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RESEARCH ARTICLE

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Formulation and Evaluation of Copper Nanoparticles Loaded Microsponges

S. Kothai, R. Umamaheswari*

Ethiraj College for Women, Chennai-600 008, Tamil Nadu, India

ABSTRACT

Microsponges become imperative in the field of targeted drug delivery and in other biomedical applications. There was a clamant need for designing microsponges incorporating with green synthesised metal nanoparticles rather than the chemical drug in order to reduce the side effects of the drug and thus increasing the effectiveness of nature of the whole material. It provokes us to design this novel approach of loading copper nanoparticles into the microsponges. Here in this work, microsponges based on ethyl cellulose and polyvinyl alcohol were synthesised by Quasi-Emulsion Solvent diffusion method in which copper nanoparticles procured from *Hibiscus rosa-sinensis* leaf extract was incorporated. The Loaded microsponges were characterised by High Resolution Scanning Electron Microscopy (HR-SEM) and Particle size distribution Analyzer (PSA). The Drug content and Entrapment Efficiency of the microsponges were found out. The antimicrobial and antioxidant activity of the loaded microsponges were evaluated.

Keywords: Copper nanoparticles, microsponges, HRSEM, PSA, antimicrobial, antioxidant.

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***Corresponding author**: Mrs. R. Umamaheswari
Address: Department of Chemistry, Ethiraj College for Women, Chennai-600 008, Tamil Nadu, India
E-mail ✉: aarthi.r90@gmail.com

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INTRODUCTION

Microsponges are polymeric delivery systems, possessed of porous polymeric microspheres that can entrap active ingredients. These are tiny sponge like spherical particles that consists of myriad of interconnecting voids with large porous surface. Usually the size of the microsponges range from 5 to 300µm. [1-2] Metal nanoparticles such as gold, silver and copper are reported as highly toxic to micro-organisms. [3-4] In recent years, it has been extensively used for the production of medical products like wound dressing because of its strong cytotoxicity. [4-6] In current scenario, the development of microsponge loaded with specific drug has been emphasised due to their controlled release of the drug. Since the microsponges prepared from synthetic polymers, it will protect the entrapped drug from any kind of degradation. These kinds of encapsulated drugs within microsponge system can significantly reduce the irritation, side effects of the drug without decreasing its efficiency. [7-8] The current work involves the formulation and evaluation of copper nanoparticles loaded microsponges and its biomedical applications. Here, microsponges were synthesised by Quasi-Emulsion

Solvent diffusion method using different proportions of ethyl cellulose and polyvinyl alcohol. Later, the green synthesised copper nanoparticles from the leaf extract of *Hibiscus rosa-sinensis* were incorporated into the microsponges. The formulated and loaded microsponges were characterised by SEM and PSA. The Drug Content and Entrapment Efficiency of the loaded microsponges were studied. The antimicrobial and antioxidant activity of the copper nanoparticles loaded microsponges were evaluated.

Hence, in the present work an attempt was made for the first time by incorporating copper nanoparticles in the microsponges rather than any chemical drug. These metal nanoparticles loaded microsponges will minimise the toxicity of the drug intake, prolong the pharmacological effect and thus improve the overall effect of the microsponges. The copper nanoparticles loaded microsponges will show enhanced activity towards biomedical applications than the copper nanoparticles alone. In future, this study would lead to a new scenario of introducing copper nanoparticles loaded microsponges for a smarter application.

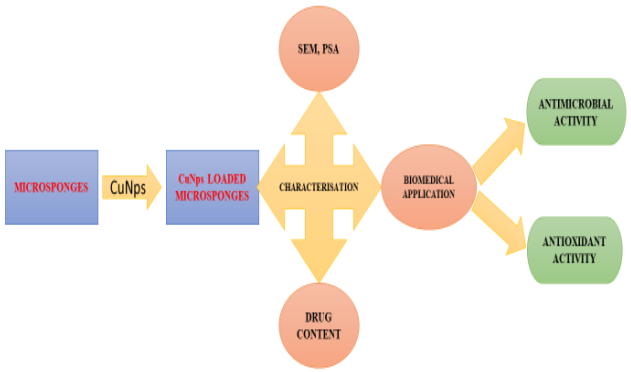

Fig. 1: Graphical Abstract of the current work

MATERIALS AND METHODS

Materials

Ethyl Cellulose (EC), Polyvinyl alcohol (PVA), Dichloromethane (DCM) of reagent grade were kindly purchased and used without purification. Copper nanoparticles were green synthesized from the leaf extract of *Hibiscus rosa-sinensis*. Double Distilled water was used throughout the study.

Green synthesis of copper nanoparticles

The Copper nanoparticles (B) were synthesized from the leaf extract of *Hibiscus rosa-sinensis*. The fresh leaves were collected and washed with distilled water to remove dust and impurities and shade-dried for 3-4 days at room temperature. About 100 g of dried and minced leaves were weighed and transferred to beaker containing 100 mL distilled water. It was then boiled at 60°C for 10-15 min. First, the prepared solution was filtered Whatmann no.1 Filter paper to get a clear solution. This filterate was known as *Hibiscus rosa-sinensis* leaf extract. 50 mL of this extract was added to 50 mL of 0.05M $CuSO_4$, kept for incubation for 3 days. After incubation, the precipitate got settled down that was confirmed by the colour change from green to black. This indicates the formation of Copper nanoparticles that was purified by repeated centrifugation at 6000 rpm for 10 min to remove unwanted materials. The synthesized CuNps were lyophilized and stored at 25°C for further use. [9]

Synthesis of copper nanoparticles loaded Microsponges

Copper nanoparticles loaded Microsponges were formulated by Quasi-Emulsion Solvent Diffusion method. Five batches of microsponges (NS_0 – NS_4Cu_B) with varying proportions of Ethyl Cellulose (EC) and Polyvinyl alcohol (PVA) were taken. The Dispersed Phase consists of Copper Nanoparticles (B -CuNps) and required amount of EC dissolved in 20 mL of Dichloromethane (DCM). It was slowly added to PVA in 150 mL of aqueous continuous phase. Then it was stirred at 1000 rpm under magnetic stirrer for 3 hours. The microsponges formed were filtered and dried in oven at 40–50°C for 24 hours. Then the dried microsponges were stored in vacuum dessicator to remove the residual solvent. The composition of the microsponge formulation was tabulated in Table 1. The Figure 2 indicates the schematic representation of microsponge formation. The prepared microsponges were characterized based upon the entrapment efficiency and particle size. [8]

Table 1: Formulation of Nanosponges with (B) CuNps

Sample Code	Copper Nanoparticles (B) mg	PVA g	EC g	DCM mL	H_2O mL
NS0	-	2	2	20	150
NS_1Cu_B	10	2	2	20	150
NS_2Cu_B	10	2	3	20	150
NS_3Cu_B	10	3	2	20	150
NS_4Cu_B	10	3	3	20	150

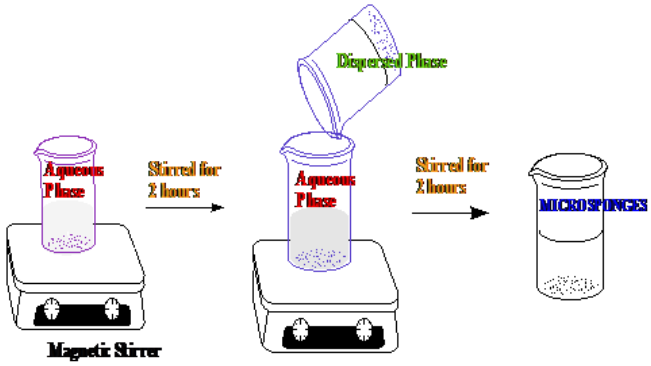

Fig. 2: The schematic representation of microsponges formation.

Characterisation of Copper nanoparticles loaded Microsponges

Microscopic studies

The morphology of the loaded microsponges and unloaded microsponges was studied by using High Resolution Scanning Electron Microscopy (HRSEM). Here we have used VEGA3 TESCAN instrument for our characterization work. A thin film of the sample

was made by placing a pinch of the sample on a carbon coated grid and then the film on the SEM grid was made to dry under mercury lamp for 5 minutes.

Particle size determination

The particle size of the copper nanoparticles loaded Microsponges was determined by using Particle Size Distribution Analyzer. Here the instrument used was HORIBA Laser Scattering Particle Size Distribution Analyzer LA-950.

Percentage Yield

The percentage yield of copper nanoparticles loaded microsponges of various batches were calculated using the weight of final product after drying with respect to the initial total weight of drug and polymer used for the preparations. [8]

Drug Content and Entrapment Efficiency

About 10 mg of microsponge from all batches were accurately weighed and dissolved in methanol in 50 mL standard flask and then made up to the volume of phosphate buffer pH 7.4. After appropriate dilution, the amount of drug was detected by a UV Spectrophotometric method at 650 nm using blank microsponges treated in the same manner. [8] The Entrapment Efficiency was calculated according to the following equation:

Entrapment Efficiency (%) = [Actual drug content in microparticles/Theoretical drug content] × 100

Preparation of Standard Calibration Curve

Preparation of Phosphate Buffer pH 7.4

Phosphate Buffer was prepared and pH was found to be 7.4 using digital pH meter. [8]

Determination of λmax of copper nanoparticles

The absorption maxima for copper nanoparticles (B) were found to be 650 nm. [9]

Standard calibration curve of Copper nanoparticles (B)

The absorbance of copper nanoparticle standard solutions having a concentration range of 100-500µg/mL in phosphate buffer pH 7.4 was plotted. The curve was found to be linear at λmax 650 nm. The calculation of the drug content and Entrapment efficiency were based on this calibration curve. [8]

In- vitro antimicrobial study

Determination of Minimum inhibitory concentration (MIC) using Resazurin Microtitre Assay

Preparation of resazurin solution

The resazurin solution was prepared by dissolving 270 mg in 40 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

Test was carried out in a 96 well Plates under aseptic conditions. A sterile 96 well plate was labelled. A volume of 100µL of sample was pipetted into the first well of the plate. To all other wells 50µL of nutrient broth was added and serially diluted it. To each well 10µL of resazurin indicator solution was added. 10µL of bacterial suspension was added to each well. Similarly, the same set up was performed for antifungal activity in which 50µL of potato dextrose broth was added and 10µL of fungal suspension was added on each well. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. The plate was incubated at 37°C for 18–24 hours. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value. [9]

Antioxidant study

Determination of scavenging activity by DPPH assay

The percentage of antioxidant activity (AA %) of each substance was assessed by DPPH free radical scavenging assay. Different concentrations of sample were added to all the tubes except blank. Then 3 mL of ethanol and 0.3 mL of 0.5 mM DPPH radical solution in ethanol was added. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL). Absorbance was read at 517 nm after 30 min of reaction. [9] The scavenging activity percentage (AA %) was calculated using the below formula

% Antioxidant activity = {(absorbance at blank) – (absorbance at test) / (absorbance at blank)} × 100

RESULTS AND DISCUSSION

Microscopic studies

From SEM studies, it was found that the samples had porous and almost spherical sponge in nature. The pores were induced by the diffusion of the solvent. [8] The SEM image of CuNps(B) were spherical and agglomerated to form clusters (Fig. 3). [9] The unloaded Microsponges shows shiny smooth surface morphology (Fig. 4). The Loaded microsponge shows porous smooth surface and spherical (Fig. 5). SEM results revealed that surface morphology has been shown to be beneficial for topical application for future studies.

Particle size

The Particle size analysis of loaded and unloaded microsponges (Fig. 6) revealed that the particle size ranges from 65µm to 93µm. NS_4Cu_B was selected for the further study in terms of lower particle size (65µm). The smaller particle size shows better entrapment efficiency in future.

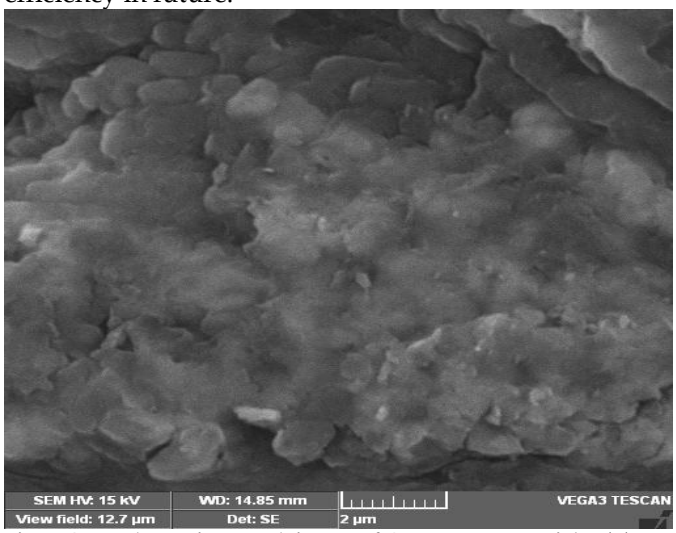

Fig. 3: SEM photomicrograph image of Copper Nanoparticles (B)

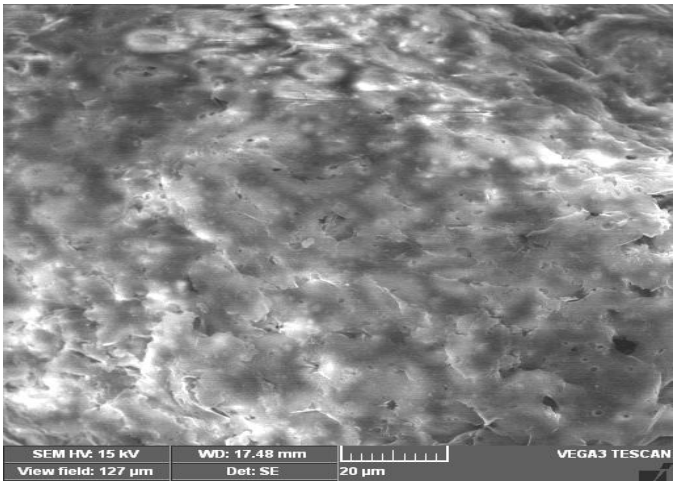

Fig. 4: SEM photomicrograph image of unloaded microsponge NS_0

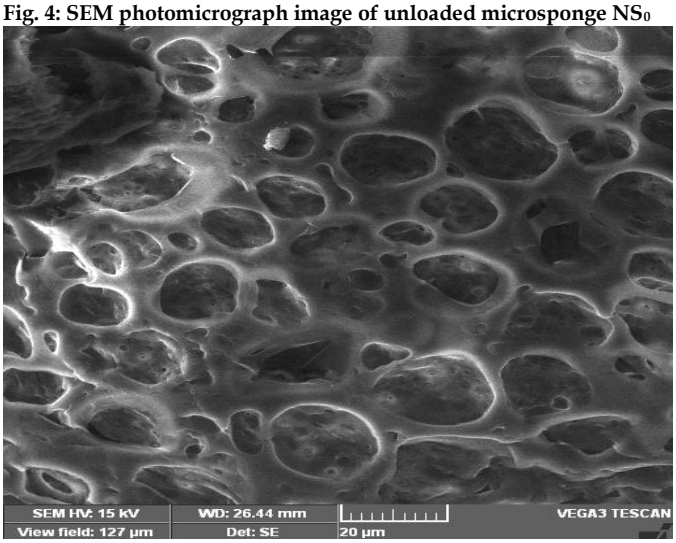

Fig. 5: SEM photomicrograph image of loaded microsponge NS_4Cu_B

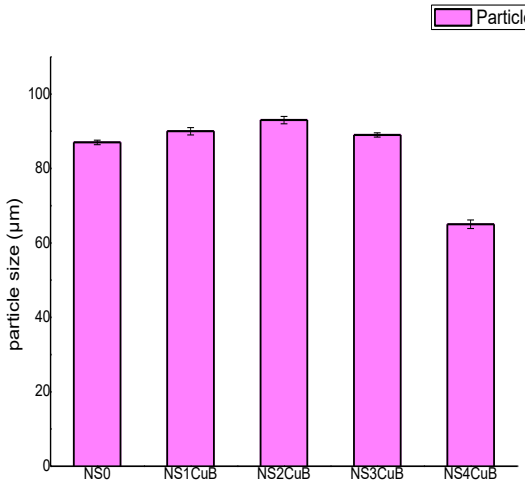

Fig. 6: Particle size of loaded and unloaded Microsponges

Production yield

The production yield of all the microsponges were calculated and shown in the Fig. 7.

Drug Content and Entrapment Efficiency

The Drug content and Entrapment Efficiency were calculated and displayed in the table 2.

Standard Calibration Curve

The standard calibration curve for copper nanoparticles (B) in phosphate buffer pH 7.4 at 650 nm was shown in Fig. 8.

Antimicrobial activity by Resazurin microtitre assay

The synthesised NS_4Cu_B microsponge formulation was selected for the biomedical applications due to its least particle size and better entrapment efficiency. The antimicrobial activity was done by Resazurin Microtitre assay (Table 3). It shows good antibacterial activity towards *E. coli* and *B. subtilis* whose MIC values are 125µg/mL and 31.2µg/mL respectively. From the results, it shows more active towards *B. subtilis.* The MIC values of copper nanoparticles loaded microsponge NS_4Cu_B is almost equal to that of the value of CuNps(B). [9] Similarly, NS_4Cu_B shows an excellent antifungal activity towards *C. albicans* whose MIC value is 62.5µg/mL whereas the MIC value of CuNps(B) was found to be 250µg/mL. [9] Hence, it is proven that the antifungal activity nature of copper nanoparticles loaded microsponge is enhanced.

(Antibacterial activity – STD- *Streptomycin*)

(Antifungal activity- STD- *Amphotericin B*)

Antioxidant activity by DPPH assay

The copper nanoparticles loaded microsponge formulation NS_4Cu_B has an antioxidant potential of 59.5% (Table 4). The percentage scavenging activity of copper nanoparticles loaded microsponge is slightly lower than the value of standard BHT (Fig. 10). The CuNps(B) showed 21.7% of scavenging activity. [9] From the results, it shows that the antioxidant activity increases in the copper nanoparticles loaded microsponge formulation (NS_4Cu_B). This indicates the successful encapsulation of drug (CuNPs) within the microsponge. Therefore, the copper nanoparticles loaded microsponge enhanced the activity of CuNps. It reveals the porous nature of the outer surface of the sponge offers control on the release of drug.

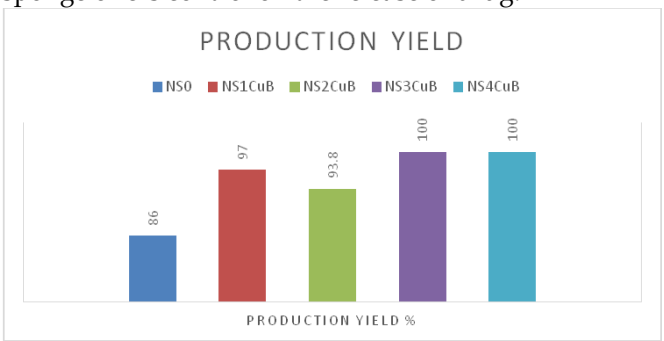

Fig. 7: Production yield of loaded and unloaded Microsponges

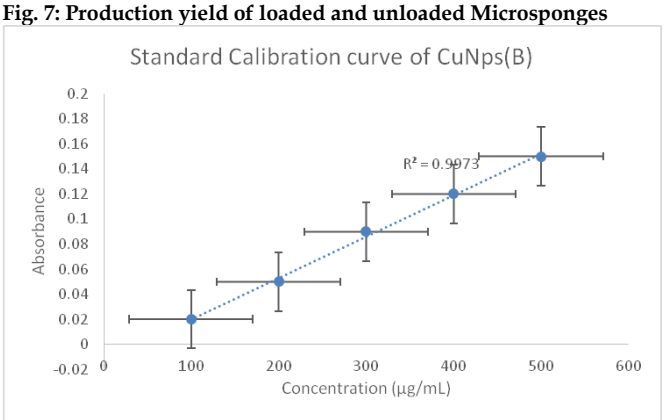

Fig. 8: Standard calibration curve of CuNps (B)

Table 2: Drug Content and Entrapment Efficiency % of Microsponge formulations

Sample Code	Absorbance	Concentration	Drug Content %	Theoretical Drug Content % in 10 mg	Entrapment Efficiency %
NS_1Cu_B	0.121	403.33	4.0333 ± 0.0183	0.0497	81.07 ± 0.3438
NS_2Cu_B	0.113	376.67	3.7667 ± 0.2269	0.0332	113.37 ± 6.8016
NS_3Cu_B	0.149	496.67	4.9667 ± 0.1836	0.0497	99.83 ± 3.6701
NS_4Cu_B	0.135	450	4.50 ± 0.0839	0.0332	135.46 ± 2.4837

Table 3: Antimicrobial activity of Copper nanoparticles(B) loaded Microsponge formulation NS_4Cu_B

S. No	Microorganisms/sample	Growth of inhibition (µg/mL)										
	$NS4Cu_B$	1000	500	250	125	62.5	31.2	15.6	7.8	STD 10	Sterile water	Culture
1	*Escherichia coli*	-	-	-	-	+	+	+	+	-	+	+
2	*Bacillus subtilis*	-	-	-	-	-	-	+	+	-	+	+
3	*Candida albicans*	-	-	-	-	-	+	+	+	-	+	+

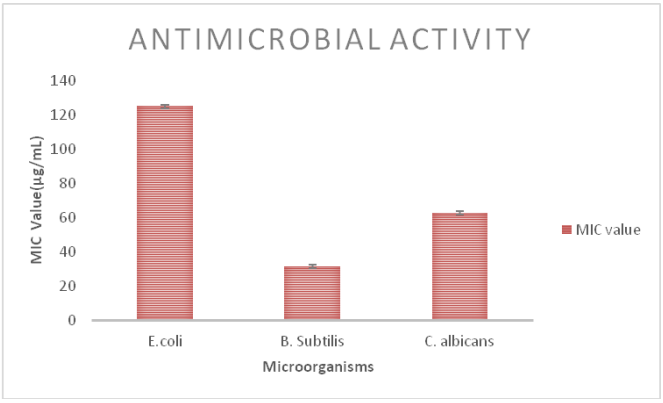

Fig. 9: Antimicrobial activity representing MIC value of NS_4Cu_B

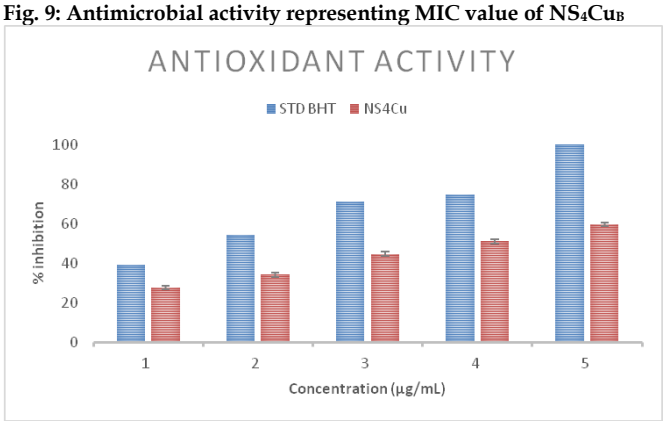

Fig .10. Antioxidant activity of NS4CuB by DPPH assay.

Table 4: Antioxidant activity of Copper nanoparticles(B) loaded Microsponge formulation NS_4Cu_B

Standard –BHT (Butylated hydroxytoluene)

Concentration	Standard BHT Blank - 0.59		NS_4Cu_B Blank – 0.47	
µg/mL	O.D	% inhibition	O.D	% inhibition
100	0.36	38.9	0.34	27.6 ± 0.15
200	0.27	54.2	0.31	34.0 ± 0.5
300	0.17	71.1	0.26	44.6 ± 0.5
400	0.15	74.5	0.23	51.0 ± 0.5
500	0.11	99.8	0.19	59.5 ± 0.7

Ethyl cellulose based microsponges loaded with copper nanoparticles green synthesised from the leaf extract of *Hibiscus rosa-sinensis* have been successfully prepared by Quasi-Emulsion solvent diffusion method. The formulated batches of microsponges were characterised by SEM and PSA. The SEM results showed smooth outer surface and porous spherical in nature. The least particle size of 65µm of NS_4Cu_B was selected for the biomedical applications.

The physicochemical parameters of the formulated microsponges including production yield, Drug content and entrapment efficiency were determined. The NS_4Cu_B with least particle size showed better entrapment efficiency of 135%. The antibacterial activity of copper nanoparticles loaded microsponge formulation NS_4Cu_B shows good activity on *B. subtilis*. The MIC values of CuNps loaded microsponge is equivalent to that of the drug (CuNps). Similarly, the antifungal activity of NS_4Cu_B towards *C. albicans* is increased when compared to that of CuNps. The antioxidant activity of NS_4Cu_B showed an enhanced activity of 59.5% to that of the CuNps (21.7%).

In this work, we have made an attempt to incorporate copper nanoparticles in microsponge for the first time. We have succeeded in our venture by encapsulating CuNps in the microsponge formulation, thereby enhancing the activity of the copper nanoparticles. The smooth and porous nature of the formulation offers good control on release of the drug and hence it can be used in topical application in future.

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