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BiTCoN: A Bimodal Therapy Against *Candida Albicans*

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Abstract

Biosynthesized thermoactive copper nanoparticles (BiTCoN) from *Tinospora cordifolia* plant extracts exhibited efficacy against biofilm formation, hyphae transition, and surface adhesion in *Candida albicans*. Adhesion is an initiator of virulence factors such as dimorphism, proliferation, stress tolerance, & antigenicity, which promote biofilm formation in the host. The thermal capacity of BiTCoN is was more compared to only aqueous plant extract. In our study, copper in nanoform enhances the colloidal solution's thermal conductivity by holding the heat. Time-killing assay, cell wall protection assay, and hemolytic assay were useful for checking the cytotoxicity of BiTCoN on cells and understanding the mechanism of action on *C. albicans*. This therapy works in a combination where nano formulation killing the Candida cells by heat effect and plant extract prevents further recurrence. Also, BiTCoN induces reactive oxygen species (ROS) production in *Candida* cells that promote free radical formation leading to oxidative damage and, consequently, death of Candida cells. The electron microscopic studies revealed that the fungal cell disintegration occurs when BiTCoN interacts with *C. albicans* cells. Therefore, various experiments proved that BiTCoN could inhibit *C. albicans* strains which are potent and safe antifungal agents.

Keywords

BiTCoN, Biofilm, Surface adhesion, *Candida albicans*, Thermal capacity

1 Introduction

The fungal infections & contagious diseases in human beings are much of the time brought about by *C. albicans,* which is one of the most dominant members of the human mycobiome and commonly causative agent in mucosal & systemic disease [1]-[2]. The presence of *C. albicans* in the buccal& the gastrointestinal pit of human being cause superficial oral, vaginal infection along with deadly systemic disease via blood circulation & organ intrusion in immune-compromised individuals [3]-[4]. *C. albicans* can colonize both abioticas well as biotic surfaces and forms 3D structures composed of yeast & hyphal forms surrounded in a self-generated matrix. Such kind of growth referred to as biofilm, which observed on medically implanted devices such as prosthetic heart valve, catheter and denture etc. [1],[5]-[7]. Development of resistance to antifungal antibiotics forms sepsis widely reported in clinical isolates from patients. Fungal and parasitic contamination isone of the most significant reasons for diseases & mortality, which shows overall lower susceptibility despite several potent drugs available in the market, such as echinocandins and azoles [8]-[9]. Therefore, full of achievement in todays world has been observed in a sector of antibiotics to lower the rate of infectious diseases like candidiasis. Also, some species of *C. albicans* shows lower susceptibility to common antifungal drugs such as fluconazole, and this situation is alarming against candidiasis [9]-[10]. Moreover, recent antibiotics also exhibit side effects in patients, and there is a need for better drugs to be discovered. There are recently developed ways for the delivery of small molecular antibiotics. One of the best-suited approaches is 'nanotechnology', which deals with nonmaterial's ("nano-antibiotics") fascinating to the researcher as they have their significant broad spectrum for anti-microbial activity [11]. Nano-antibiotics exhibit many applications such as drug delivery, induced cellular internalization, sustained drug release, solubility, and minimal side effects [12]-[16]. The nano-antibiotics are more effective towards antibiotic resistance by compromising present resistance mechanisms. Occasionally silver and gold nanoparticles are reported as antibacterial and antifungal agents, but they are less documented and approached [17]-[20]. Several combinational therapies were tested on *C. albicans* to control the spread of fungal infection [21]. The proposed bimodal therapy consists of plant extract acting like a drug and the copper nanoformulation as a heat conductor collectively referred to as BiTCoN. This nanoformulation kills the *C. albicans* due to its anti-microbial potential, heating effect and avoids its further recurrence and minimize the infection. The thermal/heat capacity of BiTCoN is more than only aqueous plant extract; it is because of the metallic nature of copper; it has higher heat capacity and conductivity [22]. The heating mechanism behind the BiTCoN is copper metal, which retains the amount of heat for longer due to its good conductance and high heat capacity. Again, as copper used in our study is in particulate form (nanoform), it is much more feasible for penetration and enhances the thermal conductivity of colloidal solution by holding the heat for a longer time, which makes it thermo active [22]-[24].The BiTCoN characterized and examined for its potential combat activities against *C. albicans* biofilm formation, adhesion, hyphal transition and dimorphism with their cell wall integrity mechanism.

2 Materials and methods

2.1 Requirements

We obtained *Tinospora cordifolia* plant from SRTM University Campus Nanded, MS, India, and then dried stem of this plant to form powder. Copper sulfate (II) pentahydrate salt ($CuSO_4.5H_2O$) (99%) was bought from Merck Chemicals Ltd. Mumbai, India & the strain ATCC90028 and GMC3 of *C. albicans* were acquired from the IMT, Chandigarh, Punjab and TB patient of GMC, Nanded, MS, India, respectively. For the preparation of inoculum and

to sustain the culture, YPD which comprises 1% of yeast separate, 2% of peptone & 2% of dextrose and the agar was inclined at 4 °C, utilized for inoculums formation. RPMI-1640 medium, 3-N-morpholine-propane sulfonic (MOPS), & 2,3-bis-(2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide *i.e.* (XTT), FBS (Fetal Bovine Serum), Menadione, and Triton-X 100 was bought from Himedia Chemicals, Mumbai, India. In next step, a single colony from the agar plate was inoculated in YPD broth near about 50 mL of stock & after that, and it kept for incubation in shaking incubator (24 h & 120 rpm) with body temperature. Centrifugation of harvested cells from the initiated culture was done at 2000 rpm for a maximum of 5 min & it was resuspended in buffer(sodium phosphate) of pH~ 7.4 after washing.

2.2 Synthesis & characterization of BiTCoN

The fresh *Tinospora cordifolia* stem processed and dried well in sunlight. The dried stem was ground into a powder, and from this aqueous powder, the extract was prepared. New 10 mM copper sulfate pentahydrate solution was prepared separately for the biosynthesis of BiTCoN. In the next step, 1.5 mL of aqueous stem extract of *Tinospora cordifolia* added 18.5 mL of aqueous copper sulphate pentahydrate solution for the biosynthesis of the BiTCoN and this mixture was warmed up to 80 ºC and kept at normal temperature for 6 h. The light yellow colour of the BiTCoN was changed to the golden brown colour in the colloidal form of solution. Later on, the formed colloidal solution was centrifuged further at 10000 rpm & the pellets were collected & washed thrice with Milli-Q water for the removal of plant derbies and dried in oven nicely [25].

The BiTCoN were visualized for its depth and surface morphology, structure, and size by Transmission Electron Microscopy (TEM) (Philips, CM-200) and scanning electron microscopy (SEM) (S-3700-N, Hitachi, Japan). The functional groups of biochemical compounds observed in BiTCoN were detected by Fourier Transforms Infrared Spectroscopy (FTIR) (Nicolet Avatar-660, USA). To identify elemental components, Dispersive Energy X-ray (EDAX) analysis was performed (Zeiss, EVO-18-EDX). The crystal lattice size of domain and peaks of the BiTCoN were analyzed with the help of X-ray diffractometer (Empyrean Multi-purpose XRD, AZo Network UK Ltd.) [26].

2.3 Study of the thermal capacity of BiTCoN

Our experiment took plant extract and BiTCoN in a separate vessel and heated for 10 minutes with a hot plate magnetic stirrer. We recorded the room temperature for both the solutions, which was 27 °C. After 10 min of heating of both the solutions (an aqueous plant extract and BiTCoN solution), it becomes 45 °C (as we provide the same amount of heat supply at given point of time). In the next step, we allowed both the solution to cool down for the next 2 minutes, and the temperature was recorded with the help of thermometer. We observed a decrease in both the temperatures, but surprisingly, the amount of heat released by the BiTCoN (43 °C) was less compared to aqueous plant extract (40 °C) after 2 minutes of cooling. We repeated this experiment thrice and recorded the observations. This study suggested that the heat holding/thermal capacity of BiTCoN is high [27]-[28]. This study helps in the understanding of biological mechanisms related to the cell membrane permeability.

2.4 Evaluation of planktonic cell growth

The planktonic cells growth impact of BiTCoN was evaluated by a standard stock micro dilution strategy dependent on the Clinical Laboratory Standards Institute (CLSI) guidelines. The concentration BiTCoN was going from 0.78-200 µg mL^{-1} was set up in the RPMI-1640 medium and transferred in non-treated well plates (Costar, USA). To get 1×10^3 cells/mL, RPMI & inoculums of 100 µL were mixed in each well separately. The optical density (absorbance) was recorded at 620 nm to examine the growth using a microplate reader (Thermo-Electron Corporation, USA) after the incubation period (at 35 °C & 48 h). The minimal concentration of BiTCoN at which there was a half decrease in the absorbance contrasted with control was supposed as the minimum inhibitory concentrations (MIC) for the development of *C. albicans* [29].

2.5 Surface adhesion test

The microplate-based experiments were utilized to study the adherence properties of *C. albicans* and check the impact of BiTCoN on it. The BiTCoN were synthesized in PBS with a concentration range of 0.78-25 µg mL^{-1}. In control wells there was no existence of BiTCoN, and to acquire 1×10^7 cells/mL & the cell suspension (50 µL) was added to each well, & the 100 µL of concentration was kept as the final concentration for the assay. The plates were kept for incubation in a shaking incubator (90 min, 37 °C & 100 rpm). The non-adhered cells were eliminated with the help of PBS after the incubation time frame over. With the use of XTT assay, the thickness of the adherence was assessed for each well by RMA (Relative Metabolic Action). If the decrease in RMA > 50%, then its correlation with the control was significant [29].

2.6 Dimorphism assay

The impact of BiTCoN over serum-incited yeast on hyphal structure progress was explored and studied concerning central venous catheter disc in a microtiter plate [30].Different groupings of the BiTCoN extending from 0.195 to 25 µg mL^{-1} were set up in 20% of serum and Milli-Q water. *Candida* cells were vaccinated to acquire 1×10^8 cells mL^{-1} in both the wells, *i.e.* test sample & control sample). These plates were incubated at body temperature in a shaking incubator (at 200 rpm & 2 h). Cells were studied minutely for the shaping of germ tubes. Out of 100 cells, the amount of transition from yeast into hyphae was counted. The focus, which indicated the restraint of hyphae with ≥ half contrasted with the control, was supposed to be the MIC level for morphogenesis or dimorphism.

2.7 Biofilm formation study

Biofilm assay of *C. albicans* was investigated with the help of 96-polystyrene well plates. The 100 µL of cell suspension *i.e.*(1×10^7 cells mL^{-1}),was added in every well formed in PBS. During the adhesion phase, 96-well plates were kept at 37 °C in shaking incubator (90 min &120 rpm) for adherence of the *C. albicans cells* to the massive surface. To eliminate non-adherent cells, wells were cleaned using sterile PBS. After that, 200 µL of RPMI-1640 medium was incubated (37 °C for 48 h) and added to form a biofilm in each well plate. Fungal cells (1×10^7 cellsmL^{-1}) were allowed to react with BiTCoN of concentration ranging from 200-0.78 µgmL^{-1}, and untreated one was referred to as control prepared in RPMI-1640 medium. Immediately after the adhesion phase, the effect of BiTCoN on biofilm proliferation was investigated [31].For

further study, BiTCoN were reacted with 24 fold mature biofilm of *C. albicans* and kept under incubation for next 48 h. Once the 48 h time frames over, non-adhered cells were washed off to maintain sterile condition. Treated well plate was visualized for biofilm observation by using an inverted light microscope (Metzer-M, 5000-ITM, India). The excellent pictures of *Candida* biofilm were captured using Labomed microphotography at 100 X of magnification, and the biofilm development was evaluated & finalized by using XTT assay.

2.8 Quantification of cell viability by XTT assay

The XTT metabolic analysis was studied to explore the biofilm growth of *C. albicans* as per given protocols. XTT reaction mixture of 1 $mgmL^{-1}$was made in Milli-Q water & kept for freezing at -20 °C. To achieve 4 µM final concentration of XTT, menadione solution designed in acetone and poured into the solution of XTT. Non-adhered cells were washed off with the help of PBS & allowed it to incubate in 100 µL of XTT-Menadione mixture under the dim condition at body temperature(5 h) [31].Water-soluble coloured formazan product was formed as a result of metabolic reaction were identified successfully. The absorption spectrain a microplate reader (Synergy HTX, Multi-Mode Reader, USA) were recorded at 450 nm. Absence of BiTCoN in the sample (control). The amount of BiTCoN responsible for ≥ 50% of reduction in relative metabolic activity was supposed as the MIC for biofilm development.

2.9 SEM analysis

The fungal biofilm was observed on the central venous catheter discs fixed in 2.5% glutaraldehyde solution made with PBS (4 °C & 24 h) and later-on fixed with 2% of osmium tetraoxide (OsO_4) solution for next 4 h. In the next step, the samples were dehydrated with ethyl alcohol and finally dried with the help of dryer. Later on, the samples were mounted on stubs & sputter coated with gold (10 sec & 11000 mA) with the influence of argon plasma. The biofilm structure was thoroughly analyzed, an SEM instrument (Hitachi, S-3000-N, Japan) was operated in a high vacuum, *i.e.* 10 kV and the SEM images were captured [30].

2.10 Sorbitol assay

Sorbitol is a product of osmotic stress condition used to investigate the growth of the fungal cell wall. RPMI medium without sorbitol considered as control used to investigate BiTCoN effect on the yeast cell wall. Experiments were done thrice to evaluate MIC in a planktonic cell by the micro-dilution method in 96-well plates. The sorbitol concentration was 0.8 M medium to implicate its impact on the cell membrane. Once the treatment over, these plates were incubated at 35°C; after that, the result was detected on 2^{nd} and 7^{th} days, respectively. The medium with an added amount of sorbitol compared to medium without sorbitol has a higher MIC value. This was considered to be one of the cell targets for the BiTCoN [32].

2.11 Growth, viability and kill-curve assay

The proliferation, time required to assess the viability and lethality of cells by BiTCoN was studied as per standard protocol and CLSI guidelines [33].

2.12 Hemolytic activity

The hemolytic assay was studied using standard protocol given for human RBC's. The RBC's from healthy and fit human beings was taken in a test tube with 2 mg mL^{-1} of EDTA as a coagulating agent. The collected sample of blood

was allowed for centrifugation (10 min& 634 × g) at 20°Cand washed thrice with buffer solution. In the next step, the buffer solution was added into a pellet to get 10% of (v/v) RBC's/PBS suspension. Later on, 10% of suspension was diluted in 1:10 ratio. Cell suspension (100 µL) was inoculated in triplicate with serial dilutions. The 2 mL of BiTCoN in the same buffer was added. Using 1%, Triton X-100 total hemolysis was performed [30].Tubes were maintained at 37°C for 1 h, and centrifugation was carried out at 20°C. Further, in Tarson Ltd. Indias microtiter plate, 150 µL of suspension was added from the supernatant fluid, and it quantified at 450 nm of optical density. By using the following formula, % hemolysis was determined (Given absorbance of 414 nm):

% hemolysis = [(Abs of the mixture – Absorbance of PBS)/ (Absorbance in 0.1 % of Triton X-100 – Absorbance of saline)] x 100

2.13 Statistical analysis

The calculated numbers were in the form of means and standard deviations (SD) obtained from three subsequent trials of samples. All the values such as control and treatment groups and results visualized in XTT were analyzed with student's t-test, and the value of $p < 0.05$ was supposed to be statistically correct [31].

3 Results

3.1 Characterization & thermal capacity of BiTCoN

The reduction of Cu^{2+} ion to Cu occurs when Copper sulfate (II) pentahydrate salt ($CuSO_4.5H_2O$) and *Tinospora cordifolia* stem extract were reacted with each other, and it leads the biosynthesis of thermoactive water-soluble BiTCoN, respectively **(Fig.1)**. BiTCoN were crystalline investigated by X-ray differentiation (XRD) **(Fig.2 a)**.A different form of peaks was visualized as (2θ) =27.64º, 32.09º, 37.50º and 46.16º with lattice plane (111), (200), (111) and (220) with cubic shape. FTIR functional groups in biochemical compounds were detected, and the wavelength ranges from 3455 nm – 1020 nm **(Fig.2 b)**. FTIR plot was showing peaks specific to various biochemical groups such as 2921, 2377,2317, 1760, 1634, 1561,1023, and 3452 wavelengths contain peaks which depict carbonyl groups (C=O), saturated aliphatic group (-CH_2-), alcoholic and phenolic group(–OH).

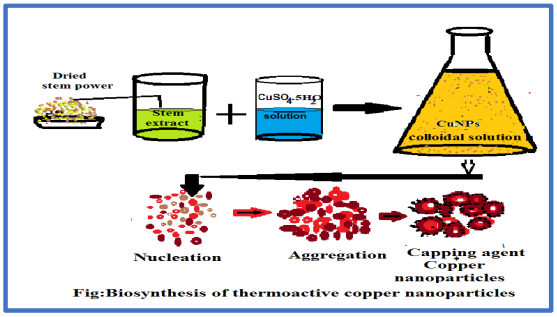


Fig.1. Synthesis of BiTCoN from stem extract & $CuSO_4.5H_2O$ solution forming dark pale-yellow colloidal solutions.

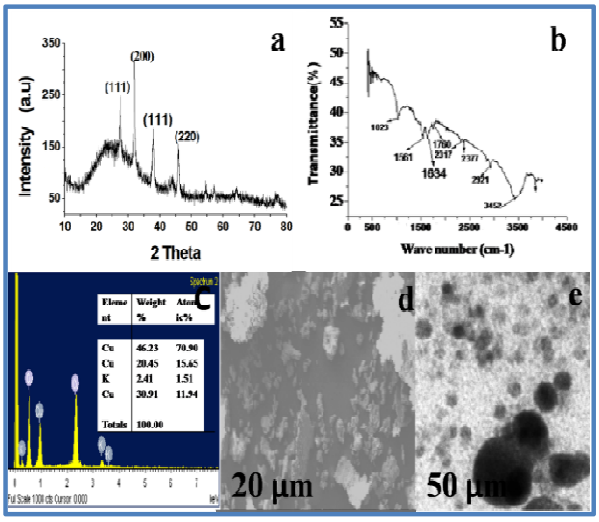


Fig.2. Characterization of BiTCoN **(a)** X-ray diffraction patterns; **(b)** FTIR analysis; **(c)** Energy Dispersive X-ray spectrum of BiTCoN; **(d)** SEM analysis of BiTCoN; **(e)** TEM image BiTCoN

2921 peak indicates the presence of aldehyde group H-C=O. Peak 1760 indicates the presence of the -CH2- of alkane and 1023 indicate C-N of amine. FTIR analysis showed capping of BiTCoN with various phytochemicals responsible for reducing Cu^{++} ion **(fig.2 b)**.The BiTCoN were spherical, cubical & polydisperse with an overall size 50 ± 30 nm were detected by SEM and TEM analysis **(Fig.2 d & e)**.Presence of copper metal was detected by the EDAX analysis, which showed a robust elementary copper peak of BiTCoN in our study**.** Other atoms such as potassium formed from the biomolecules (plant extract)help in capping & synthesis of BiTCoN; therefore, the low signals of potassium (K) were detected in EDAX analysis **(Fig. 2 c)**.

The studied experiment found that the nano form (thermoactive BiTCoN) retains the heat content for longer duration and the amount of heat released compared to plant extract was minimal **(Fig.3)**.Therefore, the decrease in temperature was recorded notably low in BiTCoN. Hence, this nano form was supposed to be thermo-active or thermo active, exploring membrane permeability, cell targeting and inhibit biofilm formation in *C. albicans*.

Fig.3. Figure showing the difference between the amount of heat released and the thermal capacity of aqueous plant extract & BiTCoN.

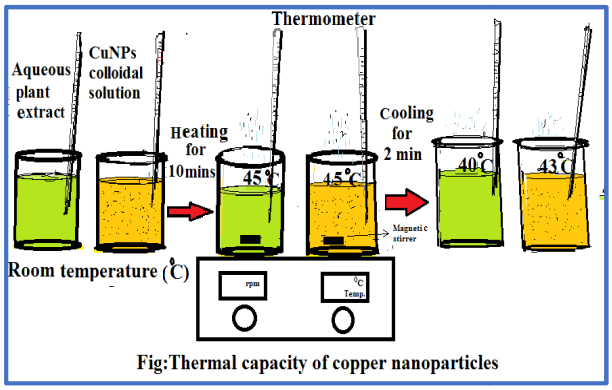


Fig:Thermal capacity of copper nanoparticles

3.2 Effect of BiTCoN on virulence activity of C. albicans

The BiTCoN inhibit virulence activities such as the development of *Candida* cells in a concentration-based manner, establishing MIC at 25 µg mL^{-1} & 50 µg mL^{-1} accordingly **(Table 1 & fig. 4 a, c-h)**. The contagious fungicidal effect was detected at 100 µg mL^{-1}&150 µg mL^{-1}, which harm planktonic cells growth very effectively. BiTCoN inhibited RPMI-1640 medium was able to elicit hyphae formation in *C. albicans* at in *C. albicans* at MIC/8. Therefore, a satisfactory result of inhibition (50% to 90%) of yeast to hyphal form shift was observed at MIC/8 successively; consequently, it leads to the inhibition of germ tube and along with this quite inhibition possibly seen in the budding formation of *C. albicans* at low concentrations **(Fig. 5)**.

The potential to avoid or restrict virulence factors such as dimorphism, without damaging a microbe, helps them skip natural selection & promotes the evolution of a drug-inhibitory population of the pathogen. Thus, BiTCoN treatment strongly affects the *C. albicans* hyphal development shown in an inverted light microscope **(Fig. 5 a, c-h)**.BiTCoN inhibited RPMI-1640 medium was able to elicit hyphae formation .

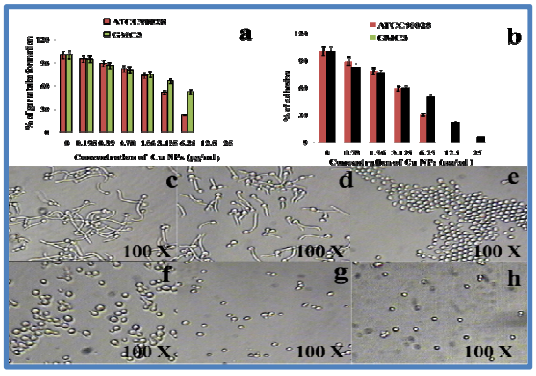


Fig.4. The effect of BiTCoN on planktonic growth of *C. albicans* ATCC90028 and GMC3 strain **(a)** Graph of BiTCoN treated with planktonic cell growth **(b)** Control **(c)** Image showing minimum inhibitory concentration (MIC) **(d)** Maximum fungicidal concentration (MFC) of BiTCoN against ATCC90028 and GMC3 strain.

Fig.5. **(a)** Image showing average growth to the hyphal transition of *C. albicans* ATCC90028 and GMC3 strains treated by BiTCoN. The *C. albicans* cells of ATCC90028 strain were treated with BiTCoN at µg mL^{-1}, **(g)** 12.5 µg mL^{-1}, **(h)** 25 µg mL^{-1},**(b)** The hyphal development of the fungal cells as adhering cells after one and half hour of BiTCoN treatment as influencing adhesion cellular metabolic activity was different concentrations for one and half hour morphogenesis in 20% serum induction, and observed in the light microscope, **(c)** Control, **(d)**-**(h)** Different concentration such as **(d)** 1.56 µg mL^{-1},**(e)** 3.25 µg mL^{-1},**(f)** 6.25 determined by XTT assay of *C. albicans* strains ATCC90028 and GMC3 treated by BiTCoN

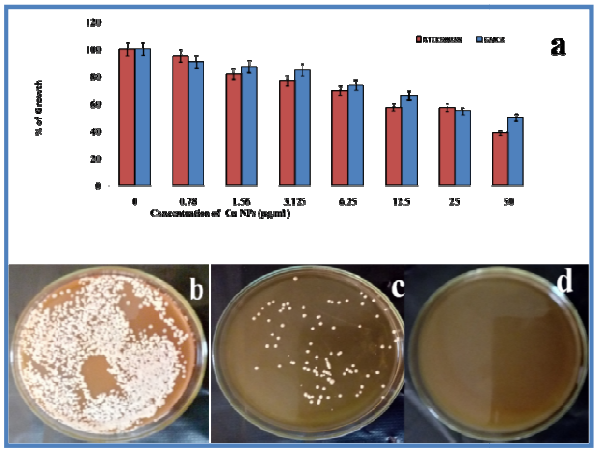
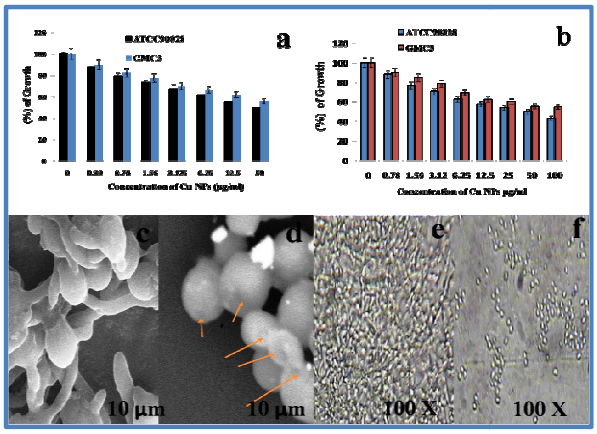


Fig.6. The study of biofilm formation of C. albicans strains ATCC90028 & GMC3 treated with BiTCoN. **(a) & (b)** Shows the graph of normal hyphal formation of the fungal cells after 24 h of incubation time treated with BiTCoN. Average growth and biofilm development of C. albicans treated with BiTCoN was observed by scanning electronic microscopy & light microscopy **(c) & (e)** Control **(d) & (f)** SEM images of Candida cells showing inhibitions and cell damage by BiTCoN.



The attaching properties of *C. albicans* on solid polystyrene surface was inhibited by BiTCoNat MIC/8 value **(Fig. 5 b)**.Further work on *C. albicans* biofilm resistant to antifungal antibiotics significant at the value of 100 µg mL^{-1}& 200 µg mL^{-1}. The MIC of biofilm formation shows double MIC value, while total deduction in biofilm production at minimum fungicidal concentrations (MFC) was discussed. The MIC value exhibit phase of yeast and mycelium formation was absent in biofilm development **(Fig. 6)**.Both the strains declined in the catabolic & anabolic (metabolic) activity as the biofilm was developing; therefore, the inhibitory concentrations of extracellular polysaccharide matrices of mature biofilm probably same. Images captured by scanning electron microscopy demonstrated that the pores were formed on the *C. albicans* cells; therefore, cell damage of *C. albicans* was confirmed **(Fig. 6 c, d)**.Similarly, the demonstration of biofilm formation on the catheter by forming discs were the well-studied model. An extensive network of hyphal filamentous & yeast cell attenuated growth by BiTCoN at 50 µg mL^{-1} on the catheter as a model was observed by using a scanning electron microscope **(Fig. 6 c, d)**.

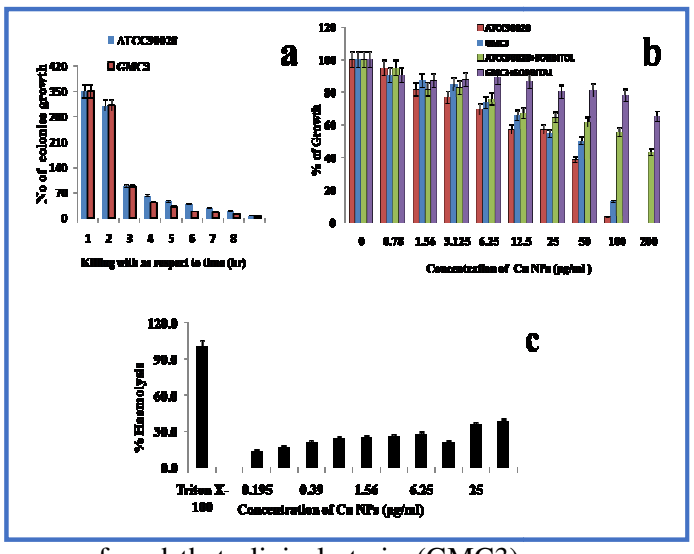
The impact of BiTCoN on the development pattern of *C. albicans* was assessed in various experiments were found to be dose-dependent manner. MIC exhibit negligible cytotoxicity, which gives us a positive approach to investigate its way of action delayed exponential phases. To explore the mechanism behind a contagious antifungal strategy of the BiTCoN on the *Candida* cell, we investigated compound of stress condition, *i.e.* sorbitol treated with BiTCoN and-non treated cell called as control of *C. albicans* on both strains. The assessment proved that thee, two folds increase in the MIC concentration, *i.e.* 200 µg mL^{-1} in a medium with sorbitol in contrast to without sorbitol at MIC value of 25 µg mL^{-1} & 50 µg mL^{-}

[1]for both strains ATCC90028 and GMC3 **(Fig. 7 b Table. 1)**.

Sorbitol acts as the osmoprotectant for the cell wall of *C. albicans*. It gives us an idea about the cell wall leakage will be the possible cell target for the checked BiTCoN.

BiTCoN showing negligible hemolysis of RBC at different concentrations, 100 µg mL^{-1} caused >30% hemolysis **(Fig. 7 c)**. The increase in the concentration of BiTCoN, indicating that the decrease in viability of *C. albicans* cells in all the experiment we performed, along with negligible cytotoxicity, was recorded. The time required to kill 100% of *Candida* cells8 to 9 hours as needed for both the strains. In most of the experiments,

Fig.7. (a) Study of BiTCoN treated with C. albicans strains ATCC90028 & GMC3 concerning time **(b)** BiTCoN treated with planktonic cell growth with the addition of sorbitol as stress molecules. **(c)** Hemolytic effect of BiTCoN on RBC's.



we found that clinical strain (GMC3) was more sensitivethanATCC90028 strain **(Fig. 7 a)**.

4 Discussions

The advantages of BiTCoN in the various fields are gaining importance due to its eco-friendly synthesis and production [34]. Green chemistry has been widely used synthesize nanomaterials. The method (chemical) used to obtain BiTCoN have a limited number of pharmaceutical and medical applications. *Tinospora cordifolia*is a well-known medicinal plant with unique properties and reported its anti-microbial activity [35]. However, there are no documents accessible on the anti-*Candida* and antibiofilm action of BiTCoN biosynthesized from *Tinospora cordifolia* (BiTCoN). The BiTCoN consists of the stem extract for synthesis, which is non-toxic, eco-friendly, cost-effective and allows rapid production in a short time. The precise tool for the synthesis of BiTCoN is not yet exact, but some are explored in gold and silver [18]-[20], [26].

The tool behind the antibacterial and antifungal activities of nanomaterials seems to be distinct, intricate and may include versatile paths which facilitate the killing of *Candida* cells. Some of these mechanisms evoke the formation of ROS, cell membrane rupture, apoptosis, ion accumulation, distorted cell membrane permeability, and electrostatic bond of attraction between charged moieties such as proteins and nanoparticles, which promotes osmotic stress in the cell were reported [36]-[39].

In addition to this, the mechanism for a rise in thermal conductivity of BiTCoN were proposed called Brownian motion [40]-[42]

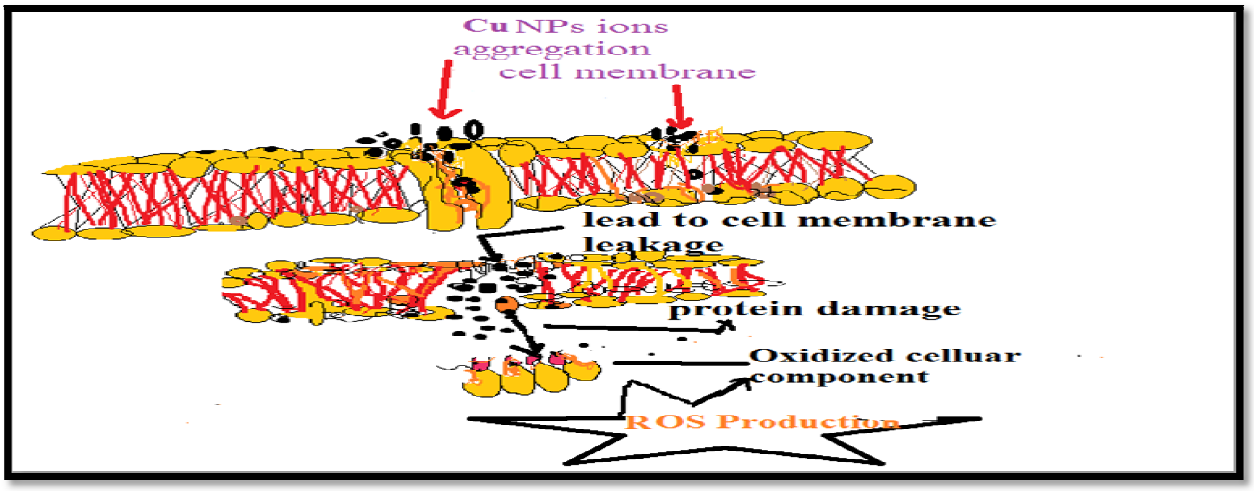


Fig:8 Schematic representation of BiTCoN activity against *Candida albicans* cell membrane

	MIC (µg mL^{-1})						MFC (µg mL^{-1})
Strains	**Planktonic Growth**	**Sorbitol stress growth**	**Morphogenesis**	**Adhesion**	**Biofilm development**	**Mature biofilm**	
ATCC90028	**50**	**200**	**3.2**	**3.2**	**100**	**100**	**100**
GMC3	**25**	**200**	**6.25**	**6.25**	**50**	**100**	**50**

Table 1.

Comparison of MICs of BiTCoN against planktonic and biofilm growth of *C. albicans* ATCC90028 and GMC3 strains.

transfer of heat in the near radioactive field [43] and aggregation of nanoparticles [44]-[46]. In contrast to this, the aqueous plant extract was made in water, and there is a loss of solidity in water molecules. With the heat rise, water molecules get evaporate by the breaking of hydrogen bonds. Hence no four bonds can be possible to form at any stage in the water molecule. The thermal energy constantly interfered in the bond formation of a water molecule [47].

The primary aim to study the thermal capacity of BiTCoN is due to its importance in the biological phenomenon as it facilitates easy penetration and changes cell surface membrane properties such as membrane permeability,

results in improvement of drug delivery and helps in the treatment of various infectious diseases [48]. The bimodal therapy where plant extract used as an adjuvant along with heat treatment of BiTCoN prevents the recurrence of fungal infection [21].

Our work's novelty includes the interaction of BiTCoN from *Tinospora cordifolia* stem extract against adhesion, planktonic, dimorphism & biofilm forms of *C. albicans* with their mechanism of inhibition as cell wall assay. The MIC of planktonic development of *C. albicans* was 50 µg mL^{-1} & 25 µgmL^{-1} for ATCC90028 and GMC3 strain, respectively. However, this interaction in GMC3 suggests a strained variation to concentrations of BiTCoN as the nanoparticles concentration was doubled in standard strain compared to clinical strain. We investigated that the hyphae transition reduced by three-fold and four-fold of MIC concentration by BiTCoN against standard ATCC90028 and GMC3 strain of *C. albicans*. The BiTCoN effective against developing mature biofilm at 50 µg mL^{-1} & 100 µg mL^{-1} of ATCC90028 & GMC3 strain. However, such interaction was compassionate and effective in the isolate GMC3 at low concentration **(Table 1)**. There are no reports on the mechanism of inhibition for *C. albicans* related to biofilm formation treated with BiTCoN.

Changes in the osmotic process may promote appropriate cellular responses. Hence, the study of the osmotic effect on cellular activities is supposed to be crucial [49-51], which may be one of the antifungal targets as cellular responses control cellular activities such as osmotic stress. The mechanism behind anti-*candida* activity suggests that osmotic pressure releasing molecule sorbitol used in sorbitol protection assay implicated that after adding sorbitol it protects the cell wall from bursting damage by maintaining osmotic stability interior of the cell which increasing MIC in three to four folds for both strains. From the assay, it suggests that the cell wall induced oxidative stress promotes cytoplasmic leakage, which is clear evidence that oxidative stress is one of the reasons for inhibition of cell growth by causing protein degradation of cells which promote ROS production and finally leads to loss of metabolic activity resulting in cell death.

Available reports suggested that the anti-microbial activity in silver and gold nanoparticles studied extensively. Still, in case of copper, there are less reports present against anti-biofilms of *C. albicans*. The information present on silver and gold primarily due to their distribution in semi permeability of cell membrane, ability to form ROS, and electrostatic attraction bond formation lead to cell death [36]-[39], [52]-[55].

The homeostasis of the cell membrane and cell content is maintained by osmotic pressure. Cell wall components responsible for maintaining rigidity, integrity, inducing proper virulence, adhesion, and protect from oxidative stress [49], [51], [56].Adhesion initiates virulence factors such as infection, proliferation (colony formation), regulating stress tolerance, morphological transition, antigenicity, and others that promote biofilm formations into the host [1]. The cell wall contents and adhesion proteins, *i.e.* chitin, mannoproteins, β-glucans and other protein forms associated with enzymes performing cross-linking, branching, and other functions are one of the targets for cell

destruction by antifungal agents; hence, sorbitol as an osmotic stabilizer which helps the cell to survive the cells in medium [38]-[49], [56].

BiTCoN are good anti-microbial agents; it reported as an antifungal and antibacterial agent. In most cases, the BiTCoN used as anticancer agents since it inhibits tumor cells [25], [34], [57]. It also inhibits the proton pump in drug-resistant bacteria [58]. There are several reports published on nanoparticles, mostly silver and gold, at high concentration. It may cause side effects such as genotoxicity, cytotoxicity, ecotoxic neurotoxicity and mitochondrial dysfunction [59-69]. Comparative analysis studies of drugs showed that hemolysis drug potential could limit the drug itself for therapeutic applications. As per our results, the BiTCoN has no effect on hemolysis at a concentration of 50 µg mL^{-1} & 100 µg mL^{-1}. Surprisingly, these were the exact concentrations necessary for the reduction of biofilms formations. The applications of BiTCoN to clinical practices were recommended; hence, it might be safe for use in various therapies.

5 Conclusions

In our study, the following conclusions can be drawn

1. The BiTCoN strongly affect and inhibit the growth of standard and clinical strains of *C. albicans*
2. The BiTCoN is a novel antifungal agent and give insights to previously unexplored targets.
3. The BiTCoN from *Tinospora cordifolia* act on cell membranes of *C. albicans* and facilitate easy penetration inside the *Candida* cells and inhibit biofilm formation and dimorphism.
4. The BiTCoN is a bimodal therapy that produces heat effect in *C. albicans*, and plant extract act as an adjuvant to prevent further infection and induce ROS formation, which causes cell death.
5. The BiTCoN is a potential agent and can be utilized in various anti-microbial biofilm formations such as candidiasis infections.

Conflict of Interest Statement

All the authors declare no conflict of interest.

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