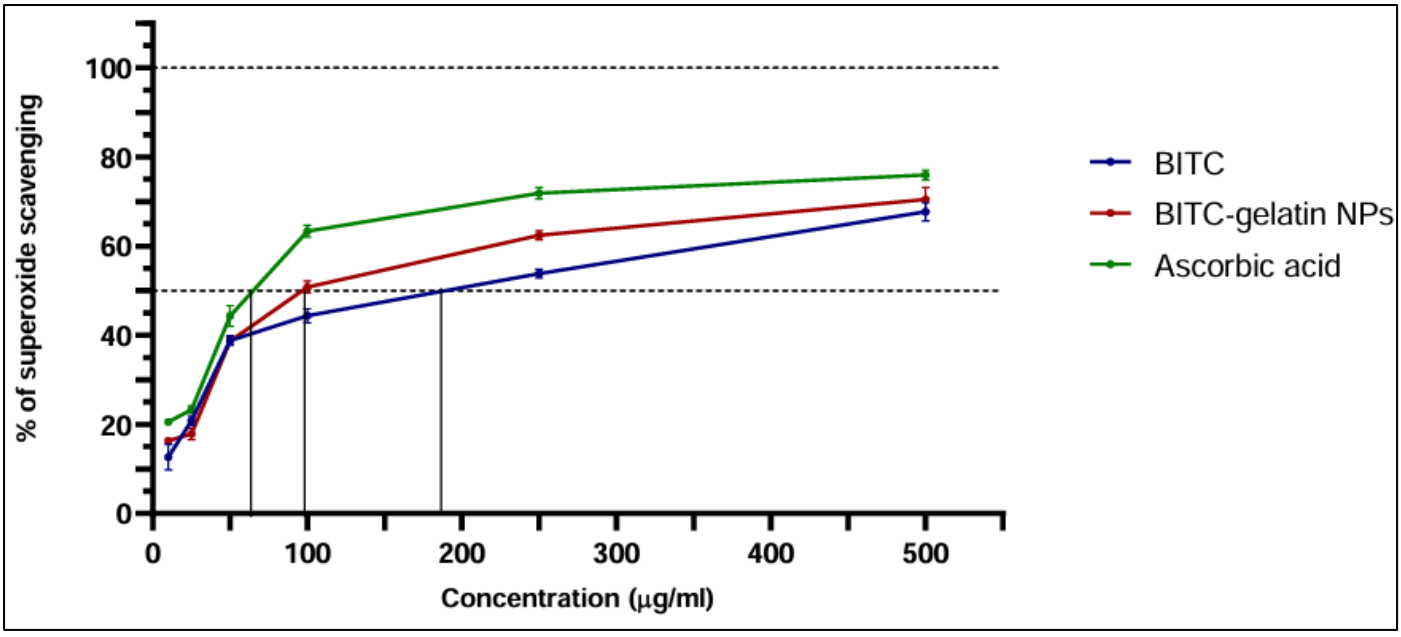

Fig 5 Superoxide anion scavenging capability of BITC, BITC-gel NPs and ascorbic acid Results are described as Mean ±SEM.

➢ *Nitric Oxide Scavenging Activity*

Nitric oxide is generated from sodium nitroprusside and it undergoes rapid oxidation and produce the stable product nitrates and nitrite in presence of oxygen [53]. The nitrates are by using the Griess reagent. In the presence scavenger, the amount of nitrate production is decreased which is reflected by less colour formation [54]. Here, the phytochemical acts as a scavenger and the extent of scavenging are proportional to the concentration. BITC -gel NP was able to scavenge more nitric oxide radical as effectively as standard antioxidant ascorbic acid. Respective IC 50 value of BITC, BITC-gel NPs and ascorbic acid was 158.73, 54.33 and 42.5 µg/ml.

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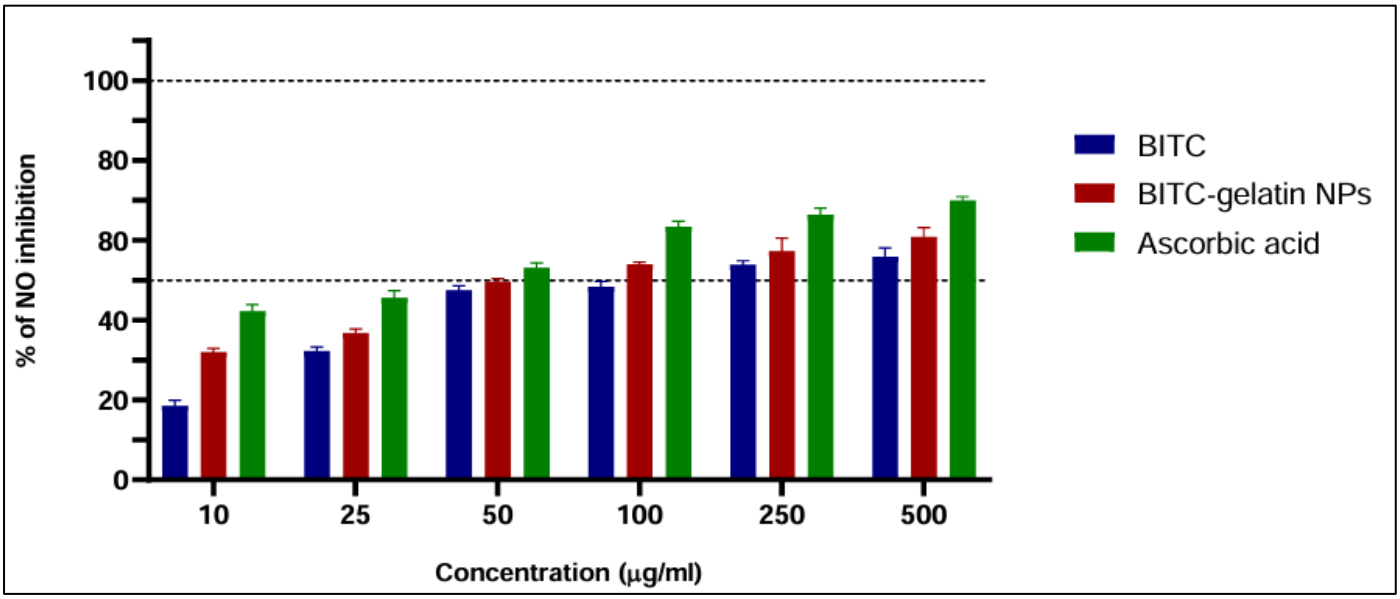

Fig 6 Nitric oxide inhibitory capability of BITC, BITC-gel NPs and ascorbic acid Results are described as Mean ±SEM.

➢ *Lipid Peroxidation Inhibition Assay*

Lipid peroxidation assays measure the ability of antioxidants to protect lipids from oxidative damage. Oxidative stress causes membrane lipid to undergo lipid peroxidation and as a result malon-di-aldehyde (MDA) is produced [55]. This assay measures the amount of malondialdehyde (MDA) and other ROOH-derived products that react with TBA to form TBA-adducts [56]. Here, BITC was able to inhibit lipid peroxidation thereby inhibiting MDA formation. figure depicts BITC-gel NPs has higher inhibitory activity than free BITC. Respective IC 50 values of BITC, BITC-gel NP and ascorbic acid was 57.14, 102.63 and 227.66 µg/ml [Figure 7].

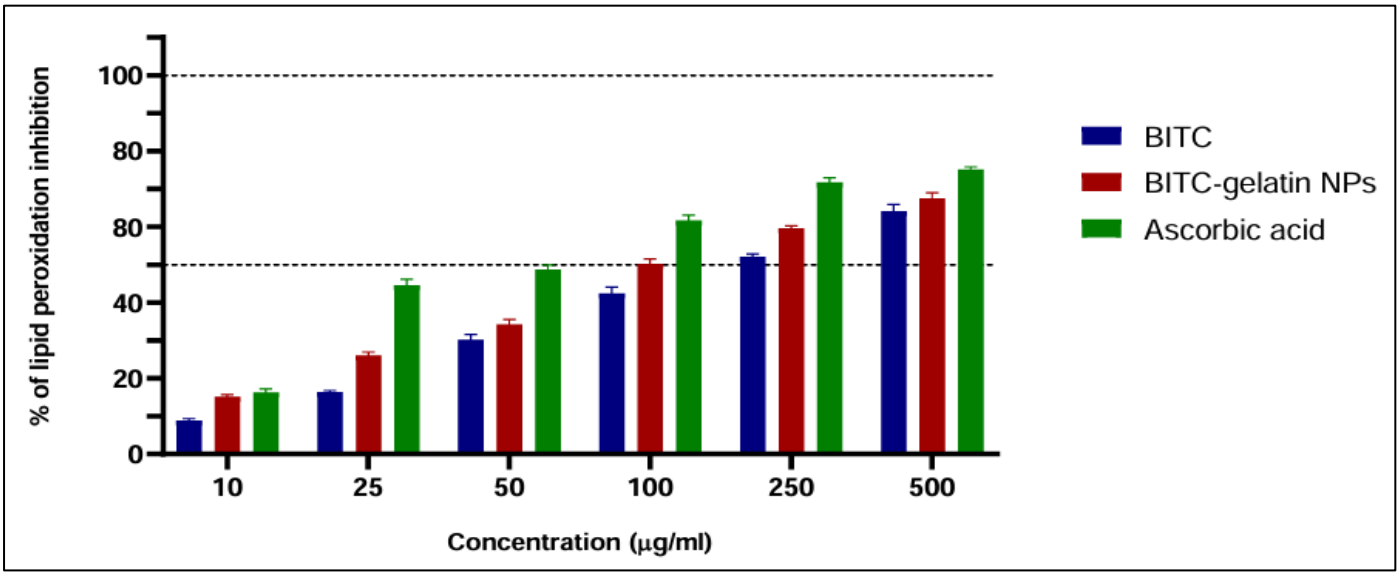

Fig 7 Lipid peroxidation inhibitory activity of BITC, BITC-gel NPs and ascorbic acid Results are described as Mean ±SEM.

IV. CONCLUSION

This study demonstrates modified two-step disolvation process to synthesize gelatin loaded benzyl isothiocyanate nanoparticles. The DLS data demonstrated formation of small uniform nanosized particles with average diameter of 197.5 nm. The UV-Vis spectra (Fig. 2) of synthesized nanoparticles shows significant absorbance within 210-280 nm which correlates with sharp absorption peak of BITC. The antioxidant capacity of the BITC loaded gelatin nanoparticle was established from the present study. BITC along with its nano formulation was able to scavenge different oxidative stress producing compounds and radicals in in-vitro condition. Nano formulated BITC effectively scavenged DPPH radical, nitric oxide, superoxide anion, hydroxyl radical and inhibited MDA formation from lipid peroxidation in a dose dependent manner. BITC and gelatin nano-formulation not only prevented ROS generation but also scavenged them effectively. These findings depict that nanoformulated BITC could be advantageous for us in battling oxidative stress related disorders.

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